

**Modulation of mammalian immune response and oxidative status by
dietary *Saccharomyces cerevisiae***

by

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Abstract

Saccharomyces cerevisiae contains multiple components within its cell wall, including β -glucan and mannans. This yeast species, along with its cell wall components, has been shown to modulate various functions of the immune system, intestinal cells, and whole animal health. In dairy cow health, much emphasis is placed on the transition from late gestation to early lactation, a period characterized by immunosuppression, inflammation, and oxidative stress. Sixty-four Holstein cows (50 multiparous, 14 primiparous) were either fed a control diet or control diet plus 18.4 g/d of *Saccharomyces cerevisiae* fermentation product (**SCFP**) from -29 ± 5 to 42 d relative to calving. Supplementation of SCFP generally did not affect measures of oxidative status, inflammation, innate immune response, or adaptive immune response. However, SCFP-supplemented cows tended to have lesser α -tocopherol concentrations in plasma 2 wk before parturition, and mean plasma retinol concentrations were greater for SCFP-supplemented primiparous cows but lesser for SCFP-supplemented multiparous cows compared to parity-matched controls. Additionally, primiparous cows supplemented with SCFP tended to have greater serum concentrations of anti-ovalbumin immunoglobulin G after subcutaneous ovalbumin vaccinations postpartum. An *in vitro* cell screening system using RAW 264.7 murine macrophages and human intestinal epithelial Caco-2 cells also identified immunomodulatory effects of live *S. cerevisiae* and *S. cerevisiae*-derived β -glucan, mannan, and zymosan, a crude cell wall preparation containing both β -glucan and mannan. D-mannose was also evaluated as the monomer of mannan. RAW cells were transfected with a vector that drives expression of alkaline phosphatase upon activation of nuclear factor κ -light-chain-enhancer of activated B cells (**NF κ B**), a major inflammatory/immune transcription factor. Messenger RNA abundance for the pro-inflammatory cytokine, IL-8, and the tight junction protein, claudin-1, was evaluated in

Caco-2 cells. RAW and Caco-2 cells were each incubated with 0.01, 0.1 or 1 mg/mL of these treatments alone or pre-incubated with these treatments followed by a lipopolysaccharide (**LPS**) challenge. Additionally, RAW cells were challenged with LPS then incubated with treatments. In RAW cells, treatment with zymosan or β -glucan alone induced NF κ B activation in a dose-dependent manner, while treatment with D-mannose, mannan, or live *S. cerevisiae* cells did not. Pre-treatment with zymosan or β -glucan followed by an LPS challenge increased NF κ B activation, whereas pre-treatment with D-mannose and mannan decreased NF κ B activation, indicating that these components may protect against LPS-induced inflammation. Post-treatment with mannan and live *S. cerevisiae* after an LPS challenge decreased NF κ B activation, suggesting that these treatments may ameliorate LPS-induced inflammation. Treatment with live *S. cerevisiae* at 1 mg/mL alone or followed by LPS challenge decreased, whereas treatment post-LPS challenge increased, measures of RAW cellular metabolism. Evaluation of live cell treatments may not be accurate with this *in vitro* screening system. In Caco-2 cells, treatment with β -glucan at 0.01 mg/mL, mannan at 1 mg/mL, and zymosan at 0.1 and 1 mg/mL increased IL-8 mRNA abundance with treatment alone or followed by an LPS challenge. Interestingly, β -glucan-induced IL-8 mRNA abundance was greater without LPS stimulation. In contrast, claudin-1 mRNA abundance did not differ among treatments. Live *S. cerevisiae* cells also increased measures of Caco-2 cell viability. Overall, *S. cerevisiae* and its cell wall components modulated innate immunity *in vitro*, whereas SCFP modulated some aspects of oxidative status and adaptive immunity in transition dairy cows.

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Chapter 1 - Immune, Intestinal, and Whole Animal Modulation by

***Saccharomyces cerevisiae* and its Cell Wall Components**

Composition of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a yeast species often used in baking and brewing, thus commonly termed “baker’s yeast” or “brewer’s yeast.” This yeast species includes several strains that can differ by genotype and source (van der Aa Kühle et al., 2005; Schacherer et al., 2009) . However, in general, the structure of *S. cerevisiae*, with particular focus on the cell wall, has been reviewed extensively (Klis et al., 2006; Lesage and Bussey, 2006; Orlean, 2012). The cell wall constitutes approximately 10 – 25% of the *S. cerevisiae* cell and contains several components with various functions. The cell wall itself can be broken down into two layers. The inner layer is composed of polysaccharides that form scaffolding for components of the outer layer, which largely consists of mannoproteins that provide physical protection for the cell.

By mass, the cell wall is 30 – 50% mannoproteins (4 – 5% proteins and remainder mannan), 30 – 45% β -1,3-glucans, 5 – 10% β -1,6-glucan branches, and 1.5 – 6% chitin (Klis et al., 2006). Due to the minor proportion of chitin in the *S. cerevisiae* cell wall, interest in this component is much less than the other components, and thus much fewer publications are available; however, a review on immunomodulation by chitin (of multiple sources) has recently been published by Komi et al. (2018). A recent publication on chitin specifically derived from *S. cerevisiae* has pointed to some immunomodulatory effects in human monocytes *in vitro* (Rizzetto et al., 2016), which could generate more interest in the component in the future. This literature review will focus on β -glucans, mannan, a commonly used β -glucan- and mannan-containing *S. cerevisiae* extract called zymosan, and whole *S. cerevisiae* cells. Additional information on physical interactions with bacteria will be provided in relevant sections.

β -Glucans

β -Glucans are polymers of glucose found in cell walls of yeast, fungi, bacteria, and some cereal grains. These carbohydrates have been classified as biological response modifiers (Müller et al., 1996; Michalek et al., 1998). β -Glucans are not produced by mammals, and thus are considered foreign and recognized by cellular pattern recognition receptors including toll-like receptors (**TLR**) and dectin-1 (Brown et al., 2003; Batbayar et al., 2012). Though containing some similarities, the structure differs by source, which can alter interactions with receptors and cellular responses (Adams et al., 2008). In *S. cerevisiae*, glucose molecules are arranged in a β -1,3-linked backbone with β -1,6-linked branches. However, β -glucans from different *S. cerevisiae* strains, sources, and preparation methods can differ by length of glucose chains, degree of branching, position of branches, molecular weight, and solubility (Bohn and BeMiller, 1995; Brown and Gordon, 2003; Orlean, 2012). Unless otherwise stated, the β -glucans discussed herein are derived from *S. cerevisiae*.

Mannan

Mannan, a polymer of mannose, can contain up to 200 mannose units on the surface of *S. cerevisiae* cell walls. These sugars are linked by α -1,6-linkages that form a backbone for α -1,2- and α -1,3-linked branches with up to three mannose residues (Peat et al., 1961; Ballou et al., 1990; Herscovics and Orlean, 1993). Further information on N-linked mannan can be found in the review by Dean (1999). These mannans are linked to proteins of various composition. Because proteins associated with mannan on the yeast cell wall are highly variable and because mannan dominates the mannoprotein fraction (Klis et al., 2006; Lesage and Bussey, 2006), the

mannan section of this literature will focus on mannan and not mannoprotein. Additionally, considerably more literature is available on immune impacts of mannans from *S. cerevisiae* vs. mannoproteins derived from *S. cerevisiae*.

Zymosan

Zymosan is a crude derivative from *S. cerevisiae* cell walls commonly used in immunology. Zymosan is often referred to as a β -glucan source from *S. cerevisiae*, but this crude extract contains only 50 – 57% glucans. This product also contains approximately 17 – 22% mannans and 13 – 17% proteins (Di Carlo and Fiore, 1958). Additionally, slightly different preparations can affect its composition (Di Carlo and Fiore, 1958; Holan et al., 1980). Di Carlo and Fiore (1958) did not specify the exact structures of the glucans in zymosan, though they are assumed to be β -1,3-linked glucose units with β -1,6-branches similar to those found on *S. cerevisiae* cell walls.

β -Glucans

Immune Effects

The immune system is categorized into innate, nonspecific and adaptive, specific branches. β -Glucans have been shown to impact functions and cells of both systems. From the innate immune perspective, β -glucans from *S. cerevisiae* modulate actions of innate immune cells, including neutrophils, macrophages, and dendritic cells (**DC**). Neutrophils act first *in vivo*, accomplishing rapid pathogen killing by phagocytosis and oxidative burst, through production of reactive oxygen species (**ROS**) such as the superoxide anion (Mayadas et al., 2014). Macrophages, which mature from monocytes, also phagocytose and oxidatively kill pathogens,

and additionally produce cytokines and chemokines to recruit more immune cells (Murray and Wynn, 2011). Lastly, DC are major professional antigen-presenting cells that capture antigens, process them, and present them to adaptive immune cells. This cell type is also capable of producing cytokines (Banchereau and Steinman, 1998).

Structures of β -glucans from a variety of sources, their immunomodulatory actions *in vitro* and *in vivo*, and their receptors have been reviewed (Chan et al., 2009; Goodridge et al., 2009b; Murphy et al., 2010). β -Glucans derived from *S. cerevisiae* impact oxidative burst functions of innate immune cells. For example, particulate glucans from *S. cerevisiae* induced ROS production by human neutrophils (Williams et al., 1986), which was shown later to rely on complement receptor 3 (**CR3**) in porcine neutrophils (Baert et al., 2015). Similarly, particulate β -glucans derived from *S. cerevisiae* stimulated ROS production by porcine neutrophils and monocytes when cells were treated *in vitro*. At the greatest concentration, however, the particulate β -glucans decreased ROS, indicating potential cytotoxicity at excessive concentrations (Sonck et al., 2010). *Saccharomyces cerevisiae* cell wall extracts containing 65% or 75% β -glucans also enhanced ROS production by murine macrophages *in vitro*, indicating enhanced bactericidal activity of these innate immune cells (Walachowski et al., 2016). Particulate β -glucans at 20 $\mu\text{g/mL}$ potentiated phorbol 12-myristate 13-acetate (**PMA**)-stimulated superoxide anion production by macrophages isolated from wounds in mice (Roy et al., 2011).

Rat peritoneal and alveolar macrophages also produced greater superoxide anion when treated with particulate β -1,3-glucan particles from *S. cerevisiae* in a dose-dependent manner; however, soluble poly-(1,6)-D-glucopyranosyl-(1,3)-D-glucopyranose glucan (**PGG-glucan**) derived from the particulate β -1,3-glucan particles did not elicit this response. Instead, only

immobilized PGG-glucan could elicit superoxide anion production (Michalek et al., 1998). These results indicate that the soluble form is unable to bind to necessary β -glucan receptors to elicit an oxidative burst response unless presented in an immobilized fashion, which authors suggested could have been due to inefficient cross-linking of receptors. Sonck et al. (2010) also observed a lack of ROS production with soluble β -glucans, though this form was derived from algae, not yeast. In contrast, pre-incubation with soluble PGG-glucan stimulated oxidative burst of human blood leukocytes and polymorphonuclear cells (consisting mostly of neutrophils) when followed by the activator formyl–norleucine–leucine–phenylalanine (fNLP) or by opsonized *Staphylococcus aureus*, but did not stimulate oxidative burst when supplied alone. Concentrations of PGG-glucan as small as 0.1 $\mu\text{g/mL}$ were able to enhance this function, which plateaued between 1 – 3 $\mu\text{g/mL}$. Enhanced oxidative burst was accompanied by increased microbicidal activity of opsonized *S. aureus* by human blood leukocytes pre-incubated with soluble PGG-glucan (Wakshull et al., 1999).

β -Glucans from *S. cerevisiae* also modulate markers of inflammation within innate immune cells. A major transcription factor involved in regulating inflammatory responses is nuclear factor κ -light-chain-enhancer of activated B cells (**NF κ B**). Pro-inflammatory cytokines are produced upon activation of inflammatory transcription factors, such as NF κ B. These cytokines, which include many interleukins (**IL**; e.g., IL-1), interferons (**IFN**; e.g., IFN γ), and tumor necrosis factor α (**TNF α**), trigger multiple functions, including recruitment of immune cells and alteration of immune cell differentiation. Soluble glucan phosphate activates NF κ B in macrophages *in vitro* (Battle et al., 1998). Soluble PGG-glucan also activated the inflammatory transcription factors NF κ B and nuclear factor-IL-6 in a murine monocytic cell line (Adams et al., 1997) and an NF κ B-like factor in human polymorphonuclear cells (Wakshull et al., 1999).

Contrary to the expected relationship, though, this activation did not induce transcription of the pro-inflammatory cytokines IL-1 β , IL-6, or TNF α in macrophages (Adams et al., 1997) or in human whole blood (Wakshull et al., 1999), though stimulation with LPS as a positive control verified the methodology of the latter experiment. Both publications identified the presence of p65 but absence of p50 in the NF κ B complex stimulated by PGG-glucan, whereas both factors were present with LPS, which is likely related to the difference in NF κ B and cytokine regulation by PGG-glucan versus LPS. Adams et al. (1997) suggested that activation of other transcription factors required for pro-inflammatory cytokine production may have not occurred. More recently, McCann et al. (2005) reported that particulate β -glucans stimulated p65 NF κ B nuclear translocation and TNF α production by RAW 264.7 murine macrophages.

Walachowski et al. (2016) also demonstrated increased NF κ B activity in RAW 264.7 murine macrophages in response to *S. cerevisiae* extracts with several levels of β -glucan content. Though these extracts were defined by their relative β -glucan composition, each contained several other components. The most purified β -glucan extracts contained approximately 75% β -glucan, 3.6% proteins, and less than 1% mannans. The next most concentrated β -glucan-containing extract contained approximately 65% β -glucans, 13% glycogen, 3.4% proteins, and 3.7% mannans. The crudest extract contained approximately 15% β -glucans, 10% glucans, 9.4% glycogen, 33% proteins, and 19% mannans. Zymosan, which was previously reported to contain between 50 and 57% glucans, including β -linked glucans, and approximately 18% mannans (Di Carlo and Fiore, 1958), was also considered a crude extract. With these compositions in mind, observations made based on β -glucan content may also be influenced by other components, of which mannan is of particular interest, discussed elsewhere in this literature review. Interestingly, extracts containing mostly β -glucan (i.e., 65 or 75% β -glucan) were much weaker

inducers of NF κ B activity than the crude extract containing 15% β -glucan or zymosan. In contrast to previous reports (Adams et al., 1997; Battle et al., 1998), soluble glucan did not elicit an NF κ B response (Walachowski et al., 2016). Production of TNF α was highly correlated with induction of NF κ B activity (Walachowski et al., 2016). Again, in contrast to previous reports (Adams et al., 1997; Wakshull et al., 1999), soluble β -glucans did not induce a TNF α response (Walachowski et al., 2016).

Interestingly, when scleroglucan, a soluble, fungal (*Sclerotium rolfsii*) β -1,3-glucan with β -1,6 branches, was co-incubated with LPS, NF κ B activation and nuclear translocation were less than when LPS was provided alone (Battle et al., 1998). This latter result provides evidence for an anti-inflammatory or antagonistic propensity of soluble β -glucans during a simultaneous pro-inflammatory challenge. In this study, though, the soluble glucan phosphate derived from *S. cerevisiae* was not tested in the LPS co-incubation model. Similarly, bone marrow-derived DC incubated simultaneously with β -glucans and TLR ligands resulted in lesser IL-12p70 production compared to incubation with TLR ligands alone. In contrast, though, TNF α production was greater with co-stimulation, indicating that responses to simultaneous pro-inflammatory challenges may differ between cytokines. These results were observed with both yeast-derived particulate β -glucan and the fungal soluble β -glucan, scleroglucan (Huang et al., 2009). In addition to macrophages and DC, neutrophils were also activated by soluble *S. cerevisiae* β -glucans as demonstrated by degranulation after 15 min and increasing elastase levels over time (Engstad et al., 2002).

β -Glucans also increase other functions of innate immune cells. Particulate β -glucans enhanced maturation of bone-marrow derived DC, demonstrated by upregulated costimulatory molecules cluster of differentiation (CD)40, CD80, and CD86, and major histocompatibility

complex-II (Qi et al., 2011). Upregulation of these proteins could lead to improved adaptive T cell response (Banchereau and Steinman, 1998). Culture supernatant from bone marrow-derived macrophages treated with *S. cerevisiae* extracts containing 65 or 75% β -glucans resulted in increased neutrophil recruitment, though a crude extract containing 15% β -glucans and zymosan more dramatically increased this activity. Consistent with these results, both the 15% β -glucan extract and zymosan strongly increased secretion of chemokine (C-X-C motif) ligand (**CXCL**) 1 and 2, which are major neutrophil-recruiting chemokines. Extracts with greater β -glucan content increased secretion of CXCL2, but to a lesser extent compared to the crude extract and zymosan; however, CXCL1 was not affected by these purer β -glucan extracts (Walachowski et al., 2016).

β -Glucans also modulate cytokine production by both macrophages and DC. Particulate β -glucans derived from *S. cerevisiae* stimulated *in vitro* TNF α production by human monocytes (Abel and Czop, 1992), RAW 264.7 murine macrophages (Berven et al., 2015; Walachowski et al., 2016), murine macrophages from wounds (Roy et al., 2011), rat peritoneal and alveolar macrophages (Michalek et al., 1998), and murine bone marrow-derived DC (Huang et al., 2009; Qi et al., 2011). Soluble β -glucans also stimulate TNF α production by innate immune cells *in vitro* (Doita et al., 1991; Berven et al., 2015), but results from other soluble preparations have not been not consistent (Walachowski et al., 2016). Soluble, aminated β -glucans also stimulated IL-1 production by human monocytes (Doita et al., 1991); however, soluble PGG-glucan did not stimulate IL-1 α , IL-1 β , IL-6, IL-8, or TNF α production in human whole blood (Wakshull et al., 1999). In contrast, particulate glucan from *S. cerevisiae* and zymosan both upregulated IL-1 β mRNA abundance in human primary macrophages via a dectin-1-dependent pathway. In contrast to LPS stimulation, addition of β -glucans also led to IL-1 β secretion by these macrophages,

demonstrating the efficacy of β -glucans as stimulants of cytokine responses (Kankkunen et al., 2010).

In human whole blood samples, soluble β -glucans derived from *S. cerevisiae* also induced significant release of pro-inflammatory cytokine IL-8 and only minor amounts of TNF α , IL-6, and the anti-inflammatory cytokine IL-10. Soluble β -glucans and LPS synergistically increased IL-8 and IL-10, but not TNF α and IL-6. Pre-treatment with β -glucans demonstrated a priming effect by increasing LPS-induced TNF α , IL-6, and IL-8 secretion (Engstad et al., 2002). Authors suggested that differential cytokine induction with β -glucan alone and with LPS could be related to clustering and cross-linking of CR3.

Phagocytosis of foreign particles is often involved in the process of pathogen recognition and immune response. Monocytes and macrophages ingest particulate yeast β -glucans *in vitro* (Williams et al., 1986; Hong et al., 2004) and degrade them into smaller bioactive β -glucan fragments that are then secreted into culture medium (Hong et al., 2004). Neutrophils were also able to phagocytose *S. cerevisiae* particulate β -glucan particles (Williams et al., 1986; Ross et al., 1987). However, phagocytosis of *Streptococcus aureus* HG001 was not affected by β -glucan supplementation (Walachowski et al., 2016). Additionally, internalization of soluble vs. particulate β -glucan compounds by macrophages and DC is not consistent in literature. Human and murine macrophages were able to internalize soluble glucan phosphate derived from *S. cerevisiae* (Müller et al., 1996). In contrast, Qi et al. (2011) found that particulate, but not soluble, β -glucans were phagocytosed by murine macrophages and DC, even though both forms were derived from the same strain of *S. cerevisiae*. Berven et al. (2015) found similar differences between soluble and particulate β -glucans using RAW 264.7 murine macrophages. The differences observed between different soluble β -glucans could be due to structural differences,

such as the presence of phosphate groups, used by Müller et al. (1996) but not Qi et al. (2011) or Berven et al. (2015).

McCann et al. (2005) also observed that phagocytosis of β -glucans by RAW 264.7 macrophages was severely reduced in the presence of an antagonist of actin-mediated internalization; however, translocation of NF κ B and production of TNF α were not affected by this antagonist. Thus, phagocytosis may not be required for β -glucans to elicit all immunomodulatory effects. Functions can also differ by receptor recognition. Phagocytosis of particulate β -glucans by macrophages was completely dependent on dectin-1, whereas phagocytosis was only partially inhibited in DC from dectin-1-deficient mice. On the other hand, DC from mice deficient in CD11b (part of CR3) were not affected (Qi et al., 2011), whereas human neutrophils and monocytes required CR3 for β -glucan internalization (Ross et al., 1987). Difference in binding affinity of different β -glucan structures could also contribute to differences in observed effects. Alveolar macrophages collected from TLR2-deficient mice were able to internalize particulate β -glucans similarly to those from wild-type mice, indicating that TLR2 was not required (McCann et al., 2005). A helical structure can be formed by glucose polymers with repeating β -1,3-linkages involving at least 7 glucose units, which could more strongly interact with dectin-1 (Adams et al., 2008). Branches formed by β -1,6-linkages can also improve dectin-1 interactions, but branches too close together could decrease interaction potential (Adams et al., 2008; Sonck et al., 2010). Thus, specific structures of β -1,3- and β -1,6-linked glucose units can affect efficacy of dectin-1-dependent pathway activation for inflammatory and immune responses.

β -Glucans also influence lymphocytes. In adaptive immunity, B and T lymphocytes recognize specific antigens and form memory based on these antigens. B lymphocytes form

immunoglobulins (**Ig**), or antibodies, in response to specific antigen recognition by receptors (LeBien and Tedder, 2008). T lymphocytes can be grouped into CD4⁺ T cells and CD8⁺ T cells that can both produce cytokines and chemokines. CD4⁺ T cells can differentiate into T helper (**Th**) cells with multiple immune functions based on different cytokine environments (Abbas et al., 1996), whereas CD8⁺ T cells elicit cytotoxicity (Harty et al., 2000). Considered part of innate immunity, natural killer (**NK**) cells are effector lymphocytes that can elicit cytotoxic effects, produce cytokines, and interact with T cells (Vivier et al., 2008).

Cytokine production and proliferation of mixtures of innate and adaptive immune cells are influenced by β -glucans. Sonck et al. (2010) isolated porcine peripheral blood mononuclear cells (**PBMC**), which include monocytes, B cells, T cells, and NK cells, and tested various particulate β -glucan sources *in vitro*. Glucan from *S. cerevisiae* dose-dependently decreased IL-1 β production but increased production of several other cytokines. In fact, all β -glucans tested, including from *S. cerevisiae*, stimulated production of pro-inflammatory cytokines IL-4, IL-6, IL-8, IL-12, and IFN γ , and glucan from *S. cerevisiae* stimulated greater TNF α release than 1 μ g/mL LPS. Xiao et al. (2004) also observed increased IFN γ production by PBMC isolated from pigs infected with porcine reproductive and respiratory syndrome virus in response to soluble yeast β -glucan, but not two insoluble forms. Previous literature reported growth suppression of porcine reproductive and respiratory syndrome virus by IFN γ in the porcine reproductive and respiratory syndrome virus-susceptible monkey kidney MARC-145 cell line (Rowland et al., 2001), and thus β -glucan treatment may improve host response to the virus. Proliferation of CD8⁺ T cells co-cultured with DC was not stimulated with 100 μ g/mL of particulate β -glucans alone but was stimulated with a combination of particulate β -glucans and ovalbumin (Qi et al., 2011). This T cell response was mediated by the co-cultured DC, as T cell proliferation was not

affected by β -glucans alone. To elicit this effect on T cells, DC required the dectin-1 receptor for β -glucan recognition (Qi et al., 2011). Only a small subset of splenic T cells expressed dectin-1 in a previous report, at much lesser levels than DC (Taylor et al., 2002). These results suggest that, while T cells may be able to recognize β -glucans, β -glucan-stimulated proliferation of T cells most likely occurs via DC. Qi et al. (2002) also found that particulate β -glucans enhanced Th1 priming of ovalbumin-specific naïve CD4⁺ T cells co-cultured with DC; however, Th17 differentiation was not stimulated by β -glucans.

Additionally, changes in cytokine production could have been related to changes in growth, as *S. cerevisiae*-derived glucan sources stimulated proliferation of PBMC at low concentrations (i.e., 50 μ g/mL). However, glucan from *S. cerevisiae* may have elicited cytotoxic effects at the greatest concentrations, as proliferation was greatly decreased at the 200 and 800 μ g/mL doses. In contrast, zymosan further stimulated proliferation at these two doses, suggesting differences in proliferative effects of this crude yeast extract compared to pure glucan (Sonck et al., 2010). These results therefore suggest that proliferative effects of β -glucans on PBMC, which include both innate and adaptive immune cells, are sensitive to the particular compound and dose. These effects could contribute to increases in cytokine production observed with β -glucan treatments, potentially resulting in enhanced immune response of the host.

Intestinal effects

As β -glucans can be orally administered, effects on intestinal epithelial cells are of great interest. Intestinal epithelial cells maintain a barrier between the external gut environment and the body, separating ingested foreign bodies and microbes from lymphatic and blood circulation. This barrier prevents entry of unwanted pathogens via maintenance of tight junction proteins

while allowing intracellular and some extracellular passage of nutrients (Peterson and Artis, 2014). In fact, the pathogenesis of enteropathogenic bacterial diseases often involves disruption of this tight junction barrier and changes in inflammation, as reviewed by Berkes et al. (2003). However, this barrier is not only physical, but also immunological, housing an ornate system of innate and adaptive immune cells. This gut-associated lymphoid tissue (**GALT**) contains Peyer's patches with multiple innate and adaptive immune cells that protect the intestinal environment and prevent pathogenic spread into circulation (Berg, 1996).

Orally administered β -glucans from *S. cerevisiae* are capable of influencing intestinal epithelial cells and GALT cells. Rice et al. (2005) observed increased expression of dectin-1 in macrophages and TLR2 in DC, but no changes in TLR4 expression in GALT cells isolated from murine Peyer's patches in response to oral administration of soluble glucan phosphate. This result demonstrated that GALT immune cells respond to soluble glucans by increasing abundance of β -glucan receptors, which could lead to increased response or awareness of these intestinal immune cells to β -glucans and other microbes or ligands that interact with these receptors, particularly TLR2. Although intestinal epithelial cells did not express dectin-1, but approximately 10% of these cells were able to internalize fluorescently-labeled glucan phosphate. Though authors were not able to identify this cell type, it is possible that they were microfold (M) cells, which can transport large molecules from the intestinal lumen to the GALT (Suzuki et al., 1989; Volman et al., 2008). In contrast, Cohen-Kedar et al. (2014) found that human epithelial cells from both ileum and colon samples, in addition to the human intestinal epithelial cell lines HT-29 and SW480, expressed dectin-1. Similarly, the Caco-2 human intestinal epithelial cell line expressed dectin-1, TLR1, and TLR6. When these Caco-2 cells were supplemented with glucan derived from *S. cerevisiae* and butyric acid, glucan increased IL-8

secretion in a dose-dependent manner (Saegusa et al., 2004). Butyric acid is present in the gut as metabolite from the intestinal microbiota, and thus comes into contact with intestinal epithelial cells *in vivo* (Hespell et al., 1987; Barcenilla et al., 2000).

Oral β -glucan administration influences intestinal barrier function against pathogenic infection. In broiler chickens challenged with *Salmonella enterica* serovar Typhimurium, the bacterial challenge decreased mRNA expression of several intestinal tight junction proteins - including claudin-1, claudin-4, and occludin - compared to the negative control group. Oral supplementation with β -glucans ameliorated this suppression by increasing mRNA expression of claudin-1 and occludin compared to the infected group without supplementation (Shao et al., 2013). *Salmonella enterica* infection decreased villus height, the ratio of villus height to crypt depth, and goblet cell number compared to the non-challenged control group, but oral β -glucan supplementation was also able to increase these three morphological characteristics compared to the challenged, non-supplemented group. Oral β -glucans also elicited adaptive immune responses within the intestine by increasing secretory IgA-expressing intestinal cells and secretory immunoglobulin A content in challenged animals (Shao et al., 2013). Mice supplemented with oral β -glucans also showed altered adaptive immune responses within the intestine, characterized by increased total number of intraepithelial lymphocytes, increased CD4⁺ and CD8⁺ T cells, and greater IFN γ release (Tsukada et al., 2003).

Whole animal effects

Similar to *in vitro* results, β -Glucans can modulate whole animal immunity. Orally-administered particulate β -glucans from *S. cerevisiae* are taken up by gastrointestinal macrophages and transported to the spleen, lymph nodes, and bone marrow. Macrophages in

bone marrow degrade these larger β -1,3 molecules into smaller, biologically active fragments, which can then bind to mature granulocytes in the bone marrow via CR3 (Hong et al., 2004). Orally administered soluble β -glucan phosphate from *S. cerevisiae* interacts with intestinal epithelial cells and GALT cells and is then transported through the systemic circulation (Rice et al., 2005). Immunomodulatory actions of dietary β -glucans from a variety of sources has been reviewed elsewhere (Volman et al., 2008; Samuelson et al., 2014). Immunomodulatory effects of other routes of administration of β -glucans from other sources also been reviewed elsewhere (Chan et al., 2009; Goodridge et al., 2009b; Murphy et al., 2010). Further pharmaceutical actions of soluble glucan phosphate from *S. cerevisiae* administered intravenously, intraperitoneally, and intramuscularly are reviewed by Williams and Browder (1994).

In vivo administration of β -glucans from *S. cerevisiae* can impact systemic cytokine profiles. Serum anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines IL-1 β , IL-2, IL-4, IFN γ , GM-CSF, and TNF α were not stimulated by oral administration of soluble β -glucans in mice; however, both pro-inflammatory cytokines IL-6 and IL-12 were elevated in serum 8 h after administration. Oral administration of particulate β -glucans also increased IL-6 concentrations in serum. These results suggest that the systemic inflammatory response to *S. cerevisiae*-derived β -glucans is modest and specific (Rice 2005). Intraperitoneal administration of soluble glucan phosphate potentiated LPS-induced IFN γ , IL-12, and IL-18 production in serum of mice. These systemic cytokine changes were similarly reflected in the spleen (Sherwood et al., 2001). Dietary *S. cerevisiae*-derived β -glucan also down-regulated gene expression of pro-inflammatory cytokines IL-1 α , IL-17A, and TNF α and anti-inflammatory IL-10 in colonic tissue, but not ileal tissue, of pigs (Sweeney et al., 2012).

Similar to *in vitro* observations, oral administration of yeast β -glucans can also impact innate immune cell responses *in vivo*. Intraperitoneal injection of particulate *S. cerevisiae*-derived β -glucans increased the proportions of neutrophils and eosinophils while decreasing the proportion of mast cells within the intraperitoneal cavity. Peritoneal macrophages also demonstrated altered morphology. When these macrophages were isolated and challenged with LPS or PMA *in vitro*, macrophages from β -glucan-injected mice had greater LPS-stimulated nitrous oxide production and greater PMA-stimulated superoxide production (Cleary et al., 1999). This observation demonstrates a priming effect of β -glucans *in vivo*. Splenic NK cells isolated from mice injected with intraperitoneal β -glucans also demonstrated greater cytotoxicity of YAC-1 target cells *in vitro* (Pelizon et al., 2005).

β -Glucans also influence populations and phenotypes of potential pathogens *in vivo*. Oral β -glucan administration decreased *Salmonella enterica* serovar Typhimurium colonization in the cecum and liver in *Salmonella*-challenged broiler chickens (Shao et al., 2013). Similarly, oral insoluble β -glucans reduced the Enterobacteriaceae population without affecting commensal *Lactobacilli* and *Bifidobacteria* populations in the ileum and colon of pigs not subjected to any additional microbial challenge (Sweeney et al., 2012). Intraperitoneal injection of β -glucans primed spleen cells to produce greater amounts of IL-12 and TNF α in response to a *Staphylococcus aureus* challenge *in vitro*. These pro-inflammatory cytokines were not induced without the challenge, though, suggesting that the β -glucans specifically primed the spleen cells and did not initiate an inflammatory response alone (Pelizon et al., 2005). Intramuscular soluble PGG-glucan administration reduced antibiotic-resistant *Staphylococcus aureus* in blood of mice after an intraperitoneal challenge of the bacteria. This reduction was accompanied by increased blood monocyte count, neutrophil count, and neutrophil oxidative burst without any changes in

serum pro-inflammatory TNF α or IL-1 β concentrations. When combined with an antibiotic, intramuscular PGG-glucan administration also improved survival rates (Liang et al., 1998). Similarly, oral gavage of soluble glucan phosphate 1 day prior to intravenous challenge with *Staphylococcus aureus* or *Candida albicans* increased long-term survival of mice (Rice et al., 2005). Intravenous injection of soluble β -glucan also improved survival in a systemic *Staphylococcus aureus* infection model, which was accompanied by decreased pathology in the kidneys (Marakalala et al., 2013). Additionally, pre- or post-treatment with intraperitoneal glucan phosphate improved survival rates in a murine polymicrobial sepsis model (Williams et al., 1999). In the same study, glucan phosphate pre-treatment or post-treatment also decreased NF κ B and NF-IL-6 nuclear binding activity and TNF α and IL-6 mRNA levels in lung and liver tissue. The inhibition of pro-inflammatory transcription factors and cytokines demonstrated an anti-inflammatory capacity of soluble β -glucans *in vivo* that may contribute to improved survival in sepsis challenges. Oppositely, pro-inflammatory effects of β -glucans in wounds and less intense microbial challenges may help to improve recovery.

Anti-tumor effects have also been observed with β -glucans derived from *S. cerevisiae*. Mice challenged with tumors had lesser tumor diameters when orally supplemented with particulate β -glucans compared to the control treatment (Hong et al., 2004; Qi et al., 2011). Oral administration of particulate β -glucans also increased macrophage and CD8a+CD11c+ DC infiltration, large amounts of IL-12 and TNF α , and low amounts of IL-6 and IL-10 within the tumor microenvironment (Qi et al., 2011). Meanwhile, mRNA levels of the anti-inflammatory cytokine transforming growth factor- β (**TGF- β**) were decreased by particulate β -glucan administration. T cells in the tumor milieu were also affected by particulate β -glucans. Specifically, CD4+ T cell differentiation into Th17 cells was decreased whereas CD8+ T cell

differentiation into IFN γ -producing effector cells was increased. These anti-tumor responses, except for increased macrophage infiltration, required dectin-1 expression (Qi et al., 2011). When oral particulate β -glucan (Hong et al., 2004) and soluble β -glucan (Qi et al., 2011) were administered along with intravenous antitumor monoclonal antibodies, additional anti-tumor effects were observed. Tumor diameter decreased and long-term survival increased with simultaneous particulate β -glucan and antibody administration, which depended on CR3 expression (Hong et al., 2004). While soluble PGG-glucan did not affect T cells *in vitro*, this compound induced neutrophil killing of opsonized tumor cells and prevented tumor progression by priming neutrophils and synergistically acting with complement-activated monoclonal antibodies via CR3. Therefore, particulate β -glucans altered the tumor microenvironment by promoting anti-tumor effects *in vitro* and *in vivo*, while soluble β -glucans elicited anti-tumor effects via neutrophils and antitumor antibodies only *in vivo*.

Mannan

Physical interactions

As mentioned previously, adherence of bacteria to epithelial cells, particularly of the gut, is considered the first step in bacterial infection (Firon et al., 1983; Berkes et al., 2003; Tiago et al., 2012). The adherence of *Escherichia coli*, which contain type 1 fimbriae and mannose-binding lectin, to mammalian cells is inhibited by D-mannose (Eshdat et al., 1978; Firon et al., 1983). D-mannose also inhibited adherence of *Salmonella* Typhimurium, another pathogen containing type 1 fimbriae, to small intestinal enterocytes from rats (Lindquist et al., 1987). Similarly, D-mannose adhered to *E. coli* and thus prevent *E. coli* from binding to intestinal epithelial cells (Duguid and Gillies, 1957). Both yeast mannan and D-mannose prevented

Shigella flexneri-induced hemagglutination of human and guinea pig red blood cells (Duguid and Gillies, 1957). Firon et al. (1983) also demonstrated the adherence of branched mannan oligosaccharides containing α -1,3 and α -1,6 linkages to *E. coli* *in vitro*. More recently, a mannan oligosaccharide product from *S. cerevisiae* was shown to agglutinate several strains of *E. coli* and *Salmonella* *in vitro*. Addition of mannose inhibited agglutination of these bacteria (Spring et al., 2000).

Immune effects

Fewer publications are available on *in vitro* effects of *S. cerevisiae*-derived mannan compared to β -glucans, but mannan has still been shown to modulate responses of innate immune cells. Mannan derived from *S. cerevisiae* increased TNF α production by human monocytes in a dose-dependent manner. This pro-inflammatory cytokine production depended on receptor availability of CD14 and TLR4, but not TLR2. Though LPS also induced TNF α production, simultaneous treatment with mannan and LPS did not result in a synergistic increase in TNF α (Tada et al., 2002). Lamkanfi et al. (2009) also found that mannan from *S. cerevisiae* stimulated TNF α and IL-1 β production by bone marrow-derived macrophages, but in this study, IL-1 β production also required the addition of ATP. In contrast, porcine alveolar macrophages incubated *in vitro* with a mannan oligosaccharide product derived from *S. cerevisiae* secreted lesser amounts of TNF α and greater amounts of the anti-inflammatory cytokine, IL-10, than negative control macrophages (Che et al., 2012).

Similar to observations with monocytes and macrophages, *S. cerevisiae*-derived mannan induced mRNA expression of pro-inflammatory cytokines IL-1 β , IL-4, IL-6, IL-12p40, IFN γ , TNF α , and GM-CSF in bone marrow-derived DC. This *S. cerevisiae*-derived mannan also

enhanced maturation of murine bone marrow-derived DC as demonstrated by increased expression of the costimulatory molecules CD40, CD80, and CD86. These DC were then able to enhance proliferation of allogenic murine T cells and peptide-specific CD8⁺ murine T cells (Sheng et al., 2006). Mannan was also able to block transmission of human immunodeficiency virus from macrophages to T cells in a macrophage-T cell co-culture by inhibiting binding of the virus to the macrophage mannose receptor (Nguyen and Hildreth, 2003).

Intestinal effects

An intensive literature search identified a large gap on the topic of *in vitro* effects of *S. cerevisiae*-derived mannan on intestinal cells as only one publication was identified. In that publication, mannan derived from *S. cerevisiae* did not affect IL-8 secretion by human intestinal epithelial Caco-2 cells when supplied at concentrations of 10, 100, or 1000 µg/mL for 7 or 24 h (Saegusa et al., 2004). A study using *ex vivo* and *in vivo* analysis provided further characterization of intestinal effects, but with phosphorylated mannans derived from *S. cerevisiae* instead (Davis et al., 2004). Pigs were supplemented with phosphorylated mannans after weaning. Macrophages isolated from the lamina propria of pigs supplemented with mannans were able to phagocytose more sheep red blood cells than those from control pigs. Additionally, mannan-supplemented pigs had greater proportions of CD14⁺ lamina propria leukocytes on d 19 after weaning, which could indicate a transient increase in lamina propria macrophages with mannan supplementation. Mannan-supplemented pigs also had lesser proportions of CD14⁺-major histocompatibility class II (MHCII)⁺ lamina propria leukocytes on d 21 after weaning, potentially indicative of accelerated maturation of lamina propria macrophage function. The ratio of CD3⁺CD4⁺ to CD3⁺CD8⁺ T lymphocytes in the lamina propria was also

lesser in mannan-supplemented pigs than control pigs 21 d after weaning, indicating a greater proportion of CD8⁺ cytotoxic T lymphocytes at this time point.

Whole animal effects

Relative to β -glucans, less published research is available on *in vivo* *S. cerevisiae*-derived mannan administration. However, available publications have shown that mannan can modulate whole animal immunity. For example, intraperitoneal injection of *S. cerevisiae*-derived mannan induced fever in rats. The initial onset of fever was accompanied by increased serum concentrations of TNF α , but not during the final stage of fever (Ataoglu et al., 2000). This pro-inflammatory cytokine is known to act as a pyrogen (Dinarello et al., 1986; Mabika and Laburn, 1999). In contrast, serum concentrations of IL-1 β and IFN γ were unaffected by mannan administration (Ataoglu et al., 2000). Interestingly, mannan from *C. albicans* did not elicit any of these effects in this study (Ataoglu et al., 2000), but Garner and Hudson (1996) observed elevated serum TNF α concentrations in mice injected with *C. albicans*-derived mannans. Mannans also modulate innate immune cells and adaptive immune response *in vivo*. Intraperitoneal injection of yeast mannan increased neutrophil abundance in the intraperitoneal cavity 6 h after injection, though this response was decreased 24 h after injection (Morikawa et al., 1984). Pigs supplemented with dietary phosphorylated mannans derived from *S. cerevisiae* had greater lymphocyte proportions but lesser neutrophil proportions in blood leukocytes compared to non-supplemented control pigs (Davis et al., 2004). Intraperitoneal or intravenous administration of *S. cerevisiae*-derived mannan enhanced maturation of lymph node and splenic DC, consistent with the *in vitro* experiments of this study (Sheng et al., 2006). Protein carrier-conjugated mannan from *S. cerevisiae* generated a specific adaptive immune response when

injected in mice, stimulating increased concentrations of anti-mannan IgG and IgM in serum (Paulovičová et al., 2005).

Mannan from *S. cerevisiae* can elicit anti-tumor effects *in vivo*. Transfer of regional lymph nodes from mice injected with yeast mannan alone, or yeast mannan and tumor cells, suppressed tumor growth in recipient mice (Suzuki et al., 1971). Additionally, partially degraded, lesser molecular weight forms of these mannans elicited lesser anti-tumor capacity, demonstrating the importance of molecular weight for this function of mannan (Matsumoto et al., 1980). Intraperitoneal injection of an acidic fraction of *S. cerevisiae* mannan also enhanced survival rates of mice challenged with tumors, and even resulted in 100% survival when injected 5 days pre- and post-tumor challenge (Hashimoto et al., 1983).

While few studies have been published on *S. cerevisiae*-derived mannan alone, much more research has been performed using *S. cerevisiae*-derived mannan oligosaccharide products, as reviewed by Spring et al. (2015). One focus of this research has been on bacterial interactions, as mannans and mannose bind several bacterial species (Firon et al., 1983; Spring et al., 2000). In a study by Fernandez et al. (2000), mannan oligosaccharide was fed to hens, then cecal contents were collected and fed to broiler chicks in mash. Cecal contents from mannan oligosaccharide-fed hens greatly reduced *Salmonella enterica* serovar *enteritidis* in broiler chicks. In contrast, cecal contents from D-mannose-fed hens elicited only minor effects in chicks (Fernandez et al., 2000). Similarly, in chicks challenged with orally-administered *Salmonella* Typhimurium 29E, chicks fed mannan oligosaccharides had lesser *Salmonella* populations in the ceca than the non-supplemented controls. In fact, *S. Typhimurium* 29E counts were reduced by approximately 25-fold with mannan oligosaccharide supplementation. *Coliform* populations also tended to be lesser in supplemented birds, but populations of *Lactobacilli*, *Enterococci*, and

anaerobes were not altered (Spring et al., 2000). As a component of the mannan polymer, mannose administered to broiler chickens in drinking water decreased the number of chickens positive for *Salmonella* Typhimurium after challenge with the bacteria by almost 75% compared to control chickens that did not receive mannose. Mannose also decreased *Salmonella* Typhimurium cell count in cecal contents (Oyofe et al., 1989).

Mannan oligosaccharide products have also been evaluated for systemic and innate immune responses. For example, mannan oligosaccharide fed to European sea bass increased the phagocytic index of head kidney leukocytes (Torrecillas et al., 2011). Che et al. (2012) found that serum concentrations of TNF α were unaffected but that serum IL-10 tended to increase with mannan oligosaccharide supplementation to pigs. Alveolar macrophages isolated from mannan oligosaccharide-supplemented pigs secreted lesser TNF α but greater IL-10 when stimulated with LPS *ex vivo*. Similar responses were observed when mannan oligosaccharide was supplemented *in vitro* to alveolar macrophages isolated from non-supplemented pigs.

In chicks and chickens, mannan oligosaccharide supplementation has also been shown to influence adaptive immunity. When chicks were simultaneously given an anticoccidial vaccine and a Newcastle disease virus vaccine, dietary mannan oligosaccharide enhanced local adaptive immune response, demonstrated by increased secretory IgA concentrations in the trachea and intestines. Mannan oligosaccharide also enhanced humoral adaptive immune response, demonstrated by greater serum antibody titers against Newcastle disease virus, and cell-mediated immunity, demonstrated by greater basophilic hypersensitivity (a measure of T cell response). These responses were accompanied by a decrease in fecal parasite count (Gómez-Verduzco et al., 2009). Shashidhara and Devegowda (2003) also found that dietary mannan oligosaccharide

supplementation enhanced infectious bursal disease virus antibody titers after vaccination in broiler chickens.

Zymosan

Immune effects

Largely composed of β -glucans (Di Carlo and Fiore, 1958), it's not surprising that zymosan has effects in innate immune cells similar to β -glucans. For example, zymosan stimulated migratory functions, myeloperoxidase production, and oxidative burst in human neutrophils (Wright et al., 1981; Williams et al., 1986; Ross et al., 1987). Similarly, zymosan induced ROS production by primary human macrophages (Kelly et al., 2010), peritoneal murine macrophages (Taylor et al., 2007), and RAW 264.7 murine macrophages in a TLR2- and MyD88-independent manner (Gantner et al., 2003). Blocking recognition of zymosan with a non-yeast soluble β -glucan source inhibited this induction, demonstrating the dependence of β -glucan recognition for this zymosan-induced function in macrophages (Gantner et al., 2003). Zymosan depleted of TLR ligands induced similar ROS production, indicating again that TLR activation was not required for inducing this oxidative burst function in macrophages (Gantner et al., 2003; Goodridge et al., 2009a). Macrophages (Sung et al., 1983) and neutrophils (Williams et al., 1986) can also phagocytose zymosan.

In addition to oxidative functions, zymosan can also activate NF κ B in macrophages (Young et al., 2001; Gantner et al., 2003; Goodridge et al., 2009a). Dectin-1 signals from zymosan stimulation were sufficient to activate NF κ B and induce TNF α production by bone marrow-derived DC but not macrophages (Goodridge et al., 2009a). The enhancement of NF κ B activation by zymosan, which contains TLR ligands, was shown to rely on TLR2 expression in

human embryonic kidney (HEK293) cells. Dectin-1 expression synergistically enhanced this zymosan-induced TLR2 response but did not initiate NF κ B activation on its own; however, these results were generated from human embryonic kidney cells, which do not endogenously express dectin-1 and were thus transiently transfected with dectin-1 (Gantner et al., 2003). On the other hand, macrophages and DC naturally expressing dectin-1 are clearly able to recognize the compound, whereas non-transfected HEK293 cells did not recognize zymosan (Gantner et al., 2003). Thus, cells with TLR but without dectin-1 are still able to mount an inflammatory response to zymosan stimulation but are not as responsive as cells that express both receptors. Zymosan depleted of TLR ligands was still able to induce NF κ B activation and TNF α production in bone marrow-derived DC (Goodridge et al., 2009a) but not bone marrow-derived or RAW 264.7 macrophages (Gantner et al., 2003; Goodridge et al., 2009a). These results suggest that macrophages require activated TLR and not just dectin-1 to respond to zymosan, whereas DC can respond to zymosan with dectin-1 alone, without TLR activation.

Consistent with NF κ B activation, zymosan also stimulates production of IL-12 and TNF α by murine macrophages (Young et al., 2001; Brown et al., 2003; Gantner et al., 2003). The absence of MyD88 and TLR2 abrogated this zymosan-stimulated pro-inflammatory cytokine production (Brown et al., 2003; Gantner et al., 2003). Zymosan also triggered TNF α and IL-1 β production by murine bone marrow-derived macrophages, but IL-1 β production also required the presence of ATP (Lamkanfi et al., 2009). Similarly, Kelly et al. (2010) found that zymosan stimulated IL-6, IL-8, and TNF α production by primary human macrophages. Taylor et al. (2007) found that zymosan stimulated TNF α production by murine peritoneal macrophages, but only when opsonized.

Interestingly, zymosan does not always induce a pro-inflammatory response. In fact, zymosan has been shown to induce production of the anti-inflammatory cytokine, IL-10, by primary human macrophages (Kelly et al., 2010). Zymosan also triggers production and secretion of IL-10 by murine and human DC isolated from blood, the spleen, and bone marrow (Rogers et al., 2005; Dillon et al., 2006; Karumuthil-Melethil et al., 2008). Zymosan can also induce secretion of the anti-inflammatory cytokine TGF- β 1 when incubated with human blood DC, murine splenic DC, and murine bone marrow-derived DC (Dillon et al., 2006; Karumuthil-Melethil et al., 2008); this was also accompanied by increased secretion of pro-inflammatory cytokines IL-2 and TNF α (Rogers et al., 2005; Karumuthil-Melethil et al., 2008), but little IL-6 and IL-12p70 (Dillon et al., 2006). Because IL-6 stimulates additional pro-inflammatory cytokine production, the lack of IL-6 production with zymosan, in addition to anti-inflammatory cytokine production, suggests zymosan can elicit anti-inflammatory responses of DC *in vitro* (Dillon et al., 2006; Karumuthil-Melethil et al., 2008). When DC and T cells were cultured together, zymosan-treated DC reduced antigen-specific T cell proliferation, which resulted from abundant IL-10 production and a lack of IL-6 production by zymosan-treated DC (Dillon et al., 2006).

Intestinal effects

As with immune cells derived from other bodily sources, zymosan triggered oxidative burst *in vitro* in ileal and colonic macrophages isolated from patients with inflammatory bowel disease. Oxidative burst of these macrophages was induced to a lesser extent in healthy patients (Mahida et al., 1989). Intraperitoneal injection of a very high dose of zymosan (750 mg/kg) increased intestinal tissue concentrations of TNF α , but also IL-10. Zymosan also induced

production of the oxidative enzyme myeloperoxidase in intestinal tissues. These observations were accompanied by histological evidence of intestinal injury, indicating that zymosan at this concentration promotes intestinal barrier dysfunction (Li et al., 2015). Similarly, intraperitoneal injection of zymosan (500 mg/kg) fragmented immunofluorescent staining of intestinal tight junction proteins, occludin and zonula occludens-1 (ZO-1), in mice. In accordance with this decreased tight junction protein staining, plasma-to-lumen ratio of fluorescent dextran, a measure of intestinal barrier permeability, was increased in zymosan-treated mice (Cuzzocrea et al., 2000).

In intestinal cell cultures, zymosan modulates cytokine production. For example, zymosan induced pro-inflammatory cytokine IL-8 and C-C motif chemokine ligand 2 (CCL2) production in human intestinal epithelial cell lines HT-29 and SW480 (Cohen-Kedar et al., 2014). Zymosan also induced IL-8 secretion in Caco-2 cells but only in the presence of butyric acid, suggesting that butyric acid may have influenced receptor recognition or downstream signaling to elicit a response to zymosan (Saegusa et al., 2004).

Whole animal effects

Similar to *in vitro* observations, zymosan can enhance anti-inflammatory or immunoregulatory processes *in vivo*. Dillon et al. (2006) injected mice intraperitoneally or intravenously with both zymosan and ovalbumin and found that ovalbumin-specific T cells secreted little or no Th1- or Th2-related cytokines. Instead, these cells secreted large amounts of IL-10. When LPS was also added to the injection, ovalbumin responses were suppressed via IL-10, TGF- β , and absence of IL-6. Zymosan also triggered a systemic TGF- β response when injected alone, which was not observed with injections of LPS alone. Spleen cells isolated from

these mice showed that this TGF- β release was most likely from splenic macrophages.

Karumuthil-Melethil et al. (2008) also demonstrated enhanced immunosuppression following zymosan treatment. Suppressor efficiency of splenic regulatory T cells was greater in zymosan-treated mice than control mice. Results from these studies suggest injection of zymosan *in vivo* promotes immune tolerance. However, when zymosan was co-administered with an HIV-1 DNA vaccine in mice, it enhanced HIV-1-specific IgG production. Zymosan co-administration *in vivo* also enhanced lysis of an HIV-1 peptide by cytotoxic T lymphocytes isolated from the spleen *in vitro* (Ara et al., 2001).

Live yeast cells

Physical interactions

As mentioned previously, adherence of bacteria to epithelial cells, particularly of the gut, is considered the first step in bacterial infection (Firon et al., 1983; Berkes et al., 2003; Tiago et al., 2012). Yeast cells can adhere to and flocculate adjacent yeast cells through binding of lectin-like proteins called flocculins to mannose residues on other cells (Teunissen et al., 1995; Kobayashi et al., 1998; Tiago et al., 2012). However, *S. cerevisiae* can also flocculate bacterial species, especially those with type 1 fimbriae. This function is of high interest for pathogen control. *Saccharomyces cerevisiae* has been shown to adhere to enterobacteria *E. coli*, *Klebsiella pneumoniae*, and *Salmonella* Typhimurium (Firon et al., 1983) and to *E. coli* JM101, *Pediococcus damnosus* 12A7, and *Staphylococcus aureus* B41 (Peng et al., 2001), but not to several other bacterial species (Peng et al., 2001). *Saccharomyces cerevisiae* also adhered to over 50% of the 45 *Salmonella* spp. isolates tested by Pérez-Sotelo et al. (2005), especially those with type 1 fimbriae.

Live and heat-killed *S. cerevisiae* UFMG 905 and live *S. cerevisiae* BY4741 adhered to enteropathogenic *Salmonella* Typhimurium and *E. coli*. Heat-killed *S. cerevisiae* UFMG 905 and live *S. cerevisiae* BY4741 were also able to fix *Salmonella* Typhi, but the live *S. cerevisiae* UFMG 905 could not. In contrast, the live strain *S. cerevisiae* W303 was not able to adhere to any of these bacterial species, demonstrating that this process can be yeast strain-specific. However, none of these yeast strains were able to fix other enteropathogenic bacteria, including *Shigella sonnei*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Bacillus cereus*, *Vibrio cholerae*, *Clostridium difficile*, or *Clostridium perfringens* (Tiago et al., 2012), suggesting that this interaction between yeast and bacteria also depends on the bacterial species and structure (Peng et al., 2001; Tiago et al., 2012). Tiago et al. (2012) also reported that individual bacteria that were not observed to be bound to *S. cerevisiae* UFMG 905 still seemed to be attracted to the yeast cells, which authors suggested may represent potential chemotaxis. These yeast-bacteria adhesions also occurred *in vivo* in monoxenic mice inoculated with *Salmonella* Typhimurium (Tiago et al., 2012). Free mannose inhibits yeast flocculation as the monomer occupies binding sites required for flocculin-mannose residue binding of adjacent cells (Teunissen et al., 1995; Kobayashi et al., 1998; Tiago et al., 2012). Thus, simultaneous administration of both mannose and *S. cerevisiae* cells could inhibit *S. cerevisiae* flocculation of pathogens *in vivo*.

Immune effects

As whole *S. cerevisiae* cells display various cell wall components such as β -glucans and mannans, whole *S. cerevisiae* cells interact with similar receptors as these components. In addition to TLR2 and dectin-1 (Tada et al., 2002; Brown et al., 2003; Jouault et al., 2006), *S. cerevisiae* DNA can also be recognized by TLR9 in macrophages; however, TLR9 deficiency

does not inhibit *S. cerevisiae*-induced immune responses, suggesting that this receptor is not required (Kasperkovitz et al., 2011). Similar to its components, whole *S. cerevisiae* cells are internalized by macrophages (Giaimis et al., 1993; Lorenz et al., 2004; Keppler-Ross et al., 2010). Heat-killed *S. cerevisiae* phagocytosis by murine macrophages was reduced with the addition of either *S. cerevisiae*-derived α -mannan or algal β -glucan, and simultaneous addition of both almost completely inhibited *S. cerevisiae* phagocytosis. These findings suggest that heat-killed *S. cerevisiae* phagocytosis is mediated by both mannose and β -glucan receptors (Giaimis et al., 1993). However, in a more recent study, mutations that affected mannans on the surface of *S. cerevisiae* decreased internalization by primary murine macrophages and the J774 murine macrophages cell line, whereas mutations to glucan or chitin did not change this function (Keppler-Ross et al., 2010). Though neutrophils were able to phagocytose live *S. cerevisiae* (Rubin-Bejerano et al., 2003), heat-killed *S. cerevisiae* did not induce superoxide burst of human neutrophils (Ross et al., 1987).

Whole *S. cerevisiae* modulate cytokine production of innate immune cells. In porcine monocyte-derived DC, live *S. cerevisiae* var. *boulardii* induced mRNA expression of pro-inflammatory cytokines TNF α , GM-CSF, and IL-6, anti-inflammatory cytokine IL-10, and C-C chemokine receptor type 7 (CCR7). However, mRNA expression of TLR2, TLR4, and TGF β were not affected. *Saccharomyces cerevisiae* var. *boulardii* did not elicit antagonistic or synergistic interactions with enterotoxigenic *E. coli* in DC (Badia et al., 2012). Human monocytes primed with heat-killed *S. cerevisiae* then challenged with LPS, Pam₃Cys₄, or *C. albicans* produced greater concentrations of TNF α and IL-6 than negative control monocytes. The experiment was replicated using several strains of *S. cerevisiae*, and some differences in

cytokine responses among strains were observed (Rizzetto et al., 2016). Authors described these observations as a mechanism of trained immunity elicited by *S. cerevisiae* cells.

Intestinal effects

Saccharomyces cerevisiae is generally considered a non-commensal and non-pathogenic yeast species (Saegusa et al., 2004; Zanello et al., 2011). When orally ingested, *S. cerevisiae* can be found in the gut (Pecquet et al., 1991; Scevola et al., 2003). In contrast, another yeast species, *Candida albicans*, can colonize the gut without pathogenic infection but can become an opportunistic pathogen in immunocompromised patients (Saegusa et al., 2004; Zanello et al., 2011). van der Aa Kühle et al. (2005) discovered that only very small proportions of several food-related *S. cerevisiae* were able to adhere to nontumorigenic porcine jejunal epithelial cells (IPEC-J2) *in vitro*. However, in this study and several others, *S. cerevisiae* were still able to modulate intestinal epithelial cells.

As with immune cells, whole *S. cerevisiae* cells modulate cytokine production in intestinal cells. Live *S. cerevisiae* increased IL-8 mRNA levels in Caco-2 human intestinal epithelial cells, but only when also supplied with butyric acid (Saegusa et al., 2004; Saegusa et al., 2007). This *S. cerevisiae*-induced IL-8 transcription was not very strong, especially compared to induction by *C. albicans*, which could imply that *S. cerevisiae* does not promote excessive inflammation but instead primes intestinal cells to fight against pathogens (Saegusa et al., 2004). Unlike live *S. cerevisiae*, heat-killed *S. cerevisiae* did not elicit IL-8 production in intestinal epithelial cells (Saegusa et al., 2004; Cohen-Kedar et al., 2014). This lack of effect could have been due to heat treatment altering or destroying cell wall components otherwise responsible for initiating immune reactions, or from metabolites produced by live but not killed

yeast cells (Saegusa et al., 2004). The presence of butyric acid also increased mRNA levels of TLR1, TLR6, and dectin-1. Dectin-1 mRNA was further increased by *S. cerevisiae*. This increased receptor expression could have contributed to the increase in IL-8 due to greater opportunity for receptor recognition of yeast cells (Saegusa et al., 2004). In contrast, *S. cerevisiae* var. *boulardii* 7103 alone did not impact expression of IL-1 α in porcine intestinal epithelial IPEC-J2 cells. However, *S. cerevisiae* var. *boulardii* 7103 greatly decreased *E. coli*-induced expression of this pro-inflammatory cytokine when IPEC-J2 cells were pre- and co-treated with the yeast and *E. coli* (van der Aa Kühle et al., 2005).

In porcine small intestinal IPI-2I cells, live *S. cerevisiae* var. *boulardii* also decreased enterotoxigenic *E. coli*-induced mRNA expression of pro-inflammatory cytokines TNF α , IL-6, GM-CSF and chemokines CCL2, CCL20, and CXCL8 (Badia et al., 2012). Similarly, live *S. cerevisiae* strain CNCM I-3856 suppressed enterotoxigenic *E. coli* induction of IL-6 and IL-8 in porcine intestinal epithelial IPEC-1 cells. The heat-killed version of this strain failed to elicit the same effects. Live *S. cerevisiae* CNCM I-3856 also reduced enterotoxigenic *E. coli*-induced transcript abundance of pro-inflammatory chemokines CCL20, CXCL2, and CXCL10 (Zanello et al., 2011). According to phase-contrast microscopy (but not scanning electron microscopy), *S. cerevisiae* was also able to agglutinate *E. coli* in IPEC-1 cell culture supernatant. This finding most likely contributed to the increased number of IPEC-1-associated *E. coli* cells and decreased non-IPEC-1-associated *E. coli* cells observed in the presence of *S. cerevisiae* (Zanello et al., 2011). Similarly, Badia et al. (2012) observed decreased adhesion of *E. coli* on IPI-21 cells in the presence of live *S. cerevisiae* var. *boulardii*.

A commonly-used measure of intestinal barrier function is transepithelial electrical resistance, which demonstrates physical barrier activity via resistance to electrical current

passage through the cells. Live *S. cerevisiae* cells did not affect transepithelial electrical resistance of Caco-2 cells after 7 h of incubation, whereas incubation with live *Candida albicans* decreased this physical barrier function at this time point. After 24 h, *S. cerevisiae* did decrease resistance, but not nearly as much as *C. albicans* (Saegusa et al., 2004). In a challenge model with enterotoxigenic *E. coli*, *S. cerevisiae* CNCM I-3856 was not able to alleviate the *E. coli*-induced decrease in transepithelial electrical resistance in IPEC-1 cells (Zanello et al., 2011). Generoso et al. (2010) tested *S. cerevisiae* impacts on intestinal barrier function by orally administering a radioactively labeled compound and measuring its appearance in blood in an intestinal obstruction model. Intestinal obstruction greatly increased appearance of the radioactively labeled compound in blood, but pre-treatment with either live or heat-killed *S. cerevisiae* 905 was able to inhibit this induced intestinal permeability.

Whole animal effects

As observed *in vitro*, *S. cerevisiae* can also agglutinate several species of bacteria *in vivo*. For example, *S. cerevisiae* UFMG 905 adhered to *Salmonella* Typhimurium in the intestines of dioxenic mice (Tiago et al., 2012). Similarly, Martins et al. (2011) observed adherence of *S. cerevisiae* 905 cells to *Salmonella enterica* Typhimurium cells in the cecum of challenged mice.

Coinciding with *in vivo* bacterial agglutination, *S. cerevisiae* can also modulate immunological responses to protect against pathogens. Martins et al. (2005) found that *S. cerevisiae* 905 was able to colonize the gut of gnotobiotic and conventional mice. This yeast strain was also found to translocate into Peyer's patches and mesenteric lymph nodes in small amounts (Martins 2007), which could prime the GALT (Berg, 1996). After *S. cerevisiae* colonization, Martins et al. (2005) challenged the mice with *Salmonella enterica* Typhimurium

and *Clostridium difficile*. *Saccharomyces cerevisiae* 905 colonized the same gastrointestinal regions where the enteropathogenic bacteria elicit infective actions. Although fecal counts of either bacteria did not differ between initially gnotobiotic control mice and initially gnotobiotic *S. cerevisiae*-colonized mice, *S. cerevisiae* 905 did improve survival rates and liver pathology of conventional mice challenged with *Salmonella* Typhimurium (Martins et al., 2005; Martins et al., 2011). Additionally, *S. cerevisiae* 905 protected colonic and cecal tissue against pathology of *Clostridium difficile* in initially gnotobiotic mice, as observed by lesser severity of lesions than initially gnotobiotic control mice (Martins et al., 2005). *Saccharomyces cerevisiae* 905 also decreased *Salmonella* Typhimurium translocation into the spleen, liver, and mesenteric lymph nodes of conventional mice (Martins et al., 2007), a process by which pathogens can initiate infection if excessive (Berg, 1996). This translocation of pathogenic species via lymph nodes is also a food safety concern in meat production (Webb et al., 2017). In pre-weaned dairy calves, a *S. cerevisiae* fermentation product decreased *S. Typhimurium* fecal shedding and colonization in the intestines after a challenge with the bacteria, demonstrating this anti-pathogenic ability of *S. cerevisiae* in livestock as well (Brewer et al., 2014).

Oral *S. cerevisiae* 905 was also shown to inhibit NF κ B p65 nuclear translocation that was stimulated by *Salmonella* Typhimurium in colonic tissues. Oral *S. cerevisiae* 905 also modulated concentrations of IL-6, TNF α , IFN γ , and IL-10, but not TGF β , in colonic tissue of *Salmonella* Typhimurium-challenged mice (Martins et al., 2011). In a murine intestinal obstruction model, live and heat-killed *S. cerevisiae* UFMG 905 increased serum IL-10 levels, but neither form was able to modulate serum IFN γ response to the challenge (Generoso et al., 2010). Similarly, IL-10 and TLR4 expression in rumen epithelium was increased by dietary live *S. cerevisiae* in late-gestation dairy cows (Bach et al., 2018). In healthy mice, oral *S. cerevisiae* RC016 decreased

TNF α concentrations and the ratio of IL-10 to TNF α in intestinal fluid. This result was accompanied by increased *ex vivo* phagocytotic activity of peritoneal macrophages (García et al., 2016).

As measures of adaptive immunity, both serum and intestinal secretory IgA were greater in gnotobiotic mice colonized with *S. cerevisiae* 905 than in control gnotobiotic mice (Martins et al., 2007). Secretory IgA was also greater in the intestinal contents from mice treated with live *S. cerevisiae* UMFG 905 compared to those fed heat-killed *S. cerevisiae* UMFG 905 or to controls (Generoso et al., 2010). Similarly, the abundance of IgA⁺ cells in the intestine was greater in healthy mice orally supplemented with *S. cerevisiae* RC016 suspensions vs. controls (García et al., 2016). Immunoglobulin A concentrations in ileal contents were also greater from *S. cerevisiae boulardii*-supplemented piglets than from antibiotic-supplemented piglets that were orally challenged with enterotoxigenic *Escherichia coli*. While supplementation did not affect T cell subpopulations in blood or mesenteric lymph nodes, it did decrease *E. coli* translocation into mesenteric lymph nodes (Lessard et al., 2009) similar to observations of *Salmonella*-challenged, *S. cerevisiae* 905-treated mice (Martins et al., 2007).

Conclusion

Saccharomyces cerevisiae and its cell wall components, β -glucans, mannans, and the extract zymosan, can modulate many factors of innate immunity, adaptive immunity, and intestinal cells, further influencing these functions in whole animal models. The majority of the previous research has been focused on *S. cerevisiae*-derived β -glucans, which have shown the most consistent and substantial immune effects. In accordance, zymosan, which is generally considered to be a *S. cerevisiae*-derived β -glucan source but also contains several other cell wall

components, elicits similar effects. However, *S. cerevisiae*-derived mannan and *S. cerevisiae* cells themselves have also been shown to modulate immune and intestinal functions. Of great interest with these two treatments is their ability to bind pathogens, therefore reducing or preventing pathogen attachment to mammalian cells that could otherwise lead to infection. Overall, review of the current literature has demonstrated several beneficial uses of *S. cerevisiae* and its cell wall components on immune and intestinal function.

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**Chapter 2 - Impact of *Saccharomyces cerevisiae* fermentation
product (SCFP) on oxidative status, inflammation, and immune
response in transition dairy cattle**

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Abstract

Dairy cattle are subjected to oxidative stress, inflammation, and immune dysfunction during the transition period. The objective of this study was to evaluate the effects of a dietary *Saccharomyces cerevisiae* fermentation product (**SCFP**; NutriTek, Diamond V, Cedar Rapids, IA) on oxidative status, inflammation, and innate and adaptive immune responses during the transition period. Holstein cows were fed a control total mixed ration (**TMR**) ($n = 30$) or SCFP TMR ($n = 34$) from -29 ± 5 to 42 d relative to calving. Blood was sampled during wk -4, -2, 1, 2 and 5 and liver at wk -3 and 2 relative to calving. Oxidative status was evaluated in plasma by retinol, α -tocopherol, and malondialdehyde (**MDA**) concentrations, glutathione peroxidase (**GPx**) activity, and Trolox equivalent antioxidant capacity (**TEAC**), and in liver by mRNA abundance of nuclear factor E2-related factor 2 (*Nrf2*), metallothionein 1E (*MT1E*), and glutathione peroxidase 3 (*GPX3*). Inflammation was evaluated in plasma by haptoglobin (**HP**) and serum amyloid A (**SAA**) concentrations and in liver by mRNA abundance of HP, serum amyloid A3 (*SAA3*), and nuclear factor kappa-light-chain-enhancer of activated B cells (*NFκB*). Innate immune response was measured by stimulated oxidative burst of polymorphonuclear cells (neutrophils; **PMN**) isolated from blood. Ovalbumin (**OVA**) was administered with adjuvant on d 7 and 21 postpartum, and adaptive immune response was evaluated by serum anti-OVA IgG content on d 28 and 35. Mixed models were used to assess effects of treatment, time, parity, and all interactions. Supplementation with SCFP did not affect overall oxidative, inflammatory, or immune parameters (all $P > 0.05$). The only treatment $\times$ wk interaction detected was for plasma α -tocopherol concentration, which tended to be greater in control cows during wk 2 ($P = 0.06$). A tendency for a treatment $\times$ parity interaction was detected for serum anti-OVA IgG titers, which tended to be greater for SCFP than for control in primiparous cows ($P = 0.08$). Plasma

inflammatory biomarkers were not impacted by SCFP but, unexpectedly, plasma HP was elevated during both prepartum timepoints and plasma SAA was elevated during wk -2 compared to the expected increases in both biomarkers postpartum. In this cohort of transition cows with low disease incidence, SCFP generally did not affect oxidative, inflammatory, or immune parameters.

Introduction

Dairy cattle experience drastic changes in metabolic and physiological demands during the transition from late pregnancy to early lactation (Goff and Horst, 1997; Drackley, 1999). This transition period is also marked by immune dysfunction (Mallard et al., 1998), inflammation (Bradford et al., 2015), and oxidative stress, which occurs when the production of pro-oxidants, such as reactive oxygen species (**ROS**), surpasses the neutralizing capacity of antioxidants (Sordillo and Aitken, 2009). These oxygen-containing free radicals are produced in normal cellular metabolism through the electron transport chain, oxidizing enzyme pathways, aerobic mitochondrial metabolism (Valko et al., 2007), and also by neutrophils and macrophages of the innate immune system (Knight, 2000; Sordillo and Aitken, 2009). Additionally, lipid stores mobilized during early lactation (Contreras and Sordillo, 2011) are broken down in tissues by β -oxidation, producing additional ROS and increasing susceptibility to lipid peroxidation (Schönfeld and Wojtczak, 2008). Oxidative stress damages lipids, DNA, proteins, and other macromolecules, ultimately disrupting cellular membranes, interfering with several cellular processes, and impairing immune defenses and increasing susceptibility to health disorders (Knight, 2000; Sordillo and Aitken, 2009). These challenges may contribute to the characteristic increase in susceptibility to metabolic and infectious diseases observed during this period (Goff and Horst, 1997; Drackley, 1999). Thus, much emphasis has been placed on improving management of periparturient cattle, particularly by manipulating nutritional and immunomodulatory strategies.

Various yeast products, including *Saccharomyces cerevisiae* fermentation products (**SCFP**), have been shown to improve rumen fermentation and milk production (Acharya et al., 2017), alter feeding behavior (DeVries and Chevaux, 2014), and increase dry matter intake

(DMI) (Dann et al., 2000; Poppy et al., 2012). Recently, yeast products have also been shown to modulate oxidative status and immune response *in vitro* and *in vivo* (Jensen et al., 2008; Magalhães et al., 2008; Liu et al., 2017), but few studies have evaluated such effects in transition dairy cattle (Zaworski et al., 2014; Yuan et al., 2015). Zaworski et al. (2014) found that supplementing SCFP during the transition period induced inflammatory biomarkers, potentially priming the immune system, but did not impact antibody production; however, the authors attributed the latter to a lack of specific vaccine challenge. When transition dairy cows were specifically challenged with an innocuous protein, increasing doses of enzymatically hydrolyzed yeast increased specific antibody production (Yuan et al., 2015). Supplementing yeast products have also been shown to modulate innate immune responses (Jensen et al., 2007; Sauerwein et al., 2007; Huff et al., 2011), inflammation (Emmanuel et al., 2007), and oxidative status (Jensen et al., 2008; Chiu et al., 2010; Liu et al., 2017) *in vitro* and in other species, but data in transition dairy cattle is lacking (Zaworski et al., 2014; Yuan et al., 2015). Thus, the objective of the current study was to evaluate the effects dietary SCFP on oxidative status, inflammation, and innate and adaptive immunity on transition dairy cattle.

We observed oxidative status in plasma by retinol, α -tocopherol, and malondialdehyde (**MDA**) concentrations, glutathione peroxidase (**GPx**) activity, and Trolox equivalent antioxidant capacity (**TEAC**), and in liver by mRNA abundance of nuclear factor E2-related factor 2 (**Nrf2**), metallothionein 1E (**MT1E**), and glutathione peroxidase 3 (**GPX3**). Inflammation was evaluated in plasma by haptoglobin (**HP**) and serum amyloid A (**SAA**) concentrations and in liver by mRNA abundance of HP, serum amyloid A3 (**SAA3**), and nuclear factor kappa-light-chain-enhancer of activated B cells (**NF κ B**). Innate immune response was measured by stimulated oxidative burst of polymorphonuclear cells (neutrophils; **PMN**) isolated from blood. Ovalbumin

(OVA) was administered with adjuvant on d 7 and 21 postpartum, and adaptive immune response was evaluated by serum anti-OVA IgG content on d 28 and 35. We hypothesized that dietary SCFP would enhance antioxidant status, innate immune response, and adaptive immune response and alleviate excessive inflammation during the transition period compared to control cows.

Materials and Methods

Experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

Design and Treatments

Sixty-four Holstein cows (50 multiparous, 14 primiparous) were enrolled in a transition study from -29 ± 5 to 42 d relative to calving. Cows were housed in a bedded pack barn prepartum and in a tie-stall barn postpartum. A total of 59 cows completed the trial. As part of the pre-determined exclusion criteria, the 5 cows removed postpartum developed chronic illness > 4 d after calving, so prepartum and calving data were included but postpartum data were not. Out of these 5 cows, 3 cows were removed due to excessive adjuvant in the adaptive immune challenge which lead to dramatic fevers irrespective of treatment diet; thus, adjuvant concentrations were decreased for the remainder of the study. The other 2 cows were removed due to severe cases of displaced abomasum that required surgery, and thus cows had severely decreased DMI and went off treatment diets. Though both of these cows were in the SCFP treatment group, the incidence of displaced abomasum was not significantly different between treatments ($P > 0.10$). Incidence of health disorders among the other 59 cows included fever ($n = 9$ control, 5 SCFP), retained placenta ($n = 2$ control, 0 SCFP), mastitis ($n = 2$ control, 1 SCFP), foot injuries ($n = 2$ control, 1 SCFP), diarrhea/digestive upset ($n = 1$ control, 0 SCFP), and

ketosis ($n = 4$ control, 12 SCFP). Incidence was not significantly different between treatments ($P > 0.10$) for any recorded problem except for ketosis, which was significantly greater for SCFP cows than for control cows (38% vs. 12%, $P = 0.02$; Fisher's exact test).

Cows were blocked by parity, expected calving date, and previous 305ME yield, then randomly assigned to treatment within block. Treatments were either control ($n = 30$) or 0.94% prepartum and 0.87% postpartum SCFP (NutriTek, Diamond V, Cedar Rapids, IA; $n = 34$), which was incorporated into a TMR. These percentages were fed based on the manufacturer's recommended dose of 19 g SCFP/d. Cows were fed 3×/d prepartum and 2×/d postpartum, and were milked 2×/d postpartum. Diets were formulated to meet NRC (2001) requirements. Prepartum diets consisted of 43% NDF and 15% starch, while postpartum diets consisted of 31% NDF and 23% starch. Diet and nutrient compositions are summarized in Table 2.1.

Sample Collection

A total of 5 jugular blood samples were collected from each cow throughout the experiment, during wk -4, -2, 1, 2, and 5 relative to calving. Samples were collected twice weekly within the following windows relative to calving: wk -4: d -28 to -24; wk -2: d -14 to -10; wk 1: d 3 to 7; wk 3: d 12 to 16; and wk 5: d 31 to 35. A total of 60 mL of blood was collected per sample, with 50 mL collected in tubes with 1 mL of 1000 U/mL heparin sodium chloride solution for polymorphonuclear cell (**PMN**; primarily neutrophils) isolation. Another 5 mL was collected in potassium EDTA tubes (ref. 368799; BD Vacutainer, Franklin Lakes, NJ) and 5 mL in glucose tubes containing sodium fluoride and potassium oxalate (ref. 367925; BD Vacutainer, Franklin Lakes, NJ) for oxidative status and inflammatory biomarker analysis. The potassium EDTA tubes and glucose tubes were centrifuged, and plasma was collected and frozen at -20°C until future analysis.

Liver samples were collected by percutaneous biopsy in the intercostal space between the tenth and eleventh ribs as described previously by Mullins et al. (2012). Briefly, the area was shaved, cleaned aseptically, and subcutaneously injected with 3.5 mL lidocaine hydrochloride (2% solution). A scalpel blade was then used to puncture the skin. A 14-gauge $\times$ 15 cm biopsy needle (SABD-1415–15-T; US Biopsy, Franklin, IN) was then passed through the incision approximately 10 times for a total of 200 mg of tissue. Liver biopsies were collected a total of 2 times per cow throughout the experiment, once during wk -3 and once during wk 2. A range of d -19 to -12 was used for wk -3 samples so that prepartum biopsies were performed no more than once per week. Biopsies for wk 2 were performed exactly 14 d postpartum. Biopsies were collected and immediately frozen in liquid nitrogen and then placed either alone or with TRIzol reagent (ref. no. 15596018; ThermoFischer Scientific, Waltham, MA) in microcentrifuge tubes and kept at -80 °C for future analysis.

Cows were challenged on d 7 and d 21 after calving with an innocuous protein, ovalbumin (0.5 mg **OVA**; Sigma-Aldrich, St. Louis, MO) along with 0.25 mg Quil-A adjuvant dissolved in 1.0 mL saline (vac-quil; Invivogen, San Diego, CA). Serum samples were collected from the coccygeal vein in serum tubes (ref. 366430; BD Vacutainer, Franklin Lakes, NJ) on d 7, 21, 28, and 35 postpartum to be analyzed for anti-OVA IgG content. Though all cows were inoculated postpartum following this protocol, only 49 cows were naïve to the ovalbumin protein as the other 10 cows had been subjected to an ovalbumin challenge on a previous study; thus, only these 49 cows (25 control and 24 SCFP) were considered in the analysis.

Sample Analyses

Plasma samples from potassium EDTA tubes were used in all analyses. All plate-based assays were read using a spectrophotometer for absorbance (PowerWave XS; BioTek

Instruments Inc., Winooski, VT) or chemiluminescent detection (Synergy HTX; BioTek Instruments Inc., Winooski, VT) using Gen5 software (BioTek Instruments Inc., Winooski, VT).

Oxidative status parameters in plasma. Retinol and α -tocopherol concentrations were measured using HPLC with some modifications to the methods of Greaves et al. (2010). Tocopherol acetate (0.03 g/L in 95% ethanol) was used as the internal standard. Samples of plasma (0.5 mL) were mixed with 0.5 mL of internal standard. Hexane (1.0 mL) was added to extract the vitamins. After centrifugation at $1,000 \times g$, the hexane layer was recovered and dried at room temperature with nitrogen gas. Samples were reconstituted in the mobile phase (0.4% acetic acid in acetonitrile) and separated isocratically by HPLC at 0.5 mL/min through a Kinetex 2.6 μ m XB-C18, 100 mm $\times$ 4.6 mm column with a guard column (cat. no. 00D-4496-E0; Phenomenex, Torrance, CA). Retinol, α -tocopherol, and tocopherol acetate were detected by absorption at 290 nm (Acutect 500 UV/VIS Detector) with retention times of 3.5, 12.8, and 15 min, respectively. α -Tocopherol was also evaluated as a ratio of plasma α -tocopherol to plasma cholesterol to account for changes in lipid mobilization, and thus changes in α -tocopherol transport (Herdt and Smith, 1996). Plasma cholesterol was measured with an enzymatic kit (item no. 10007640; Cayman Chemical, Ann Arbor, MI).

Glutathione peroxidase (**GPx**) enzymatic activity was measured using a colorimetric assay kit (#K762-100; Biovision, Milpitas, CA, USA) based on the methods of Paglia and Valentine (1967). Available GPx in the plasma samples reduced cumene hydroperoxide added in the assay by oxidizing glutathione. The oxidized glutathione was then converted back to its reduced form by glutathione reductase, a reaction that required NADPH. The decline of NADPH was proportional to the GPx activity of the plasma sample. For this assay, 40 mM NADPH solution, glutathione reductase, and reduced glutathione were added to the plasma samples to

deplete the oxidized glutathione. Cumene hydroperoxide solution was then added to begin the GPx enzymatic reaction, and absorbance was measured at 340 nm at 0 and 5 min. Activity of GPx activity was calculated by subtracting the 5-min absorbance value from the initial value.

Trolox equivalent antioxidant capacity (**TEAC**) was measured by a colorimetric assay kit (item no. 709001; Cayman Chemical, Ann Arbor, MI). This assay utilized the antioxidants in the plasma samples to prevent the oxidation of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (**ABTS**), a colorless compound, to its oxidized, blue-green form, $ABTS^{\cdot+}$, by metmyoglobin and hydrogen peroxide. Extent of the reduction in absorbance, measured at 705 nm, was equal to the concentration of total antioxidants in the plasma samples. The antioxidant concentration was compared against Trolox, an α -tocopherol analogue commonly used as an antioxidant standard, and was expressed as millimolar Trolox equivalents (Miller et al., 1993).

Malondialdehyde (**MDA**) concentration was measured using colorimetric detection with a commercial kit (#ab118970; Abcam, Cambridge, MA, USA). Plasma samples were prepared with 42 mM sulfuric acid and phosphotungstic acid solution, then pelleted and resuspended in water and butylated hydroxytoluene. Thiobarbituric acid (**TBA**) was then added and samples were heated to 95° C for 60 minutes, then allowed to cool on ice. This process allowed MDA and TBA to condense and form an MDA-TBA adduct, which was measured by absorbance at 532 nm.

Inflammatory biomarkers in plasma. Serum amyloid A (**SAA**) concentration was measured in plasma using a sandwich ELISA kit (cat no. TP-802; Tridelata Development Ltd., Kildare, Ireland). Plate wells were coated with an SAA-specific monoclonal antibody. Horseradish peroxidase-labelled anti-SAA antibody was then added to the coated wells, followed by samples, and plates were incubated at 37 °C for 1 h. After washing, 3,3',5,5'-

tetramethylbenzidine (**TMB**) substrate was added and allowed to incubate for 15 minutes, followed by stop solution to end the reaction. The intensity of blue color generated was directly proportional to the amount of SAA in the samples. Absorbance was measured at 450 nm with 630 nm as a reference. Controls purchased from the same company (cat no. TP-802-CON; Tridelata Development Ltd., Kildare, Ireland) were run on each ELISA plate.

Haptoglobin (**HP**) concentration was measured in plasma based on differences in hydrogen peroxidase activity through measuring absorbance of complexes formed by HP and hemoglobin (Cooke and Arthington, 2012). Briefly, 10 μ L of plasma was added to an O-dianisidine solution at pH 4.1 in a 16 $\times$ 100 mm borosilicate tube. Immediately afterwards, 25 μ L of hemoglobin solution was added to the tube. Following a 45-minute incubation at 37 $^{\circ}$ C, 100 μ L of a freshly prepared hydrogen peroxide solution was added. Tubes were then incubated at room temperature for 1 h. A 200 μ L aliquot from each tube was transferred to a well in a 96-well plate, and absorbance was measured at 450 nm. A blank was run using water in place of plasma, which was subtracted from all optical density values of plasma samples. Plasma samples from a previous study were analyzed using a bovine-specific HP ELISA kit (cat. no. HAPT-11; Life Diagnostics, Inc., West Chester, PA, USA), and the sample with the greatest HP concentration was used as a stock standard. This stock standard was serially diluted and used as the standard curve on each plate for the biochemical assay.

Quantitative real-time PCR. Liver biopsy samples were evaluated for mRNA abundance of oxidative stress response mediators (nuclear factor E2-related factor 2 [**Nrf2**], metallothionein 1E [**MT1E**], glutathione peroxidase 3 [**GPX3**]), inflammatory mediators (haptoglobin [**HP**], serum amyloid A3 [**SAA3**], and nuclear factor kappa-light-chain-enhancer of activated B cells [**NF κ B**]), and 2 internal control genes (beta actin [**β -actin**] and 40S ribosomal

protein S15 [***RPS15***]). The Direct-zol RNA mini prep kit (cat. no. r2072; Zymo Research, Irvine, CA) was used to isolated RNA from the liver tissue. Quantitative real-time PCR was performed as previously described (Yuan et al., 2013). Briefly, 2 µg of total RNA per sample was used as a template for the reverse transcriptase reaction using random primers (High-Capacity cDNA RT Kit; Applied Biosystems, Foster City, CA). Quantitative real-time PCR was run in duplicate using 5% of the cDNA product on 96-well plates with 200 nM of gene-specific primers (Table 2.3) and SYBR green reagent (cat. no. 172_5120; BioRad Laboratories, Hercules, CA). All target genes in samples were normalized against the geometric mean of control genes *β-actin* and *RPS15*. Messenger RNA abundance was calculated as $2^{-\Delta C_t}$ and data were log transformed for statistical analysis. Results were back-transformed and scaled such that the wk -3 control mean equaled 1 for each transcript.

Innate immunity. Polymorphonuclear cells were isolated from jugular blood samples as reported previously by Oh et al. (2008) with some modifications. Briefly, whole blood was first layered with Ficoll-Paque (cat. no. 17-1440-03; GE Healthcare Biosciences, Uppsala, Sweden) in another tube. These tubes were then centrifuged at $1,700 \times g$ for 45 min at 4 °C. The top layers containing plasma, peripheral blood mononuclear cells, and Ficoll-Paque were discarded and the remaining PMN were mixed with cold Hanks' Balanced Salt Solution without calcium or magnesium (**HBSS**; cat. no. 14175079; ThermoFischer Scientific, Waltham, MA). Tubes were centrifuged again at $2,500 \times g$ for 5 min at 4 °C and the supernatant was discarded. Cell pellets were then mixed with Ammonium-Chloride-Potassium lysing buffer (cat. no. A1049201; ThermoFischer Scientific, Waltham, MA) to lyse red blood cells, followed by another centrifugation at $2,500 \times g$ for 5 min at 4 °C. Cell pellets were resuspended in more lysing buffer and HBSS and centrifuged with the same conditions again. Cells were resuspended in HBSS,

centrifuged with similar conditions, and then resuspended in HBSS with calcium and magnesium (cat. no. 14025126; ThermoFischer Scientific, Waltham, MA) and 2% bovine serum albumin (cat. no. BP9704100; Fischer Scientific, Hampton, NH).

Isolated PMN were then counted using Countess Automated Cell Counter (cat. no. C10281; Invitrogen, Waltham, MA). To assess treatment effects on innate immune function, isolated PMN were used to determine oxidative burst under the stimulation of phorbol 12-myristate 13-acetate (**PMA**) essentially as described by Moya et al. (2008), modified as a plate-based assay using a kit (kit #ABEL-16MM; ABEL, Plymouth, UK). Briefly, 50 μ L of PMN cell suspension at 1×10^6 cells/mL was added to each of 6 wells in a black-walled, 96-well plate. All 6 wells also received 60 μ L of the assay buffer, 20 μ L of an adjuvant to increase chemiluminescent signal, and 50 μ L of Pholasin, a photoprotein that emits light in the presence of ROS. To activate PMN to produce ROS through the NADPH oxidase system, 20 μ L of PMA solution was added to half of the wells, referred to as PMA-stimulated PMN oxidative burst. The other half of the wells was left untreated, referred to as unstimulated PMN oxidative burst. The plate was incubated at 37°C for 10 minutes and chemiluminescence was measured. Additionally, concentrations of tumor necrosis factor α in basal and PMA-stimulated PMN media samples were analyzed using a commercial kit (kit no. EBTNF; Thermo-Scientific, Frederick, MD), but concentrations were below the limit of detection.

Adaptive immunity. Serum samples collected on d 7, 21, 28, and 35 postpartum were analyzed for anti-OVA IgG content by ELISA using a protocol previously described (Yuan et al., 2015). Briefly, 96-well plates were coated with 100 μ L of a coating solution that consisted of 15.4 mg OVA (cat. no. A5503; Sigma-Aldrich, St. Louis, MO) diluted in 11 mL of 0.05 M carbonate-bicarbonate buffer (pH 9.4). This buffer was prepared by mixing 4 mL of a 0.2 M

anhydrous sodium carbonate solution with 46 mL of a 0.2 M sodium bicarbonate solution and 150 mL of water. Plates were incubated with the coating solution overnight. The next day, plates were washed 5 times with a wash buffer, which consisted of PBS and 0.05% Tween solution (pH 7.4). Next, 200 μ L of blocking buffer, which consisted of 4% bovine serum albumin and 5% sucrose in PBS filtered at 0.2 μ m, was added to each well and incubated at room temperature for 2 h. Plates were then washed again with the wash buffer.

Afterwards, serum samples diluted 1:200 in PBS, blank controls (wash buffer), negative controls (pooled serum samples from d -7 diluted 1:200), and positive controls (pooled serum samples from d 28 diluted 1:200) were added to duplicate wells. Plates were incubated at room temperature for 1 h then washed 5 times again. Anti-bovine IgG peroxidase (cat. no. A5295; Sigma-Aldrich, St. Louis, MO) diluted in 10 mM PBS at 1:30,000 was then added in 100 μ L aliquots to each well. After a 1 h incubation, plates were washed 5 times with wash buffer and 100 μ L of 3,3',5,5'-tetramethylbenzidine substrate solution (TMB solution; Thermo Fischer Scientific, Inc., Waltham, MA) was added to each well. After 5 min of incubation at room temperature, 100 μ L of stop solution (0.18 M sulfuric acid) was added to each well. Absorbance was measured by optical density (**OD**) adjusted for the blank control at 450 nm. Final OD was corrected between plates to reach a positive control OD of 0.5 by multiplying the original OD by 0.5 and then dividing by the positive control OD for that plate.

Statistical Analyses

Results were analyzed using the Mixed Procedure of SAS. Models included fixed effects of treatment, time, parity, and 2- and 3-way interactions with treatment, and the random effects of block and cow. Interactions with parity were tested and removed from the model when $P > 0.20$. Repeated measures within cow were modeled with autoregressive or heterogeneous autoregressive

covariance structures when data points were equally-spaced, selected based on the least Bayesian Information Criterion value. Unequally spaced data points (e.g., plasma data) were modeled with spatial power covariance structures. Outliers were excluded when the Studentized residual exceeded an absolute value of 4. Significance was declared when $P < 0.05$, and tendencies at $0.05 \leq P < 0.10$. When treatment interactions were $P < 0.05$, the slice option of SAS was used to test treatment effects at each measurement time or within parity. Anti-ovalbumin IgG content was analyzed using d 7 values as co-variants. Beyond treatment effects, regression techniques in JMP were used to explore relationships between immune, oxidative, and inflammatory parameters. Outliers were removed from analysis based on Cook's D Influence. If distributions were not normal, data were natural log transformed for analysis.

Results and Discussion

Oxidative Status in Plasma and Liver

As general outcomes, average prepartum DMI was 11.52 and 11.74 ± 0.50 kg/d ($P = 0.70$) for control and SCFP-supplemented cows, respectively. Average postpartum DMI was 20.58 and 20.69 ± 0.52 kg/d ($P = 0.84$) for control and SCFP-supplemented cows, respectively. Though treatment with SCFP did not alter DMI, SCFP supplementation increased meal count per day and decreased time between meals. Additionally, SCFP supplementation did not affect milk yield; however, cows supplemented with SCFP also produced greater overall milk fat concentration (Olagaray et al., 2018).

Relative to oxidative status, antioxidants can be classified as enzymatic, such as GPx, or non-enzymatic, such as α -tocopherol and retinol (Valko et al., 2007). In our study, all plasma antioxidants differed by wk ($P < 0.01$) and results are summarized in Table 2.2. In general, SCFP treatment did not affect oxidative status (all $P > 0.05$). Overall plasma concentrations of retinol

and α -tocopherol, stemming from lipid-soluble dietary vitamins A and E, respectively (Goff and Stabel, 1990), were not impacted by treatment ($P = 0.66$ and 0.56 , respectively). Plasma concentrations were similar to or greater than those previously reported in transition dairy cows (Goff and Stabel, 1990; LeBlanc et al., 2004; Debier et al., 2005; Pilotto et al., 2016). Both vitamins were supplied in the diet above NRC (2001) recommended levels (average of 256 IU/kg of BW for supplemental retinol and 3.75 IU/kg of BW for supplemental α -tocopherol in the prepartum diets; average of 398 IU/kg of BW for supplemental retinol and 5.83 IU/kg of BW for supplemental α -tocopherol in the postpartum diets). The addition of 18.4 g/d of SCFP most likely was not able to contribute directly to overall concentrations in circulation. However, a tendency for a treatment $\times$ wk interaction was observed for α -tocopherol as control cows tended to have greater α -tocopherol concentrations 2 weeks prior to parturition compared to cows supplemented with SCFP ($P = 0.06$; Figure 2.1). Supplementation with SCFP could have decreased mobilization of α -tocopherol or increased free radical consumption of the antioxidant. This explanation is unlikely, though, as this difference was only a tendency and only observed at one time point. This difference in α -tocopherol concentrations between treatments at wk -2 is most likely not biologically relevant.

Parity also affected α -tocopherol concentrations, as multiparous cows tended to have greater α -tocopherol concentrations than primiparous cows (5.02 vs. $4.34 \pm 0.23 \text{ }\mu\text{g/mL}$; $P = 0.05$). Multiparous cows had greater DMI than primiparous cows throughout the study (22.9 vs. $18.4 \pm 0.62 \text{ kg/d}$; $P < 0.001$; Olagaray et al., 2018), and thus consumed a greater amount of dietary vitamin E. Greater intake of the vitamin could have led to greater plasma α -tocopherol concentrations compared to primiparous cows (Goff and Stabel, 1990). Additionally, multiparous cows are generally presumed to have more adipose tissue than primiparous cows.

Lipid-soluble vitamins can be stored in adipose tissue as well as in liver (Bjørneboe et al., 1990; Frey and Vogel, 2011), and thus multiparous cows may have had greater adipose vitamin E stores able to be mobilized compared to primiparous cows. In addition, α -tocopherol is generally associated with lipoproteins for transport (Bjørneboe et al., 1990); thus, when lipid mobilization is increased, such as during the transition period, α -tocopherol is mobilized more rapidly into circulation (Herdt and Smith, 1996). Using cholesterol as a biomarker of lipoprotein content, the ratio of plasma α -tocopherol to plasma cholesterol has been suggested to more accurately reflect actual changes in concentration versus changes in lipid mobilization (Herdt and Smith, 1996). In our study, the ratio of α -tocopherol to cholesterol tended to be greater for control cows than SCFP cows (0.0038 vs. 0.0034 ± 0.0002 ; $P < 0.10$). However, control cows also had lesser plasma cholesterol concentrations than SCFP cows ($P = 0.02$; Olagaray et al., 2018), complicating the interpretation of this result.

A treatment $\times$ parity interaction was observed for retinol ($P < 0.001$) as plasma concentrations were greater in primiparous cows supplemented with SCFP (0.37 vs. 0.33 ± 0.02 $\mu\text{g/mL}$; $P = 0.02$), but lesser in multiparous cows supplemented with SCFP (0.30 vs. 0.33 ± 0.01 $\mu\text{g/mL}$; $P < 0.01$), compared to parity-matched controls. Plasma retinol also showed a treatment $\times$ wk $\times$ parity interaction ($P < 0.001$; Figure 2.2) as concentrations were greater in primiparous SCFP cows during wk -2 ($P = 0.02$) but lesser in multiparous SCFP cows during wk 2 ($P < 0.01$), compared to parity-matched controls. Supplementation of SCFP could have increased retinol absorption, storage, or overall availability, or decreased its consumption in the neutralization of pro-oxidants in primiparous cows, with opposite effects in the multiparous cows. Primiparous cows have also been reported to have greater plasma concentrations of vitamin A than multiparous cows, potentially due to increased vitamin A absorption or greater

storage (Debier 2005). Plasma retinol is known to decrease at parturition and increase during the first week after calving, most likely due to greater secretion into colostrum and lesser secretion into milk (Debier et al., 2005). Multiparous cows in our study had greater milk yield and milk fat concentrations than primiparous cows (both $P < 0.001$). Therefore, SCFP supplementation could have also increased secretion of retinol into colostrum or milk in multiparous cows. Though the reason for this observation was unclear, these minute changes in plasma retinol concentrations are most likely not biologically relevant.

In addition to dietary sources, the body also produces its own antioxidant defense systems. Glutathione peroxidase is an important endogenous antioxidant enzyme produced by multiple organs, including the liver. This enzyme protects against oxidative damage by reducing lipid peroxides and hydrogen peroxide (Arthur, 2000; Valko et al., 2007). In our study, levels of GPx activity in plasma did not differ between SCFP-supplemented and control cows ($P = 0.32$; Figure 2.3). Similarly, feeding a SCFP did not affect plasma GPx activity in dairy calves during weaning (Alugongo et al., 2017). In contrast, Liu et al. (2017) reported increased GPx activity in plasma of weanling piglets fed a *Saccharomyces cerevisiae* cell wall extract compared to controls; however, supplemented piglets also had lesser concentrations of plasma MDA, a marker of lipid peroxidation, compared to control piglets. Supplemented transition dairy cows from our trial and supplemented dairy calves from Alugongo et al. (2017) did not have different plasma MDA concentrations compared to controls ($P > 0.05$). Less oxidative stress may have prevented an increase in induced plasma GPx activity by *Saccharomyces cerevisiae* products in our trial and Alugongo et al. (2017).

During wk 1 postpartum in our study, GPx activity increased while α -tocopherol and retinol concentrations decreased. The secretion of retinol and α -tocopherol into colostrum during

the first few days after calving could have accounted for the loss in these vitamin-derived antioxidants during wk 1 (Goff and Stabel, 1990). Glutathione peroxidase has also been found in bovine milk (Debski et al., 1987) and human colostrum (Guerra et al., 2018), but is also produced within mammary cells and tissues (Lane and Medina, 1983; Imai and Nakagawa, 2003). This enzyme is also inducible by oxidative stress (Tüzün et al., 2002; Bierl et al., 2004), unlike dietary vitamins. Thus, a loss of other antioxidants, particularly from exogenous sources, could induce compensatory GPx production. At wk 2 postpartum, however, the vitamin-derived antioxidants increased while GPx fell. The increase of vitamin-derived antioxidants could have been due to increased DMI during wk 2 and therefore greater vitamin intake, or less vitamin excretion as colostrum production ceased (Goff and Stabel, 1990). Less need for GPx production because of decreased oxidative stress and/or increased availability of other antioxidants could have led to a decrease in GPx activity.

In our study, GPx activity was affected by parity as multiparous cows had greater GPx activity than primiparous cows (1.10 vs. 0.83 ± 0.08 mU/mL, $P = 0.03$). Multiparous cows generally experience more severe negative energy balance and mobilize more body energy stores at the onset of lactation compared to primiparous cows (Friggens et al., 2007). Severe negative energy balance, compared to moderate negative energy balance, has been linked to an upregulation of several antioxidative Nrf2 target genes in the spleen (Morris et al., 2009). Greater lipid mobilization for energy, which can be observed by greater ketone bodies in the blood, in multiparous cows could also subject the animal to greater lipid peroxidation and ROS production compared to primiparous cows with less lipid mobilization (Friggens et al., 2007; Wathes et al., 2007; Schönfeld and Wojtczak, 2008). Additionally, multiparous cows are diagnosed with diseases during the transition period more often than primiparous cows

(Markusfeld, 1987; Erb and Grohn, 1988). This could be related to oxidative stress, which has been shown to be greater in primiparous women compared to multiparous women, indicated by oxidative stress markers in cord blood (Mutlu et al., 2012). Thus, greater oxidative stress potentially experienced by multiparous cows compared primiparous cows would induce greater production of the endogenous GPx enzyme (Tüzün et al., 2002; Bierl et al., 2004). However, we did not observe any parity differences for TEAC as an overall measure of oxidative capacity ($P = 0.21$). A potential caveat to our finding, though, is that multiparous cows in our study were relatively healthy. Literature to solidly support the relationship of greater oxidative stress with increasing parity is lacking.

Consistent with previous reports in plasma and erythrocytes (Bernabucci et al., 2005; Pilarczyk et al., 2012) and mammary tissue (Aitken et al., 2009), GPx activity was greater postpartum than prepartum. Oxidative stress increases after parturition (Bernabucci et al., 2005; Bionaz et al., 2007; Sordillo and Aitken, 2009; Celi, 2011), which could induce greater production of GPx (Tüzün et al., 2002; Bierl et al., 2004). Several reports of different scenarios support this idea. Sordillo et al. (2007) observed an increase in activity of GPX1, a cytosolic GPx isoform, while total antioxidant potential and activity of thioredoxin reductase, another antioxidant enzyme, declined. Authors suggested that GPX1 activity was able to recover more quickly during the oxidative stress of early lactation than other antioxidants. Deficiency of catalase, another endogenous antioxidant, increased GPx and glutathione-S-transferase activity in goldfish brains (Bagnyukova et al., 2005). These results suggest that total or selenium-independent GPx activity may be able to compensate for deficiencies in other antioxidants. The antioxidant system is redundant, and thus decline or depletion of one antioxidant does not necessarily lead to a decline in overall antioxidant neutralizing capacity (Abuelo et al., 2014).

When exogenous antioxidants decline during oxidative stress, unable to fully be replaced by the body, inducible endogenous antioxidants such as GPx are relied upon as major antioxidant sources.

Retinol and α -tocopherol at wk -4 significantly and negatively predicted GPx activity during wk 1 postpartum ($R = -0.46$; $P < 0.001$ and $R = -0.29$; $P = 0.03$, respectively). Cows with lesser antioxidant vitamin concentrations nearing the start of the transition period had greater GPx activity 1 week postpartum. Lesser antioxidant availability prepartum could have signaled the body to produce more endogenous antioxidants for early lactation.

The liver plays a major role in producing antioxidants for itself and the whole body. A major transcription factor stimulated by pro-oxidants in the liver is Nrf2, which induces several antioxidant and cytoprotective target genes (Ma, 2013). Hepatic oxidative stress response mediators are summarized in Table 2.4. In our study, SCFP did not affect hepatic mRNA abundance of *Nrf2*, *MT1E*, or *GPX3* (all $P > 0.20$). Though mRNA abundance of *Nrf2* did not differ by wk, abundance of two of its target genes, *MT1E* and *GPX3*, increased postpartum, suggesting that cows were experiencing some level of oxidative stress 2 weeks after calving at the hepatic level. Similarly, hepatic mRNA abundance of *GPX3*, *MT1E*, and other *Nrf2* target genes were shown to increase from 3 weeks antepartum to 1 week postpartum (Gessner et al., 2013). Though *Nrf2* was not affected by wk in our study, upregulation of its target genes is a more important outcome as the target genes directly translate into proteins that function as antioxidants (Ma, 2013). Additionally, Han et al. (2018) reported that total protein abundance of Nrf2 from mammary tissue did not differ from d -30 prepartum to d 1, 15, and 30 postpartum but phosphorylated Nrf2 and the ratio of phosphorylated Nrf2 to total Nrf2 increased postpartum. Hepatic mRNA abundance of *GPX3* and *MT1E* also tended to be greater in multiparous cows

than primiparous cows (2.62 vs. 1.12 ± 0.48 , $P = 0.06$ and 1.83 vs. 1.03 ± 0.31 , $P < 0.10$, respectively). Because transcription of these antioxidant genes is induced by Nrf2 signals from oxidative stress (Gessner et al., 2013; Ma, 2013; Han et al., 2018), these differences between parities suggests that multiparous cows underwent more oxidative stress than primiparous cows.

As both variables were measured at wk -3 and wk 2, we also explored the relationships between the hepatic transcript abundance and plasma enzyme activity of GPx. Oddly, hepatic *GPX3* mRNA abundance was not correlated with plasma GPx activity during wk -3 ($R = -0.19$; $P = 0.20$). During wk 2 postpartum, however, hepatic *GPX3* mRNA abundance tended to correlate with plasma GPx activity ($R = 0.23$; $P = 0.06$). This enzyme exists as many isoforms, with GPX3 as an extracellular form found abundantly in plasma (Arthur, 2000). Thus, other isoforms in the liver could more accurately reflect total GPx activity in plasma. Aitken et al. (2009) also observed inconsistent shifts between mRNA abundance and activity of the cytosolic isoform *GPX1* in mammary tissue during the transition period. Similarly, rats fed copper-deficient diets had decreased GPx mRNA abundance but no changes in total GPx activity in liver (Lai et al., 1996). These findings suggest that levels of specific GPX isoform transcription may not always match the activity of their respective isoform, at least within tissues. Additionally, transcriptional, translational, and potentially post-translational factors can all affect GPx production (Bierl et al., 2004; Ottaviano et al., 2009). Thus, assessing hepatic mRNA abundance of *GPX3* alone likely does not accurately predict total plasma GPx enzymatic activity.

Trolox equivalent antioxidant capacity represents total antioxidant capacity of both lipid- and water-soluble antioxidants using Trolox, an α -tocopherol analogue, as a standard (Miller et al., 1993). This measurement more accurately reflects antioxidant capacity within an animal compared to individual antioxidant measurements, as deficiency of one antioxidant may not

decrease overall neutralizing capacity (Abuelo et al., 2014). Levels of TEAC in our study were greater than previously observed in periparturient cows (Gessner et al., 2015; Winkler et al., 2015). In our study, supplementation with SCFP did not affect overall TEAC concentrations ($P = 0.54$); however, we detected a treatment $\times$ wk $\times$ parity interaction ($P < 0.001$; Figure 2.4) with greater TEAC concentrations in primiparous control cows than primiparous SCFP cows during wk 1 ($P = 0.02$). Similarly, weanling piglets supplemented with a yeast cell wall extract had slightly decreased plasma TEAC compared to controls; however, concentrations of reactive oxygen metabolites were not different, indicating that this decrease was most likely not biologically meaningful (Sauerwein et al., 2007). In an *in vitro* trial, pre-treatment with SCFP decreased intracellular oxidation in human erythrocytes challenged with hydrogen peroxide, indicating some protection from oxidative damage at least *in vitro* (Jensen et al., 2008).

Many different antioxidants in blood have been used to assess antioxidant status in dairy cattle. However, TEAC more accurately reflects antioxidant capacity compared to individual antioxidants (Abuelo et al., 2014). As multiple antioxidants contribute to TEAC, we explored the contributions of the individual antioxidants measured in our study to the overall antioxidant measure. Retinol, α -tocopherol, and GPx did not strongly predict TEAC during weeks -4, 1, or 5 (all $R^2 < 0.20$). However, α -tocopherol and GPx were stronger predictors during wk -2 and 2 ($R^2 = 0.26$; $P < 0.001$ and $R^2 = 0.31$; $P < 0.001$, respectively; Figure 2.5). Though both α -tocopherol and GPx contribute to TEAC (Miller et al., 1993) and thus were expected to be positive predictors, observed relationships with TEAC were negative. This finding is not consistent with the biology of either antioxidant (Miller et al., 1993). During wk 2 postpartum, GPx activity below 0.5 mU/mL appeared to reflect deficient TEAC at this time point. Above 1.0 mU/mL of GPx activity, though, TEAC did not change. One interpretation of this quadratic relationship is

that GPx activity ≥ 0.8 mU/mL may be necessary for optimal TEAC. Because GPx is an inducible, endogenous enzyme (Tüzün et al., 2002; Bierl et al., 2004; Sordillo and Aitken, 2009), it may have more accurately represented total antioxidant status in the animal compared to the dietary antioxidants during the oxidative stress of early lactation. A positive correlation between TEAC and GPx activity in plasma was observed in humans diagnosed with a pulmonary disease, but neither measure correlated with other antioxidants, including tocopherol and retinol (Sadowska et al., 2005). While other antioxidants contribute to TEAC, the antioxidants measured in our study were moderately predictive of TEAC during weeks -2 and 2. However, measuring just one antioxidant is not sufficient to infer the full oxidative status of the animal. Additionally, though plasma samples were deproteinized with ethanol before determination of retinol content by HPLC in the current study, determination of free vs. RBP-bound retinol was not verified as literature using this vitamin extraction preparation for HPLC has not distinguished between these forms.

If antioxidants are unable to neutralize pro-oxidants, lipid peroxidation can occur, producing MDA. Determination of MDA has been criticized for being non-specific but is still considered a sensitive biomarker of oxidative stress (Celi, 2011; Abuelo et al., 2014). Consistent with a lack of changes in overall antioxidants, plasma MDA was not affected by SCFP supplementation ($P = 0.52$; Figure 2.6). Similarly, supplementing dairy calves with SCFP during weaning did not affect different plasma MDA concentrations (Alugongo et al., 2017). In our study, concentrations of MDA increased during wk -2, indicating increased lipid peroxidation at this prepartum time point. This result contradicts previous reports that observed lesser MDA during late pregnancy compared to early lactation (Bernabucci et al., 2005; Liu et al., 2013). This prepartum time point was also characterized by an unexpected spike in plasma SAA, an

inflammatory biomarker. Though both plasma MDA and SAA shared similar unexpected increases during wk -2, the two were not significantly correlated at this time point that was not significant ($R = -0.20$; $P = 0.13$). Also unexpectedly (Bernabucci et al., 2005; Castillo et al., 2006), MDA levels decreased 1 wk after calving, suggesting that cows either experienced less lipid peroxidation even with elevated lipid mobilization, or that antioxidants were able to detoxify lipid peroxides. This pattern was opposite of what Castillo et al. (2006) reported, though large standard errors 1 wk before and after calving prevented significant differences from being observed by the authors. In our study, plasma MDA did not increase after wk 2, most likely because cows were adapting to the increased metabolic demands approaching peak lactation (Castillo et al., 2006); however, postpartum levels did not return to wk -4 values, suggesting the cows were still experiencing some lipid peroxidation.

α -Tocopherol acts as a scavenger of free radicals, particularly protecting against lipid peroxidation (Benzie, 1996; Sordillo and Aitken, 2009), and MDA is a product of lipid peroxidation (Celi, 2011). However, both α -tocopherol and retinol had strong positive relationships with MDA during wk -2 ($R = 0.43$, $P < 0.001$ and $R = 0.55$, $P < 0.001$, respectively), which contradicts the expected nature of the antioxidant/pro-oxidant relationship. In contrast, GPx and MDA were negatively correlated ($R = -0.30$; $P = 0.02$), which was a more expected result as selenium-dependent GPx also prevents lipid peroxidation (Arthur, 2000).

Effects of SCFP supplementation on oxidative status in transition dairy cattle have not been reported previously. Our cohort of transition cows was relatively healthy with low infectious disease incidence, and results could have been different with a more challenged group. Additionally, SCFP supplementation could have impacted oxidative status within mucosal tissues, such as the uterus (Yuan et al., 2015) or gut (Magalhães et al., 2008), as yeast products

have been shown to modulate these tissues. Vitamin antioxidant concentrations and lipid peroxidation markers in tissues can also differ from those in plasma (Bouwstra et al., 2008). More research is needed in this area, but overall, SCFP supplementation generally did not affect oxidative status in our study.

The three-way interactions observed for retinol and TEAC are difficult to interpret and may have been due to chance, given the sheer number of comparisons made in this study. We also demonstrated that hepatic mRNA abundance and corresponding proteins in plasma do not always correlate, particularly during the transition period. Antioxidants are also impacted differently throughout the transition period, and measuring total antioxidant capacity, such as with TEAC, may be a more accurate measurement of oxidative status within the animal compared to measuring individual antioxidants.

Inflammatory Biomarkers in Plasma and Liver

The acute phase response is activated in response to inflammation, pathogen exposure, or tissue damage. This response includes the production of acute phase proteins (**APP**), including SAA and HP, mainly by the liver (Cray et al., 2009). These proteins aid in recruiting immune cells to the site of infection or damage and may also act to restore homeostasis, in addition to other unique functions (Wang et al., 2001; Cray et al., 2009; Eklund et al., 2012; Ye and Sun, 2015). Both SAA and HP are class I APP, as they are both induced by IL-1 and IL-6 (Baumann and Gauldie, 1990; Raynes et al., 1991; Yoshioka et al., 2002; Bode et al., 2012; Eklund et al., 2012). The major inflammatory transcription factor NF κ B also regulates SAA transcription (Edbrooke et al., 1989; Huang and Liao, 1994), as well as expression of IL-6 (Shimizu and Yamamoto, 1994), thus indirectly regulating HP as well. Nuclear factor κ B, SAA, and HP are all considered biomarkers of inflammation, and are summarized in plasma and in liver in Tables 2.1

and 2.3, respectively. Plasma SAA and HP are also displayed in Figures 2.7 and 2.8, respectively.

Some inflammation is expected after parturition (Zaworski et al., 2014; Bradford et al., 2015). Hepatic mRNA abundance of both *HP* and *NFκB* was greater postpartum than prepartum ($P = 0.01$ and $P < 0.05$, respectively), as expected from previous reports in transition dairy cattle (Gessner et al., 2013; Han et al., 2018). However, in contrast to previous reports (Gessner et al., 2013; Saremi et al., 2013), hepatic mRNA abundance of *SAA3* was not significantly different prepartum versus postpartum ($P = 0.10$). This APP exists as 4 known isoforms. In cattle, *SAA3* is produced after inflammatory stimulus both hepatically and extrahepatically, such as by mammary and adipose tissues (Berg et al., 2011; Saremi et al., 2013). Across species, *SAA1* and *SAA2* are mainly produced in the liver in response to inflammation (Jensen and Whitehead, 1998; Eklund et al., 2012). The ELISA performed to measure plasma SAA concentrations in our study was not specific to one SAA isoform (cat no. TP802; Tridelta Development Ltd., Kildare, Ireland). Therefore, other hepatic isoforms of SAA may have been more relevant to plasma SAA concentrations than *SAA3* in our study.

Supplementation of SCFP has been shown to enhance production of SAA and HP in beef cattle (Emmanuel et al., 2007) and transition dairy cattle (Zaworski et al., 2014), as well as splenic *NFκB* gene expression in broilers (Chou et al., 2017). In our study, SCFP treatment did not affect plasma concentrations of SAA ($P = 0.33$; Figure 2.7) or HP ($P = 0.18$; Figure 2.8) or hepatic mRNA abundance of *SAA3*, *HP*, or *NFκB* ($P = 0.55$, 0.92 , and 0.96 respectively). Patterns of both plasma SAA and HP were unlike other reports in transition dairy cattle (Humblet et al., 2006; Bionaz et al., 2007; Zaworski et al., 2014; Yuan et al., 2015). Plasma SAA differed by wk ($P < 0.001$), but spiked 2 weeks before calving, instead of within the first week

postpartum as was expected (Humblet et al., 2006; Zaworski et al., 2014). In contrast, plasma HP was elevated throughout the transition period compared to previous observations (Humblet et al., 2006; Bionaz et al., 2007; Zaworski et al., 2014; Yuan et al., 2015). Because basal prepartum levels were elevated, no spike in HP was observed at wk 1 postpartum, though concentrations were similar to those previously reported at this time point (Bionaz et al., 2007; Yuan et al., 2015). Plasma HP remained elevated at wk 2 and 5 postpartum, unlike previous observations where plasma HP declined after wk 1 (Humblet et al., 2006; Zaworski et al., 2014; Yuan et al., 2015). These unusual patterns and values in our study could have contributed to a lack of treatment effects on these inflammatory biomarkers in plasma.

In addition to inflammatory stimuli during the transition period, previous reports have shown that certain inflammatory challenges, such as traumatic injury or infusion of lipopolysaccharide, stimulate simultaneous increases in plasma SAA and HP concentrations (Ansari-Lari et al., 2008; Vels et al., 2009). In our study, plasma HP and SAA concentrations were moderately correlated during wk 1 postpartum ($R = 0.34$, $P = 0.01$), following previously reported trends in transition dairy cattle and during inflammation (Humblet et al., 2006; Bionaz et al., 2007; Ansari-Lari et al., 2008; Vels et al., 2009; Zaworski et al., 2014). However, these inflammatory biomarkers were not strongly correlated at any other time point (all $R < |0.14|$, $P > 0.33$). Interestingly, hepatic mRNA abundance of *HP* and *SAA3* were positively correlated during wk -2 ($R = 0.41$; $P = 0.002$) but not during wk 2 ($R = 0.10$; $P = 0.46$).

Though often generalized under the acute phase response, SAA and HP have unique individual functions. SAA is an apolipoprotein that is involved in cholesterol metabolism (Bode et al., 2012; Eklund et al., 2012). During cases of acute SAA production, SAA can also associate with HDL (Eriksen and Benditt, 1980; Liang and Sipe, 1995; Eklund et al., 2012). Haptoglobin,

on the other hand, has antioxidant properties, specifically by binding free hemoglobin to prevent kidney damage (Dobryszczyka, 1997; Bode et al., 2012). Differences in functions could have affected signaling, production, and clearance of SAA and HP differently in plasma and liver. Additionally, an alternative classification of APP based on fold-change differences after stimuli categorizes SAA in class II, with quicker and more dramatic increases above baseline, and HP in class III, with slower and less dramatic increases above baseline (Petersen et al., 2004). These two proteins share the transcription factor, CCAAT enhancer binding protein, or C/EBP (Li et al., 1990; Wang et al., 2001). Though SAA and HP share the transcription factors NF κ B and C/EBP, SAA can also be regulated by SAA-activating factor, or SAF (Ray and Ray, 1999), and HP by certain Smad proteins (Zauberman et al., 2001). These different regulators could have also contributed to different patterns of the two proteins in our study. Though both APP share similar promoters and stimuli, results from our study suggest that they are not regulated identically.

A dissimilarity between plasma APP kinetics during an acute phase response has been observed previously, as both plasma HP and C-reactive protein, another APP, increased but did not significantly correlate (Körmöcz et al., 2006). Differences in turnover rates could have contributed to the weak relationships between SAA and HP. Plasma concentrations of SAA increase before that of HP after an acute inflammatory stimulus, while HP generally remains in circulation longer than SAA. The peak of SAA occurs around 2-3 days after stimulus, while HP concentrations peak around day 3-5 (Malle and De Beer, 1996; Sorensen et al., 2006; Stenfeldt et al., 2011). In dairy cattle, plasma SAA increased 8 h and plateaued 24-57 h after an LPS challenge. Later on, plasma HP increased 12 h and plateaued 48-72 h after the challenge (Vels et al., 2009). Increases in plasma concentrations are also greater for SAA than HP in response to acute inflammatory stimuli, increasing 100-1000-fold for SAA and 3-8-fold for HP (Malle and

De Beer, 1996; Dobryszczycka, 1997). This dissimilarity further supports some differences in regulation and function.

To evaluate the utility of individual hepatic transcript abundance measurement as a proxy for plasma concentrations, we also explored relationships between the two APP in plasma and their respective mRNA abundances in liver. Plasma HP did not significantly correlate with its hepatic transcript abundance during wk -2 ($R = 0.08$; $P = 0.54$) or wk 2 ($R = -0.13$; $P = 0.34$). Plasma SAA also did not significantly correlate with hepatic transcript abundance of *SAA3* at wk -2 ($R = -0.22$; $P = 0.12$) or wk 2 ($R = -0.05$; $P = 0.72$). Vels et al. (2009) observed a lag time in hepatic mRNA expression and protein abundance in blood of SAA and HP in dairy cattle after an LPS challenge. Kalmovarin et al. (1991) reported HP in multiple extrahepatic tissues, which could contribute to plasma HP, particularly during peak acute phase response (Wang et al., 2001). Extrahepatic tissues could have contributed to the elevated plasma HP concentrations we observed throughout the transition period; however, our cows were most likely not experiencing peak acute phase response for this long of a period. Steel et al. (1993) also detected a disconnect between mRNA and protein abundance of SAA in human hepatoma cells, suggesting control by post-translational events. Analysis by qPCR showed 3-fold greater hepatic mRNA expression of *SAA1* at d -14 versus d -65 relative to calving date, though plasma concentrations were not analyzed (Loor et al., 2005). Though plasma SAA and hepatic *SAA3* mRNA abundance were not correlated at wk -2 in our study, the increase of another isoform of SAA could have contributed to the spike in plasma SAA at wk -2. Multiple extrahepatic tissues produce SAA as well (Ray and Ray, 1999; Upragarin et al., 2005), and could have contributed to plasma SAA concentrations, particularly during the spike in wk -2. Our cattle could have been experiencing acute inflammation during wk -2, or throughout the transition period, but other hepatic and

plasma biomarkers do not support this explanation. Liver biopsies were performed around the same time as blood collections, but Vels et al. (2009) found that liver biopsies did not cause an acute phase response in dairy cattle. We also demonstrated that measuring hepatic mRNA encoding inflammatory biomarkers may not describe the total body acute phase response.

Retinol binding protein (**RBP**) is produced in the liver and allows retinol to be transported in circulation (Chew, 1987). During inflammation and infection, RBP production decreases (Rosales et al., 1996), while production of SAA and HP increases (Ansari-Lari et al., 2008; Vels et al., 2009). In addition, Derebe et al. (2014) found that SAA production requires vitamin A and that SAA can tightly bind retinol. The authors suggested that SAA could compensate for decreased production of RBP during infection and act as a carrier protein for retinol, permitting its transport into circulation. In our study, the overall relationship between plasma SAA and retinol was significant but negative and only explained 5% of the variance for plasma SAA ($R = -0.22$, $P = 0.002$). Plasma HP tended to predict plasma retinol, but again the relationship was negative and explained only 1% of the variance in plasma HP ($R = -0.10$; $P < 0.10$). During wk -2 when plasma SAA unexpectedly spiked, neither plasma HP or SAA were correlated with retinol concentrations ($R = -0.05$, $P = 0.67$ and $R = -0.04$, $P = 0.86$, respectively).

Instead, plasma retinol concentrations predicted hepatic HP mRNA abundance ($R = -0.44$; $P = 0.001$). Both act as antioxidants (Dobryszczyka, 1997; Montilla et al., 1998), and low retinol concentrations in plasma may signal the liver to increase HP production to compensate. Lesser retinol availability may also lead to increased inflammation 2 wk postpartum, as HP is also a biomarker of inflammation.

Innate Immune Response: PMN Oxidative Burst

Polymorphonuclear (PMN) leukocytes, primarily consisting of neutrophils, are important innate immune cells that respond quickly to infection or tissue damage. Though not a target-specific process, these cells phagocytose pathogens and produce ROS as a killing mechanism. This process of oxidative burst is used as a measure of PMN function (Root and Metcalf, 1977; Moya et al., 2008). Oxidative burst of PMN is summarized in Table 2.5. Treatment with SCFP did not affect oxidative burst of unstimulated PMN ($P = 0.28$; Figure 2.9), indicating basal function was not different between treatments. Overall, SCFP treatment did not affect innate immune response as measured by PMA-stimulated oxidative burst of neutrophils ($P = 0.47$; Figure 2.9). In contrast, SCFP supplementation to immune cells *in vitro* decreased ROS production by stimulated neutrophils (Jensen et al., 2007; Jensen et al., 2008). Similarly, supplementation of 0.3% yeast cell wall extract decreased PMN oxidative burst in weanling piglets compared to controls during and after the supplementation period (Sauerwein et al., 2007). However, supplementation of 0.03% of this same yeast product oppositely increased respiratory burst 2 weeks after supplementation ended, highlighting the effects of dosage (Sauerwein et al., 2007). Supplementation of *Saccharomyces cerevisiae* cells to groupers (Chiu et al., 2010) and a yeast extract to turkeys (Huff et al., 2011) has also been shown to increase leukocyte functions. Though previous reports have described impaired neutrophil and macrophage innate immune cell function in periparturient cows, particularly around calving (Kehrli et al., 1989; Mehrzad et al., 2002; Hammon et al., 2006), PMN oxidative burst did not show differences throughout the transition period in our study ($P = 0.35$). Liang et al. (1998) reported that glucan purified from *Saccharomyces cerevisiae* had no effect on rats before a *Staphylococcus aureus* challenge, but increased neutrophil oxidative burst after the challenge.

Thus, SCFP treatment in this study may have not been able to impact PMN oxidative burst due to a lack of challenge to PMN function.

Polymorphonuclear cells require glucose as an energy source for oxidative burst and phagocytosis (Borregaard and Herlin, 1982; Weisdorf et al., 1982). These cells can take up extracellular glucose or use intracellular glucose stores (Weisdorf et al., 1982). Decreased blood glucose, as occurs after calving, could result in less energy available for PMN function and thus increase susceptibility to infection (Galvão et al., 2010; Sordillo and Raphael, 2013). However, blood glucose was not related to PMN oxidative burst in our study ($R = -0.008$; $P = 0.89$). Lab analysis for PMA-stimulated oxidative burst in our study did not replicate blood glucose available to PMN *in vivo*.

Additionally, these innate immune cells have been suggested to rely on their own glycogen stores instead of extracellular glucose uptake for phagocytosis (Borregaard and Herlin, 1982; Weisdorf et al., 1982). Blood glucose was related to PMN glycogen content in periparturient dairy cows but only explained 5% of the variation (Galvão et al., 2010). Though PMN function was not directly evaluated, Galvão et al. (2010) reported that PMN of cows that developed uterine diseases contained less glycogen compared to PMN from healthy cows. Preventing glucose storage or uptake, as in glycogen storage disease in humans, reduced neutrophil number and function (Kim et al., 2017). Cows with more severe negative energy balance prior to calving had depressed PMN function compared to cows in greater energy balance (Hammon et al., 2006). In our study, energy balance tended to positively correlate with PMA-stimulated oxidative burst during wk 2 postpartum ($R = 0.24$; $P = 0.07$), but not at any other time point (all $P > 0.19$). Thus, energy balance probably relates to PMN function when

severely depressed, as lesser energy balance 2 weeks postpartum related to lesser PMN oxidative burst, but the measure does not account for all changes in function.

Kehrli et al. (1989) reported changes in multiple immune cell functions over time within the transition period, but these changes did not all follow the same pattern. In our study, other functions could have been affected by week, even though PMN oxidative burst did not significantly differ over time. In dairy calves during weaning, supplementation with yeast culture tended to increase neutrophil phagocytosis of a pathogenic strain of *Escherichia coli* (Magalhães et al., 2008). However, phagocytosis of a nonpathogenic strain was not altered (Magalhães et al., 2008). Additionally, yeast cell wall extract affected neutrophil phagocytosis and respiratory burst differently over weaning in piglets (Sauerwein et al., 2007). These results provide evidence for SCFP altering some PMN functions but not others. Treatment with SCFP could also have a greater influence on mucosal immunity, as demonstrated previously in the gut (Shen et al., 2009; Zanello et al., 2011) and uterus (Yuan et al., 2015) with other yeast products.

Another factor that can affect PMN function is parity (Mehrzhad et al., 2002; Moya et al., 2008). Though no treatment $\times$ parity interactions were observed ($P > 0.20$), primiparous cows tended to have greater PMN oxidative burst than multiparous cows (7441 vs. 5572 ± 784 mV relative light units (**RLU**); $P < 0.10$). This result was consistent with other transition period reports (Mehrzhad et al., 2002; Moya et al., 2008). One possible explanation is that primiparous cows also had less severe negative energy balance compared to multiparous cows ($P < 0.01$; Olagaray et al., 2018). Moya et al. (2008) suggested that primiparous cows have a lesser metabolic load during the transition period compared to multiparous cows, as primiparous cows had lesser milk production and greater energy balance. Thus, the PMN of primiparous cows

could have had greater energy availability and/or less stress *in vivo* and thus may have been able to exhibit greater oxidative burst.

Adaptive Immune Response: Anti-ovalbumin IgG Response

The adaptive immune system consists of cytotoxic and humoral responses to defend against and remember specific antigens. The humoral pathway consists of B lymphocytes that produce antibodies, or immunoglobins (**Ig**), that bind specific antigens of pathogens or other foreign bodies for effective recognition and killing (Butler, 1998). Adaptive immunity results from the 49 naïve cows in our study are summarized in Table 2.5. Anti-OVA IgG titers differed by day ($P < 0.001$). This result was expected as cows were naïve to the OVA protein before the subcutaneous injections on d 7 and 21 postpartum, and thus antibody production is expected to increase after these challenges (Butler, 1998; Yuan et al., 2015).

Supplementing SCFP did not affect overall anti-OVA IgG titers in serum ($P = 0.25$). No treatment $\times$ day interaction was detected ($P = 0.31$; Figure 2.10), and treatment contrasts were not significant at any time point (all $P > 0.10$). Similarly, SCFP supplementation did not alter serum Ig titers in transition dairy cows (Zaworski et al., 2014); however, cows in this study were not subjected to a specific challenge, such as vaccination. When dairy calves were challenged with OVA vaccination, supplementation of *Saccharomyces cerevisiae* culture did not affect antibody response (Magalhães et al., 2008). In transition dairy cows also challenged with OVA vaccines, increasing dose of enzymatically hydrolyzed yeast at 0, 30, 60, and 90 g/d linearly increased anti-OVA IgG titers (Yuan et al., 2015). This yeast product was different from the SCFP fed in our study, and the daily dosage was also greater than the 18.4 g/d fed in our study. Dose also impacted Ig levels in weanling piglets, as yeast cell wall extract fed at 0.03% increased serum IgA titers but supplementation at 0.3% had no effect (Sauerwein et al., 2007).

Though an overall treatment effect was not detected in our study, we did observe a tendency for a treatment $\times$ parity interaction ($P = 0.07$) as anti-OVA IgG titers tended to be greater in primiparous SCFP cows compared to primiparous control cows (0.36 vs. 0.28 ± 0.08 optical density (**OD**); $P = 0.08$). Anti-OVA IgG titers were not affected by treatment among multiparous cows ($P = 0.61$). Previous publications on effects of yeast supplementation on antibody production did not report parity interactions (Zaworski et al., 2014; Yuan et al., 2015). Supplementation of SCFP may have enhanced B lymphocyte activation, thus increasing antibody production, in primiparous cows. Though there were no differences in energy balance between treatments, primiparous cows had greater energy balance than multiparous cows ($P < 0.001$; Olagaray et al., 2018). Natural antibody production was positively correlated to energy balance in early lactation dairy cows (van Knegsel et al., 2007). B lymphocytes from primiparous cows could have had more energy to produce anti-OVA IgG in response to SCFP stimulation.

Transition dairy cattle fed mannan oligosaccharide, a component of yeast cell walls, during the last 3 weeks of the dry period had greater antibody response to rotavirus vaccinations at parturition; however, the product did not affect serum IgA or total serum Ig titers (Franklin et al., 2005). Various yeast products have been shown to enhance antibody production and B lymphocytes in other species as well. In broilers, yeast increased antibody response to Newcastle disease virus after vaccination (Muthusamy et al., 2011). This finding was further investigated by Chou et al. (2017), who found that yeast increased mature B cell markers in the spleen and mature T cell subset counts in the thymus. Additionally, SCFP increased secretory tonsil IgA counts in broilers challenged with pathogenic *Eimeria tanella* (Gao et al., 2009). *In vitro* trials with human immune cells showed that incubation with SCFP enhanced B lymphocyte activation and natural killer cell cytotoxic response (Jensen et al., 2007; Jensen et al., 2008).

Treatment with SCFP may be able to impact the adaptive immune response in the gut as well. Though supplementation with *Saccharomyces cerevisiae* culture did not impact serum anti-OVA IgG titers in dairy calves, Magalhães et al. (2008) still observed decreased incidence of mortality due to diarrhea in supplemented calves compared to control-fed calves. In transition dairy cattle, hydrolyzed yeast fed at low and medium doses increased fecal IgA, the main immunoglobulin from mucosal tissues of the digestive tract (Yuan et al., 2015). Additionally, interferon- γ concentrations in the gut, but not plasma, were greater in nursery pigs fed SCFP than in control pigs and pigs treated with antibiotics (Shen et al., 2009). Treating porcine intestinal epithelial cells with live *Saccharomyces cerevisiae* inhibited enterotoxigenic *Escherichia coli*-induced upregulation of pro-inflammatory cytokine transcripts *in vitro*; however, treatment did not prevent challenge-induced loss of membrane integrity (Zanello et al., 2011). Yeast cells from this study were also able to agglutinate enterotoxigenic *Escherichia coli*. In addition, many studies have demonstrated the ability of yeast cells and components to bind to and interact with pathogens, thus potentially preventing them from invading intestinal tissues and causing infection (Badia et al., 2012; Ganner and Schatzmayr, 2012; Tiago et al., 2012; García et al., 2016). Supplementation of SCFP may have altered other aspects of the adaptive immune system not measured in this study, as demonstrated previously with T cells (Davis et al., 2004; Chou et al., 2017) and natural killer cells (Jensen et al., 2008).

Conclusion

In general, SCFP supplementation did not impact measures of oxidative status, inflammation, innate immunity, or adaptive immunity in this relatively healthy cohort of transition dairy cattle. Plasma α -tocopherol tended to be greater for control cows compared to SCFP-supplemented cows only during wk -2. We also observed treatment $\times$ wk $\times$ parity

interactions for plasma retinol and TEAC, but differences were most likely not biologically relevant. Individual antioxidants generally did not strongly predict TEAC as an overall measure of total antioxidant capacity, but α -tocopherol and GPx at wk -2 prepartum and GPx at wk 2 postpartum were stronger predictors. Uncharacteristic patterns of inflammatory biomarkers in plasma were observed, but were not affected by SCFP. No depression in PMN oxidative burst during the transition period was observed, nor did SCFP affect this variable. SCFP supplementation may enhance adaptive immune response in primiparous cows, as antibody production tended to be greater for SCFP-supplemented primiparous cows compared to control primiparous cows. Future research is warranted to determine effects and mechanisms of SCFP supplementation on oxidative status, inflammation, and immune responses during the transition period, particularly in more challenged dairy cattle.

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Tables and Figures

Table 2.1: Ingredient and nutritional composition of the prepartum and postpartum diets.

	Prepartum		Postpartum	
	Control	SCFP ¹	Control	SCFP
Ingredient, % of DM				
Alfalfa hay ²	-		9.75	
Alfalfa hay ³	-		9.70	
Grass hay	38.03		1.62	
Corn silage	19.83		22.32	
Wet corn gluten feed ⁴	18.44		23.76	
Cotton seed	-		4.05	
Ground corn	8.28	8.23	17.92	17.87
Micronutrient premix ⁵⁻⁸	15.43	15.48	10.88	10.94

Nutrient, % of DM (unless otherwise specified)				
DM, % as-fed	63.3		59.7	
CP	12.9		17.0	
ADF	25.0		17.8	
aNDF	43.1		31.3	
NFC	30.1		37.6	
Starch	15.3		22.6	
Crude fat	5.1		6.3	
NE _L , Mcal/kg	1.42		1.66	

¹Saccharomyces cerevisiae fermentation product

²Lower quality alfalfa with 22.1% CP

³Higher quality alfalfa with 23.9% CP

⁴Sweet Bran (Cargill Inc., Blair, NE)

⁵Prepartum control micronutrient premix consisted of 37.5% SoyChlor (Anionic feed supplement (West Central Cooperative, Ralston, IA), 34.3% soybean meal, 7.51% calcium propionate, 6.44% calcium sulfate, 5.36% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ), 2.68% Reashure (28.8% choline chloride, Balchem Corp., New Hampton, NY), 2.14% vitamin E (20 kIU/g), 1.29% stock salt, 1.03% Niashure (65% niacin, Balchem Corp.), 0.54% magnesium oxide, 0.33% 4-Plex (Zinpro Corp., Eden Prairie, MN), 0.21% vitamin A premix (30 kIU/g), 0.19% selenium,), 0.15% Zinpro 120 (Zinpro Corp.), 0.10% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.09% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), 0.06% vitamin D premix (30 kIU/g), 0.02% ethylenediamine dihydriodide premix (3.65% I).

⁶Prepartum SCFP micronutrient premix consisted of 37.2% SoyChlor (Anionic feed supplement (West Central Cooperative, Ralston, IA), 34.0% soybean meal, 7.44% calcium propionate, 6.37% calcium sulfate, 5.31% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ), 2.66% Reashure (28.8% choline chloride, Balchem Corp., New Hampton, NY), 2.12% vitamin E (20 kIU/g), 1.27% stock

salt, 1.02% Niashure (65% niacin, Balchem Corp.), 0.94% SCFP (NutriTek; Diamond V, Cedar Rapids, IA), 0.53% magnesium oxide, 0.33% 4-Plex (Zinpro Corp., Eden Prairie, MN), 0.21% vitamin A premix (30 kIU/g), 0.19% selenium,), 0.15% Zinpro 120 (Zinpro Corp.), 0.10% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.09% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), 0.06% vitamin D premix (30 kIU/g), 0.02% ethylenediamine dihydriodide premix (3.65% I).

⁷Postpartum control micronutrient premix consisted of 59.9% expeller soybean meal (SoyBest, Grain States Soya, West Point, NE), 12.0% limestone, 10.5% sodium bicarbonate, 7.48% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ), 2.40% magnesium oxide, 2.14% 1.50% stock salt, 1.50% trace mineral salt, 1.50% potassium chloride, 1.50% vitamin E (20 kIU/g), 0.94% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), 0.25% selenium premix (0.06%), 0.23% 4-Plex (Zinpro Corp., Eden Prairie, MN), 0.15% vitamin A premix (30 kIU/g), 0.12% Zinpro 120 (Zinpro Corp.), 0.06% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.04% vitamin D premix (30 kIU/g), 0.01% ethylenediamine dihydriodide premix (3.65% I).

⁸Postpartum SCFP micronutrient premix consisted of 59.4% expeller soybean meal (SoyBest, Grain States Soya, West Point, NE), 11.9% limestone, 10.4% sodium bicarbonate, 7.42% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ), 2.37% magnesium oxide, 1.48% stock salt, 1.48% trace mineral salt, 1.48% potassium chloride, 1.48% vitamin E (20 kIU/g), 0.93% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), 0.87% NutriTek (Diamond V, Cedar Rapids, IA), 0.25% selenium premix (0.06%), 0.23% 4-Plex (Zinpro Corp., Eden Prairie, MN), 0.15% vitamin A premix (30 kIU/g), 0.12% Zinpro 120 (Zinpro Corp.), 0.06% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.04% vitamin D premix (30 kIU/g), 0.01% ethylenediamine dihydriodide premix (3.65% I).

Table 2.2: Oxidative status parameters and inflammatory biomarkers in plasma of control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from 29 ± 5 days prepartum to 42 DIM by treatment.

Variable ²	Control	SCFP	SEM ¹	P-values					
				Trt	Wk	Trt × Wk	Parity	Trt × Parity	Trt × Wk × Parity
Retinol, µg/mL	0.33	0.34	0.01	0.66	< 0.001	0.21	0.11	< 0.001	< 0.001
α-Tocopherol, µg/mL	4.75	4.61	0.21	0.56	< 0.001	0.06	0.05	0.36	0.07
MDA, µmol/L	25.9	26.6	0.84	0.52	< 0.001	0.74	0.14	NS ³	NS
TEAC, mmol/L	0.69	0.68	0.01	0.54	< 0.001	0.21	0.21	0.10	< 0.001
GPx, mU/mL	0.93	0.99	0.07	0.32	< 0.001	0.44	0.03	NS	NS
SAA, ng/mL	10.0	12.0	1.72	0.33	< 0.001	0.72	0.62	NS	NS
HP, ng/mL	514	575	44.0	0.18	0.07	0.51	0.75	0.09	NS

¹ Pooled standard error of the mean

² MDA = malondialdehyde; TEAC = Trolox equivalent antioxidant capacity; GPx = glutathione peroxidase, SAA = serum amyloid A, HP = haptoglobin

³ NS = Not Significant; Interaction not included in final model because $P > 0.20$.

Table 2.3: Gene primers for quantitative reverse-transcriptase PCR of liver samples

Transcript ¹	Primer	Primer Sequence (5' to 3')	Accession Number ²
<i>Nrf2</i>	Forward	CTCAGCATGATGGACTTGGAG	NM_001011678.2
	Reverse	CTCATGCTCCTTCTGTCTGTTG	
<i>MT1E</i>	Forward	TCAGATCGGGTGTCTCTTCT	NM_001078134.2
	Reverse	GGAGCAATTGGGGTCCATT	
<i>GPX3</i>	Forward	ACACTCCCATGCTAGACCCT	NM_174077.4
	Reverse	GCAGAGCAGTACGGACACAA	
<i>HP</i>	Forward	TGGGTCGTGTGGGTTATGTG	NM_001040470.2
	Reverse	TACGGGGCTCTTAGCTGTCT	
<i>SAA3</i>	Forward	GCTGGCCTGCCTAACAAGTA	NM_001242573.1
	Reverse	CTGTCCTTGGGGACTCACAG	
<i>NFκB</i>	Forward	ACCTCCTTCCGCAAACCTCAG	NM_001076409.1
	Reverse	ATAGGTCCTTCCTGCCCGTA	
<i>β-actin</i>	Forward	ACGACATGGAGAAGATCTGG	NM_173979.3
	Reverse	ATCTGGGTCATCTTCTCACG	
<i>RPS15</i>	Forward	GGCGGAAGTGGAACAGAAGA	NM_001024541.2
	Reverse	GTAGCTGGTCGAGGTCTACG	

¹ *Nrf2* = nuclear factor E2-related factor 2; *MT1E* = metallothionein 1E; *GPX3* = glutathione peroxidase 3; *HP* = haptoglobin; *SAA3* = serum amyloid A; *NFκB* = nuclear factor kappa-light-chain-enhancer of activated B cells; *β-actin* = beta actin; *RPS15* = 40S ribosomal protein S15

² From NCBI Nucleotide Database (<https://www.ncbi.nlm.nih.gov/nucleotide/>)

Table 2.4: Hepatic mRNA abundance of oxidative stress mediator genes and inflammatory mediator genes was measured in control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from 29 ± 5 days prepartum to 42 DIM by treatment by wk. All target genes in samples were normalized against the control genes β -actin and RPS15. Messenger RNA abundance was calculated as $2^{-\Delta C_t}$ and data were log transformed for statistical analysis. Results were back-transformed and scaled such that the wk -2 control mean equaled 1 for each transcript.

					P-values					
Variable ²	Treatment	Wk -2	Wk 2	SEM ¹	Trt	Wk	Trt × Wk	Parity	Trt × Parity	Trt × Wk × Parity
Oxidative stress mediator genes										
<i>Nrf2</i>	Control	1	1.68	0.31	0.79	0.11	0.22	0.53	NS ³	NS
	SCFP	1.47	1.58	0.36						
<i>MT1E</i>	Control	1	2.10	0.48	0.74	0.02	0.73	0.10	0.42	0.10
	SCFP	0.98	1.71	0.43						
<i>GPX3</i>	Control	1	3.42	0.68	0.57	< 0.001	0.45	0.06	NS	NS
	SCFP	0.71	3.56	0.66						
Inflammatory mediator genes										
<i>HP</i>	Control	1	2.69	1.15	0.92	0.01	0.41	0.22	NS	NS
	SCFP	0.62	3.97	1.44						
<i>SAA3</i>	Control	1	1.96	0.50	0.55	0.10	0.58	0.63	NS	NS
	SCFP	0.97	1.35	0.40						
<i>NFκB</i>	Control	1	1.66	0.38	0.96	< 0.05	0.91	0.17	NS	NS
	SCFP	1.04	1.64	0.39						

¹ Pooled standard error of the mean.

² NS= Not Significant; Interaction not included in final model because $P > 0.20$.

³ *Nrf2* = nuclear factor E2-related factor 2; *MT1E* = metallothionein 1E; *GPX3* = glutathione peroxidase 3; *HP* = haptoglobin; *SAA3* = serum amyloid A; *NFκB* = nuclear factor kappa-light-chain-enhancer of activated B cells; β -actin = beta actin; RPS15 = 40S ribosomal protein S15

Table 2.5: Innate immune response was measured by PMA-stimulated oxidative burst of polymorphonuclear cells (PMN) isolated from blood of control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from 29 ± 5 days prepartum to 42 DIM by treatment. Adaptive immune response was measured by anti-ovalbumin immunoglobulin G (IgG) in serum of control cows and cows supplemented with SCFP from 29 ± 5 days prepartum to 42 DIM by treatment. Cows were challenged on d 7 and d 21 after calving with an innocuous protein, ovalbumin, with adjuvant. Serum samples were collected on d 7, 21, 28, and 35 postpartum. Only the 49 naïve cows (25 control and 24 SCFP) were included in this analysis.

	Control	SCFP	SEM ¹	<i>P</i> -values					
Variable				Trt	Wk	Trt × Wk	Parity	Trt × Parity	Trt × Wk × Parity
Innate immune response									
PMA ² -stimulated oxidative burst, mV RLU ³	6684	6204	641	0.47	0.35	0.31	< 0.10	NS ⁴	NS
Adaptive immune response									
Anti-ovalbumin IgG, optical density	0.29	0.32	0.03	0.25	< 0.001	0.31	0.51	0.07	NS

¹ Pooled standard error of the mean

² Phorbol 12-myristate 13-acetate

³ Relative light units

⁴ NS = Not significant; interaction not included in final model because $P > 0.20$.

Figure 2.1: Plasma α -tocopherol concentration was not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d -29 ± 5 relative to calving through 42 DIM ($P = 0.56$). There was an effect of wk ($P < 0.001$). A treatment $\times$ wk tendency was detected as α -tocopherol concentrations tended to be greater for control cows compared to SCFP cows during wk -2 ($P = 0.06$). Treatment differences are indicated by * ($P < 0.05$) and † ($0.05 \leq P < 0.10$).

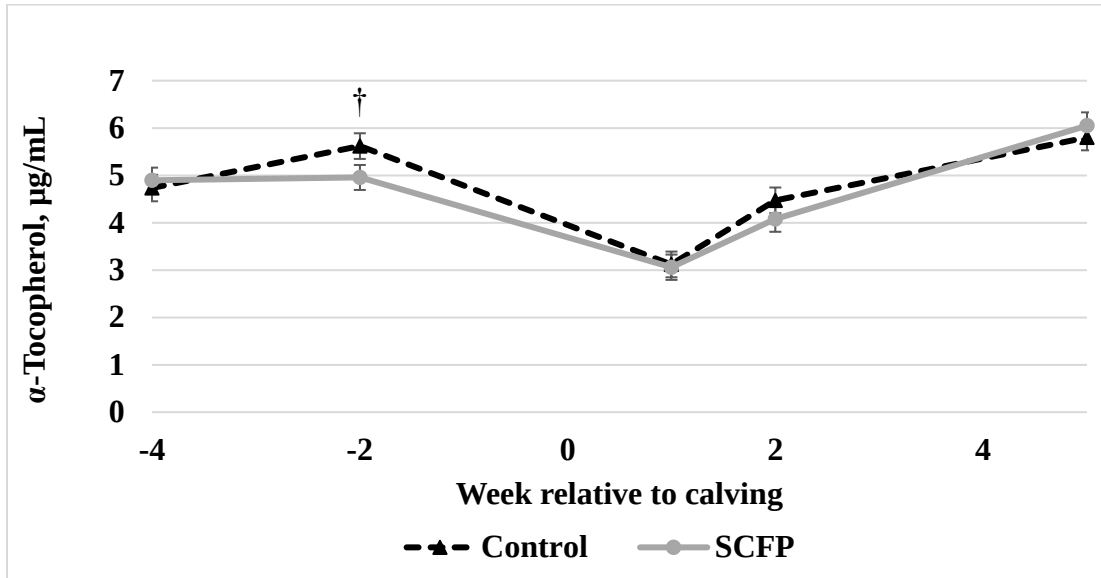


Figure 2.2: Plasma retinol concentrations were not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 ± 5 relative to calving through 42 DIM ($P = 0.66$). There was an effect of week ($P < 0.001$), but no treatment × wk interaction ($P = 0.21$). Though parity did not affect retinol concentrations ($P = 0.11$), there was an interaction of treatment × parity ($P < 0.001$). Additionally, there was a treatment × wk × parity interaction ($P < 0.001$). Treatment differences are indicated by * ($P < 0.05$).

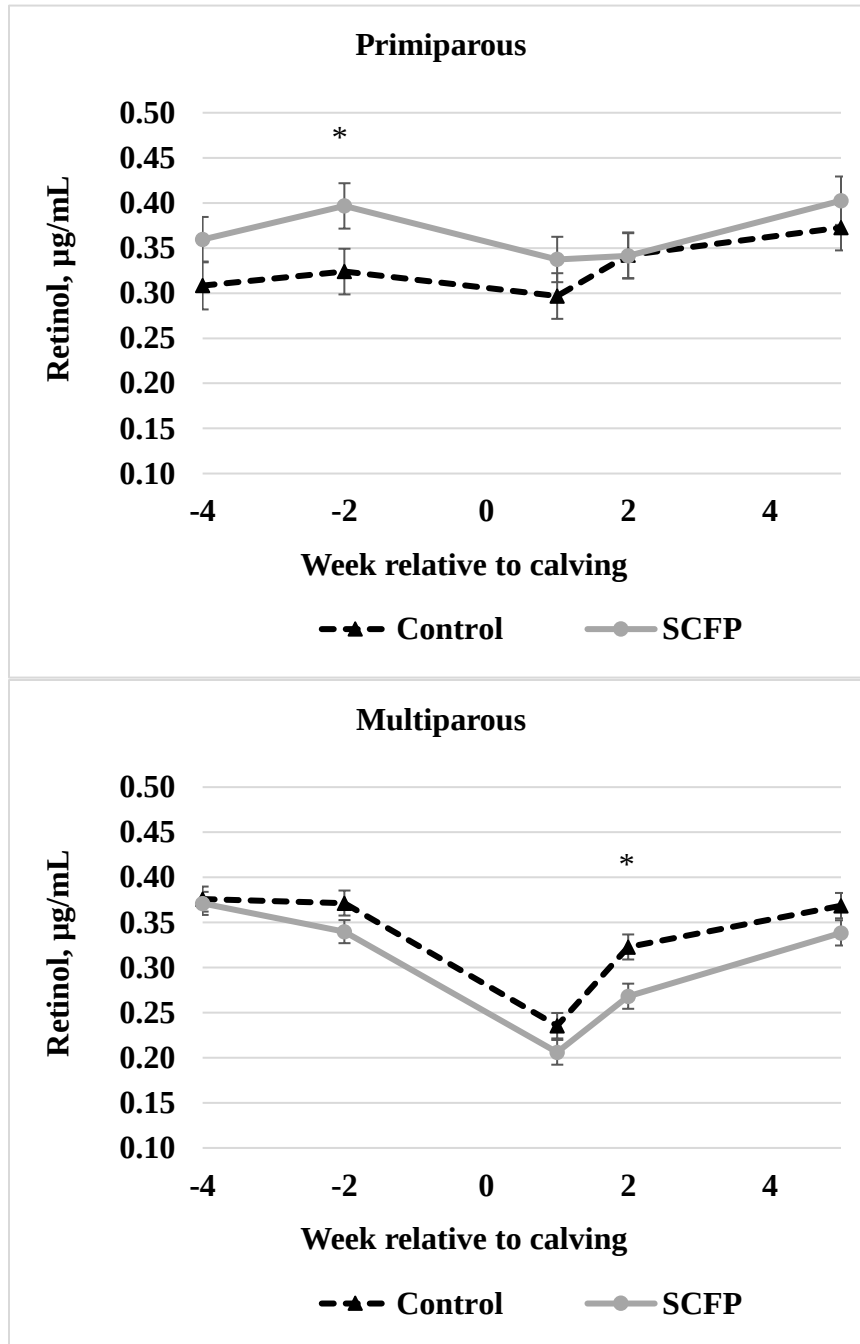


Figure 2.3: Plasma glutathione peroxidase (GPx) activity was not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 ± 5 relative to calving through 42 DIM ($P = 0.32$). There was an effect of week ($P < 0.001$), but no treatment × wk interaction ($P = 0.44$). GPx activity was greater in multiparous cows than primiparous cows (1.10 vs. 0.83 ± 0.08 , $P = 0.03$).

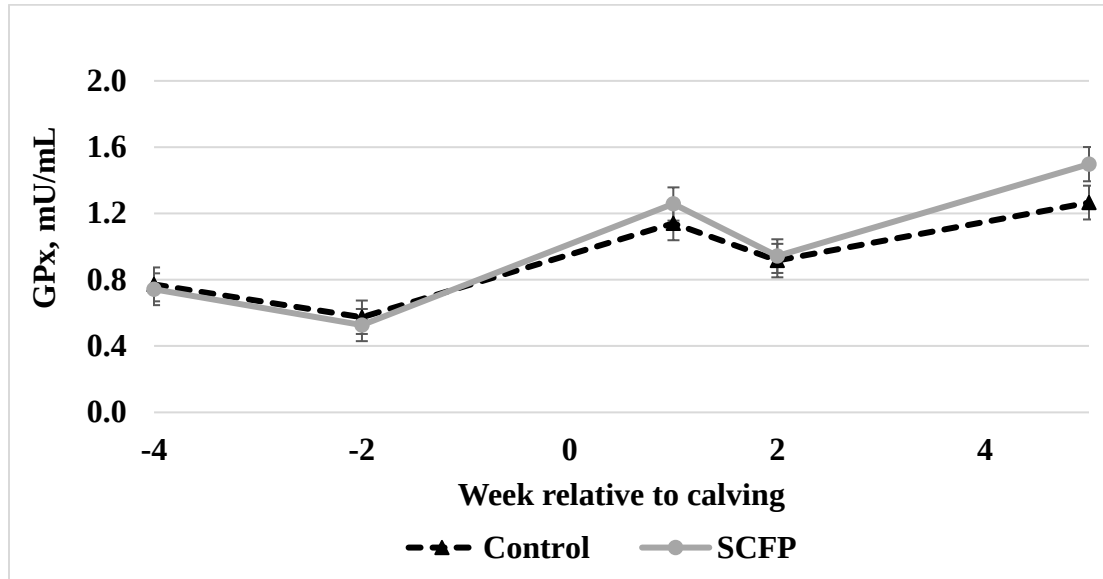


Figure 2.4: Plasma Trolox Equivalent Antioxidant Capacity (TEAC) concentrations were not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 ± 5 relative to calving through 42 DIM ($P = 0.54$). There was an effect of week ($P < 0.001$), but no treatment × wk interaction ($P = 0.21$). Despite no parity effect ($P = 0.21$) and no treatment × parity interaction ($P = 0.10$), there was a treatment × wk × parity interaction ($P < 0.001$). Treatment differences are indicated by * ($P < 0.05$).

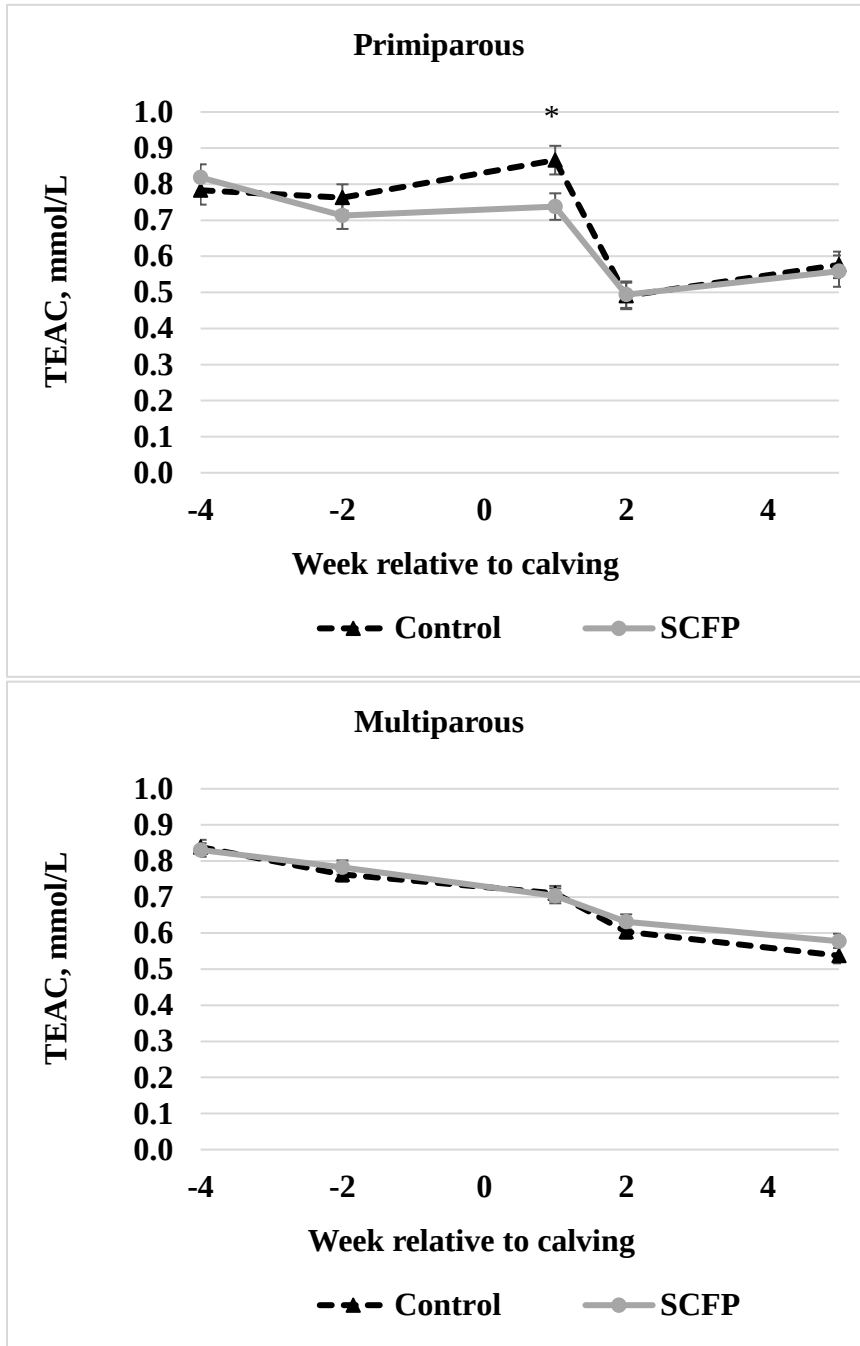


Figure 2.5: Plasma glutathione peroxidase (GPx) enzyme activity significantly predicted plasma Trolox Equivalent Antioxidant Capacity (TEAC), a measure of total antioxidant capacity in blood, during wk 2 postpartum ($R^2 = 0.31$; $P < 0.001$). At this timepoint, GPx activity below ~ 0.8 mU/mL was associated with decreased TEAC, suggesting that GPx activity ≥ 0.8 mU/mL may be necessary for optimal antioxidant capacity.

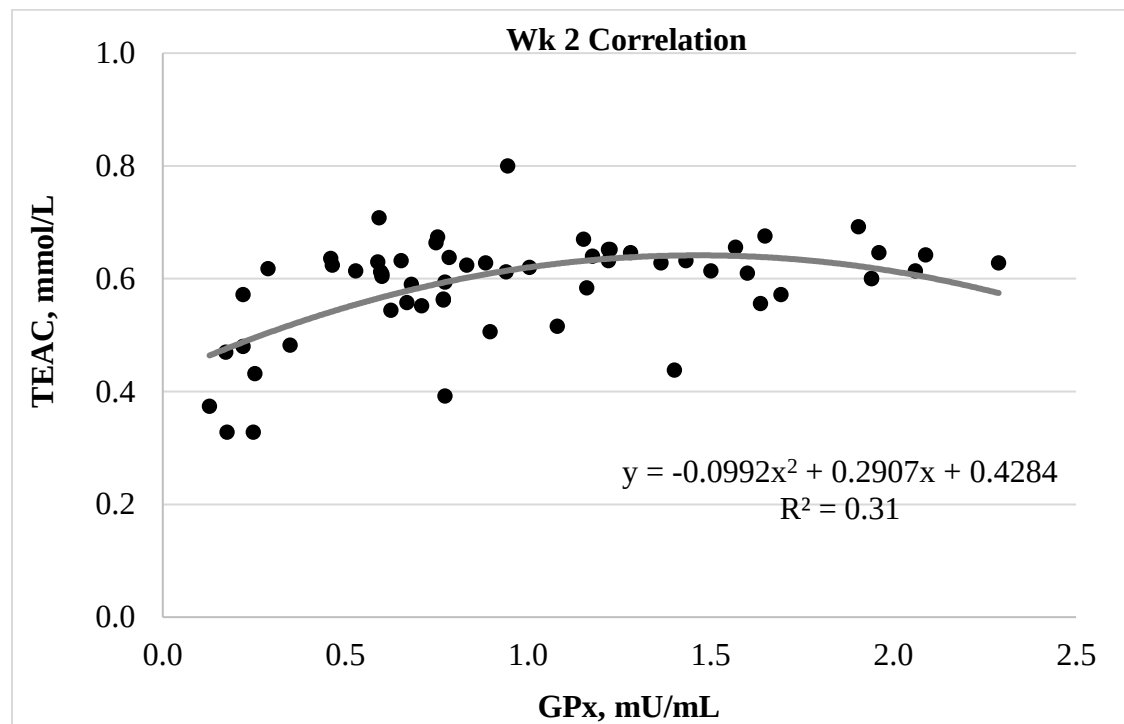


Figure 2.6: Plasma malondialdehyde (MDA) concentration was not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 $\pm$ 5 relative to calving through 42 DIM ($P = 0.52$). There was an effect of week ($P < 0.001$), but no treatment $\times$ wk interaction ($P = 0.74$).

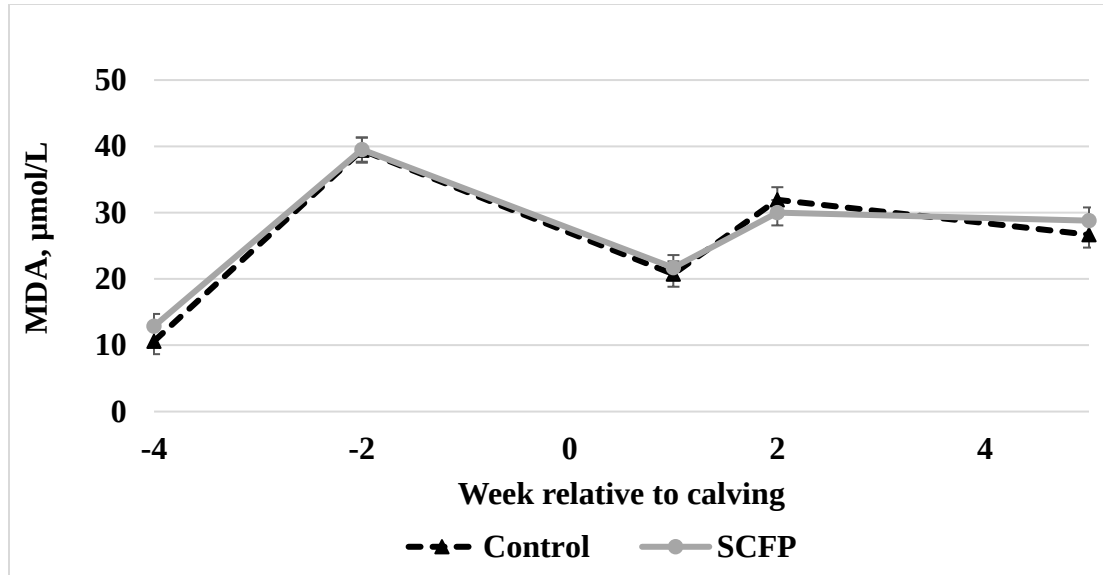


Figure 2.7: Plasma serum amyloid A (SAA) concentration was not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 ± 5 relative to calving through 42 DIM ($P = 0.33$). There was an effect of wk ($P < 0.001$), but no treatment × wk interaction ($P = 0.72$).

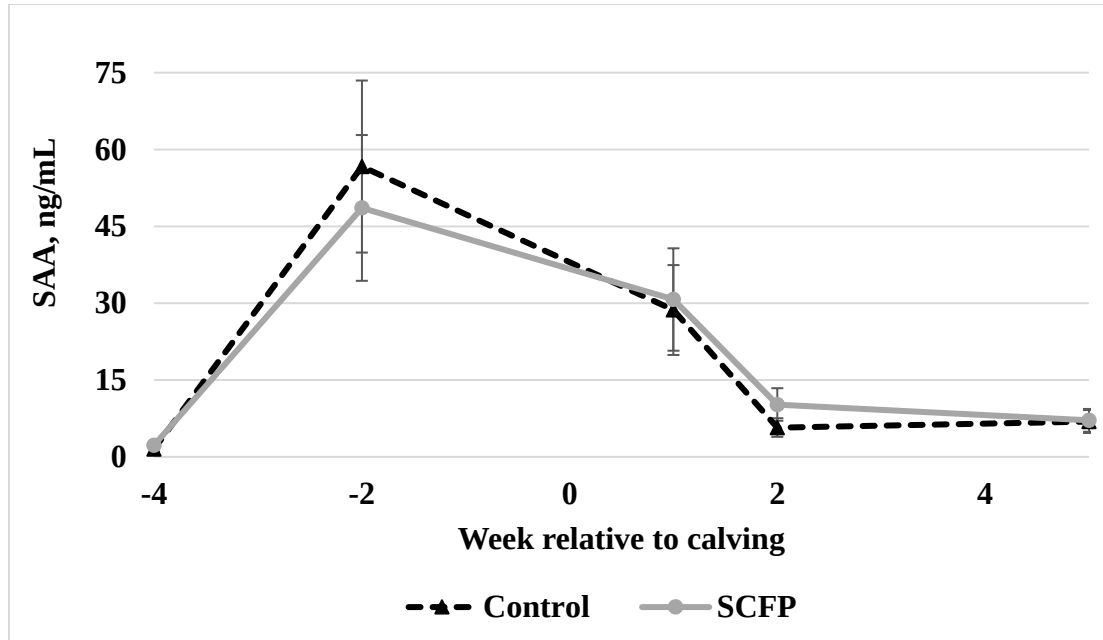


Figure 2.8: Plasma haptoglobin (HP) concentration was not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 ± 5 relative to calving through 42 DIM ($P = 0.18$). Concentrations tended to be different by wk ($P = 0.07$), and there was no treatment × wk interaction ($P = 0.51$).

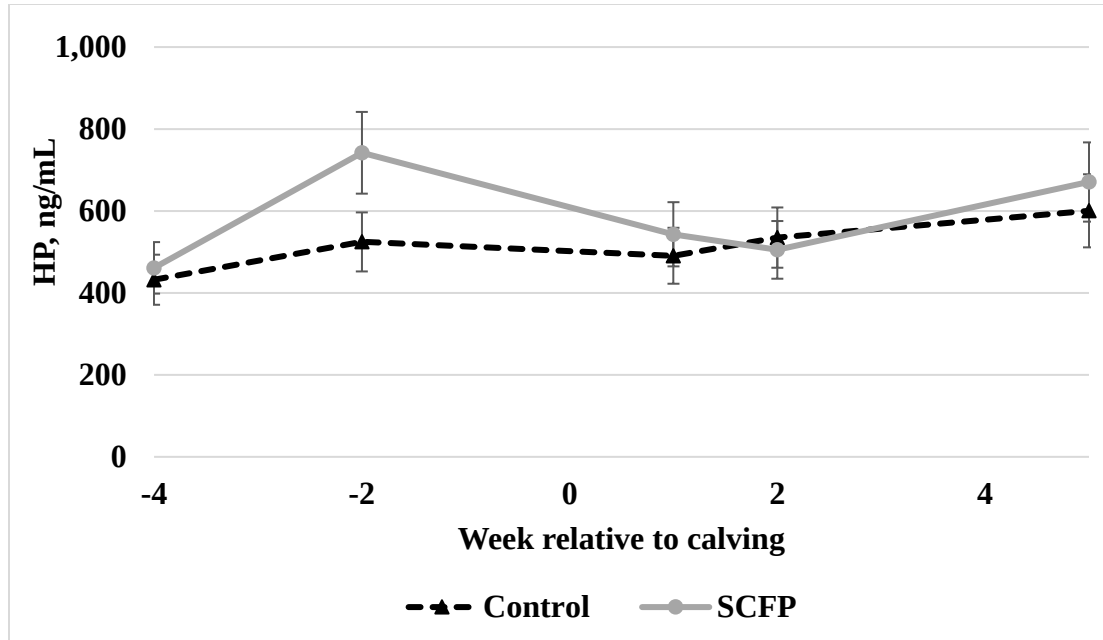


Figure 2.9: Unstimulated oxidative burst of polymorphonuclear cells (PMN) was not different between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 ± 5 relative to calving through 42 DIM ($P = 0.28$). PMA-stimulated oxidative burst of PMN was also not different between treatments ($P = 0.47$). There was no effect of week ($P = 0.35$), and no treatment × wk interaction ($P = 0.31$). However, stimulated oxidative burst of PMN tended to be greater in primiparous cows than multiparous cows (7441 ± 784 mV relative light units (RLU); $P < 0.10$).

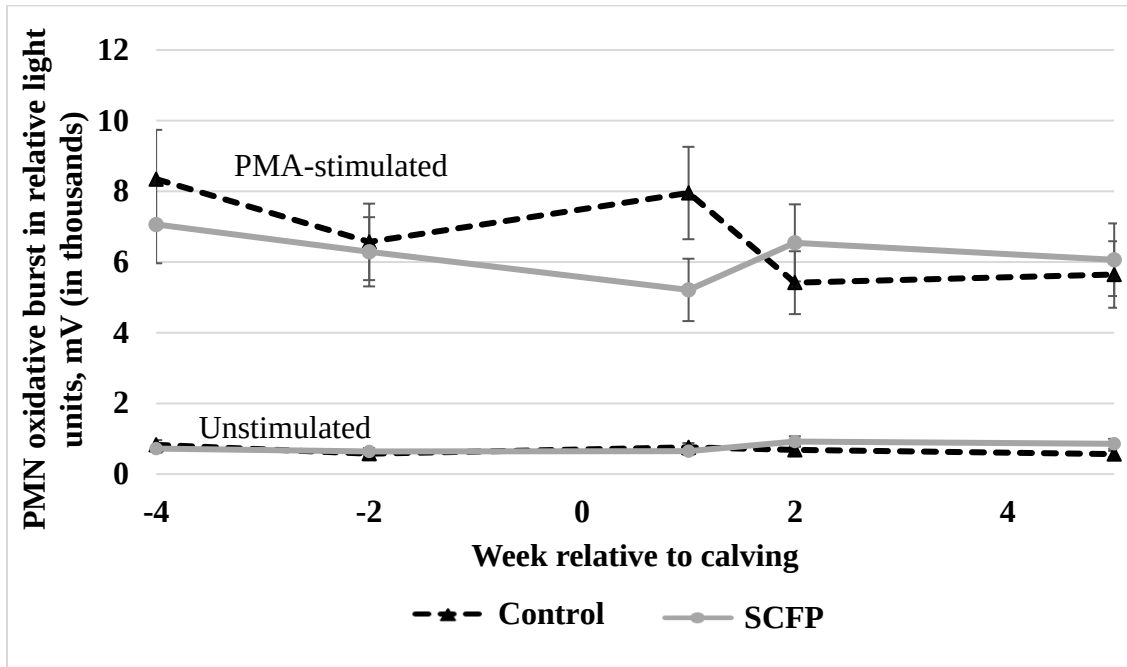
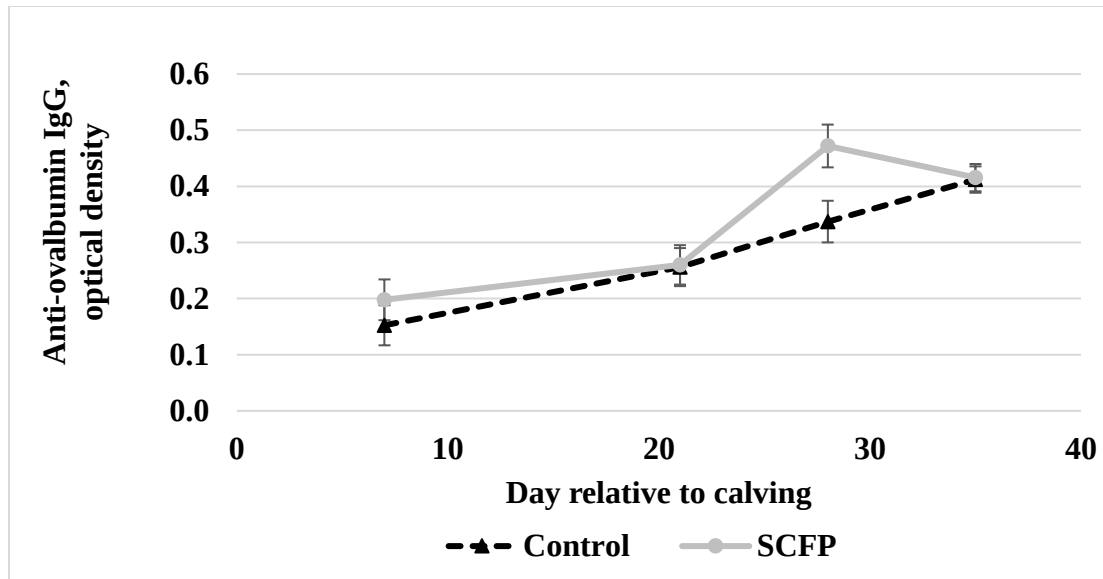


Figure 2.10: Cows were challenged on d 7 and d 21 after calving with an innocuous protein, ovalbumin. Serum anti-ovalbumin IgG levels did not differ between control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from d - 29 ± 5 relative to calving through 42 DIM ($P = 0.25$); treatment contrasts were not significant at any time point (all $P > 0.10$). There was an effect of day ($P < 0.001$), but no treatment × day interaction ($P = 0.31$). However, anti-ovalbumin IgG levels tended to be greater in primiparous SCFP versus primiparous control cows (0.36 vs. 0.28 ± 0.08 optical density; $P = 0.08$). Only the 49 naïve cows (25 control and 24 SCFP) were included in this analysis.



**Chapter 3 - Determining innate immune-modulating components of
Saccharomyces cerevisiae using an *in vitro* macrophage and
intestinal epithelial cell screening system**

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Abstract

While feed components capable of modulating the immune system are highly sought after and marketed, often little *in vivo*, or even *in vitro*, evidence is available to support functional immune response claims. Thus, a high-throughput *in vitro* cell screening system was developed to test these nutrients for innate immune signaling effects, using *Saccharomyces cerevisiae* and its cell wall components as a model. This screening system utilized RAW 264.7 murine macrophages and Caco-2 human intestinal epithelial cells to assess live *S. cerevisiae* cells and *S. cerevisiae*-derived cell wall components β -glucan, mannan, and zymosan (a crude cell wall preparation containing both β -glucan and mannan). D-mannose was also evaluated as the monomer of mannan. RAW cells were transfected with a vector that drives expression of alkaline phosphatase upon promoter activation of nuclear factor κ -light-chain-enhancer of activated B cells (**NF κ B**), a major inflammatory/immune transcription factor. Intestinal epithelial cells contain tight junction proteins that regulate intestinal immunity, and messenger RNA abundance for the pro-inflammatory cytokine IL-8 and the tight junction protein claudin-1, was evaluated in Caco-2 cells. RAW and Caco-2 cells were each incubated with 0.01, 0.1 or 1 mg/mL of yeast compounds alone or were pre-incubated with these treatments followed by a lipopolysaccharide (**LPS**) challenge (protective mechanism). Additionally, RAW cells were challenged with LPS then incubated with yeast compounds (remedial mechanism). In RAW cells, treatment with zymosan or β -glucan alone induced NF κ B activation in a dose-dependent manner, whereas treatment with D-mannose, mannan, or live *S. cerevisiae* cells did not. Pre-treatment with zymosan or β -glucan followed by an LPS challenge increased NF κ B activation, whereas pre-treatment with D-mannose and mannan decreased NF κ B activation, indicating that these components may protect against LPS-induced inflammation. Post-treatment with mannan

and live *S. cerevisiae* after an LPS challenge decreased NF κ B activation, suggesting that these treatments may ameliorate LPS-induced inflammation. However, treatment with live *S. cerevisiae* at 1 mg/mL alone or then followed by LPS challenge decreased, while treatment post-LPS challenge increased, RAW cellular metabolism, demonstrating that evaluation of metabolic activity in the presence of live probiotics may not be accurate with this system. In Caco-2 cells, treatment with β -glucan at 0.01 mg/mL, mannan at 1 mg/mL, and zymosan at 0.1 and 1 mg/mL increased IL-8 mRNA abundance. Interestingly, β -glucan-induced IL-8 mRNA abundance was greater without LPS stimulation. In contrast, claudin-1 mRNA abundance did not differ among treatments. Effects of treatments on the innate immune response generally followed trends reported previously in literature. Overall, this cell screening system using RAW macrophages and Caco-2 cells was effective, high-throughput, and sensitive to *S. cerevisiae* and its cell wall components at different concentrations and with different LPS challenges, indicating that these yeast components modulated innate immune signaling *in vitro*.

Introduction

Nutrients with immune-modulating capabilities are of increasing interest, but *in vivo* data are expensive and time-consuming to obtain, and thus evidence for nutrient-mediated immune effects is often lacking. Therefore, development of an innate immunity *in vitro* cell screening tool would be useful for determining which nutrients to investigate *in vivo*. To develop this screening system, *Saccharomyces cerevisiae* and its cell wall components were used as a model. *Saccharomyces cerevisiae* is a yeast species commonly used in baking and brewing, and is also popularly supplemented in many mammalian diets. The *S. cerevisiae* cell wall, which is 10 – 25% of cell mass, is mostly composed of β -glucans and mannoproteins consisting of 4 – 5%

protein and 25 – 45% mannan (Klis et al., 2006; Lesage and Bussey, 2006; Orlean, 2012). Zymosan, a crude *S. cerevisiae* cell wall extract, contains approximately 50 – 57% glucans, 17 – 22% mannan, and 13 – 17% proteins (Di Carlo and Fiore, 1958), and is a commonly used immune stimulant. β -Glucans of the *S. cerevisiae* cell wall consist of β -1,3-linked glucose backbones with β -1,6-linked glucose branches (Klis et al., 2006) while cell wall mannan, which can include up to 200 mannose residues, consists of α -1,6-linked mannose backbones with α -1,2- and α -1,3-linked branches with up to three mannose residues (Peat et al., 1961; Ballou et al., 1990). These components, along with *S. cerevisiae* cells themselves, impact several aspects of immunity and intestinal health both *in vitro* and *in vivo* (Ganner and Schatzmayr, 2012), and thus serve as a model of interest for development of this *in vitro* screening system.

One central way nutrients can influence immunity is through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (**NF κ B**). Nuclear factor κ B acts as an inducible central regulator of inflammatory responses involved in most innate immune receptor signaling pathways, triggering expression of pro-inflammatory cytokines such as interleukin-8 (**IL-8**) and tumor necrosis factor α (**TNF α**). The canonical pathway of NF κ B activation involves signaling via pattern recognition receptors, including Toll-like receptors (**TLR**) and C-type lectin-like receptors (e.g., mannose receptor; **MR**) found on mammalian cells (Stahl and Ezekowitz, 1998; Newton and Dixit, 2012). After receiving these signals, the inhibitor of kappa B (I κ B) becomes phosphorylated, thus leading to its degradation, and allowing nuclear translocation of NF κ B (Liu et al., 2017). Reviews on NF κ B signaling in inflammation and immunity have been published previously (Li and Verma, 2002; Lawrence, 2009; Liu et al., 2017).

The widely-used RAW 264.7 murine macrophage cell line (Bognar et al., 2013; Merly and Smith, 2017) is useful for assessing broad impacts on inflammatory/immune signaling,

because the cells are armed with a wide variety of pathogen-associated molecular pattern receptors. We used RAW cells stably transfected with a vector that drives expression of an alkaline phosphatase (**AP**) reporter gene upon activation of the response elements for NFκB and activator protein 1.

The Caco-2 human intestinal epithelial cell line can also be used as an *in vitro* gut model, similar to that described by Gonzalez et al. (2013), Khalil et al. (2016), and Koltes and Gabler (2016) using a porcine model. The intestinal epithelium is a critical physical and immunologic barrier that protects the host and plays an important role in maintenance and regulation of gastrointestinal homeostasis (Berkes et al., 2003; Peterson and Artis, 2014); therefore, a gut epithelial cell line was used to more closely model the *in vivo* impacts of dietary compounds on the mucosal immune system and barrier function. Intestinal barrier function is regulated by expression of tight junction proteins, including the claudin family, occludin, and junctional adhesion molecule A (Berkes et al., 2003; Peterson and Artis, 2014). Innate immune activation was monitored by measuring mRNA abundance of interleukin-8 (**IL-8**; a product of NFκB activation) and claudin-1 (**CLDN1**; a tight junction protein) by quantitative real-time polymerase chain reaction (**qPCR**).

The primary objective of the current study was to determine immune-modulating properties of *S. cerevisiae* components in RAW 264.7 murine macrophages and human intestinal epithelial Caco-2 cells. The secondary objective was to develop a high-throughput innate immune *in vitro* cell screening tool capable of identifying potential immune-modulating nutrients. Our hypothesis was that *S. cerevisiae* cells and their major cell wall component, β-glucan, would elicit the strongest immune modulation in both macrophages and intestinal cells.

Materials and Methods

RAW 264.7 Murine Macrophages

Cell Culture Protocol

RAW-Blue™ cells were derived from RAW 264.7 macrophages (cat. no. raw-sp; Invivogen, Manassas, VA). These cells were stably transfected with a vector that drives expression of an AP reporter gene upon activation of the response elements for NFκB and activator protein 1. RAW cell initial growth media consisted of Minimum Essential Media with 4.5 g/L glucose (**MEM**; cat no. 11095080; Thermo Fisher Scientific, Waltham, MA), 10% heat-inactivated fetal bovine serum (**FBS**; various sources), 100 µg/mL Normocin (cat. no. ant-nr-1; Invivogen), and 2 mM L-glutamine (cat. no. 25030081; Thermo Fisher Scientific, Waltham, MA). RAW cell maintenance media consisted of MEM with 4.5 g/L glucose, 10% heat-inactivated FBS, 2 mM L-glutamine, 1% antibiotic-antimycotic (cat. no. 15240062; Thermo Fisher Scientific), 100 µg/mL Normocin, and 200 µg/mL Zeocin (cat. no. ant-zn-1; Invivogen). RAW cell freeze media consisted of 90% FBS and 10% dimethyl sulfoxide (DMSO).

RAW cells were maintained in 75 or 150 cm² culture flasks at 37°C with 5% CO₂ in a humidified incubator. Cells were passaged when they reached approximately 80% confluency, which generally occurred every 2 – 3 days. To passage cells, media was removed and cells were then washed with PBS. A 3-min incubation with 10 mL of trypsin was used to detach cells, followed by the addition of 15 mL of maintenance media. Cells still attached were then gently detached with a cell scraper. All cells were transferred to a 50-mL conical tube and centrifuged at 1500 × *g* for 7 min. After resuspension in maintenance media, cells were returned to culture flasks and placed in the incubator. Alternatively, cells for experiments were resuspended in 1 mL of maintenance media. A 10-µL aliquot of this cell suspension was then transferred to an

microcentrifuge tube and mixed with 90 μ L of media. This mixture was then combined with 100 μ L of Trypan Blue (cat. no. 15250061; ThermoFisher Scientific). Using a hemocytometer (cat. no. 02-671-54; Hausser Scientific, Horsham, PA), RAW cells were counted under a microscope at 10 $\times$ magnification. Cells were resuspended in the appropriate volume of maintenance media to be plated at 1×10^5 cells (180 μ L of cell suspension) per well in clear 96-well plates for AP activity (cat. no. 353072; Corning, Corning, NY) or black-sided, clear-bottom 96-well plates for resorufin formation (cat. no. 3603; Corning). Cultures were terminated when they reached the 20th passage, as recommended by ATCC.

Treatments and Assays

Compounds were added in a dose-titration (log scale) progression, with the objective of determining the concentration at which 50% activation was achieved. This is important for assessing the potential of a feed component to have meaningful impacts at cost-effective doses *in vivo*. Treatments were 0.01, 0.1, or 1 mg/mL of β -glucan (cat. no. 34-621-025MG; Fisher Scientific), D-mannose (cat. no. M6020-25G; Sigma-Aldrich, St. Louis, MO), mannan (cat. no. M7504-250MG; Sigma-Aldrich), live yeast cells (type II, cat. no. YSC2-500G; Sigma-Aldrich), or zymosan (includes cell wall protein-carbohydrate complexes, mannans, and β -glucans; cat. no. Z4250-250MG; Sigma-Aldrich). β -Glucan, mannan, and zymosan were specifically derived from *S. cerevisiae*. The live *S. cerevisiae* cell treatment was dried by the manufacturer to produce 90% viable, active cells. Compounds were first made into stock solutions or suspensions at 10 mg/mL, then serially diluted to 1 and 0.1 mg/mL to achieve final concentrations of 1, 0.1, and 0.01 mg/mL after 1:10 dilution in media. Compounds were dissolved in phosphate-buffered saline (**PBS**), except for β -glucan, which was suspended in 0.1 mM sodium bicarbonate. This

solution did not alter NFκB activation or cell viability compared to PBS diluent (data not shown). In addition, treatment solution pH was either maintained at 7 or adjusted to 3 with HCl to mimic the acidic pH of the stomach and assess potential alterations in treatment activity. Sample size was 6 wells per treatment for all RAW cell experiments.

RAW macrophages were plated at 1×10^5 cells (180 μL of cell suspension in media) per well in 96-well plates. The endotoxin lipopolysaccharide from *Escherichia coli* O111:B4 (**LPS**; cat. no. L4391; Sigma Aldrich) was used as a positive control at a final concentration of 1 μg/mL (200 μL final volume per well). Unstimulated RAW cells were used as negative controls. Treatments were applied for 18 h, then 50 μL of supernatant was transferred to another 96-well plate containing 150 μL of AP substrate solution per well (QUANTI-Blue; cat. no. rep-qb1; Invivogen). This plate was incubated for another 2 h. RAW cells were maintained at 37°C with 5% CO₂ in a humidified incubator for both incubation periods. To assess NFκB/activator protein 1 activation, AP activity was quantified by determining optical density (**OD**) at 620 nm with a colorimetric plate reader (Synergy HTX; BioTek Instruments Inc., Winooski, VT) heated to 37°C, and calculations were performed using Gen5 software (BioTek Instruments Inc.).

In addition to measuring AP activity as an indicator of NFκB activation, cellular metabolism was assessed by resazurin metabolism (Riss et al., 2004). Some compounds can impair cellular metabolism, which is often related to cell viability, thus confounding measurement of capacity for NFκB activation. Resazurin, a non-fluorescent blue compound, is converted to resorufin, a fluorescent pink compound, by viable, metabolically active cells. Dead, metabolically inactive cells are unable to convert resazurin into resorufin, and therefore no fluorescence is emitted.

In black-walled, clear bottom 96-well plates, 20 μ L of a 0.15 mg/mL solution of resazurin sodium salt (cat. no. R7017-5G; Sigma-Aldrich) was added directly to RAW cells in 200 μ L of media following the 18 h incubation with treatments. These plates were then incubated for another 4 h. Cell viability was assessed by measuring fluorescent resorufin formation in relative fluorescent units (**RFU**) after 2 and 4 h of incubation with excitation at 560 nm and 590 nm emission using a fluorometric plate reader (Synergy HTX; BioTek Instruments Inc.) and Gen5 software.

Furthermore, because some compounds may not activate but rather block immune signaling, we also tested compounds in the presence of the endotoxin LPS. The same dose-titration was used with the objective of determining the concentration at which 50% inhibition of LPS-induced NF κ B activation is achieved, if inhibition occurred. To maintain the total volume per well at 200 μ L while incorporating LPS solutions, yeast treatments were initially dissolved or suspended at 20 mg/mL. These initial concentrations were then serially diluted to 2 and 0.2 mg/mL and added at 10 μ L per well. Lipopolysaccharide was added to plated RAW cells at 10 μ L per well to achieve either 0.1 or 0.03 μ g/mL final concentrations.

Both protective (pre-treatment) and remedial (post-treatment) impacts of yeast components were evaluated with respect to LPS challenge. To assess the capacity of the various yeast compounds to prevent LPS-induced NF κ B activation (protective/pre-treatment), RAW cells were first incubated with yeast treatments for 18 h. Lipopolysaccharide was then added at final concentrations of either 0.03 or 0.1 μ g/mL per well directly to cells with yeast treatments and incubated for 6 h and AP activity was analyzed as described previously. To assess the capacity of yeast compounds to aid RAW cells in recovering from an LPS challenge (remedial/post-treatment), RAW cells were first cultured in 96-well plates for 18 h. Cells were

then incubated with either 0.03 or 0.1 µg/mL LPS (final concentration) for 6 h. Because LPS is known to stimulate NFκB activation, all 180 µL of media was then replaced to remove the confounding AP already secreted into cell supernatants. The previously described yeast treatments were then added directly to the fresh media on top of RAW cells and incubated for 4 h before AP measurement. In parallel black-walled, clear bottom 96-well plates, yeast treatments and resazurin were simultaneously added directly to the fresh media on top of RAW cells. These plates were then incubated for 4 h and resorufin formation was measured by fluorescence as described previously.

Process optimization

Additional developments in the RAW cell culturing process were required. This included testing multiple sources of FBS because certain FBS sources had background AP activity (Figure 1), despite the fact that all FBS sources were heat-inactivated. To test this background variation, 5 µL of FBS was added to 45 µL of MEM to replicate 10% of FBS in RAW maintenance media, as would be present in 50 µL of RAW cell supernatant when transferred to QUANTI-Blue AP substrate media. This 50 µL mixture was then added to 150 µL of AP substrate media and incubated for 2 h. Absorbance was then measured as previously described. In addition to differences across suppliers, FBS sources from the same company but from different lot numbers had different AP activity. Observed AP activity of media containing GIBCO exosome-free FBS did not differ from that of PBS or MEM alone ($P > 0.05$; Figure 3.1) and was thus used for RAW cell experiments.

Additionally, multiple plates and batches of RAW cells were required for experimentation over time, and some variation by plate was expected. Thus, RAW cells

stimulated with 1 µg/mL of LPS as a positive control on each plate were used to normalize both NFκB activation and cell viability data across plates. The coefficient of variation for NFκB activation was 16.9% before normalization but 14.7% after normalization, whereas the coefficient of variation for cell viability at the 4 h reading was 16.5% before normalization but 5.71% after normalization. Thus, values normalized by the positive control were used for statistical analysis.

The manufacturer for the QUANTI-Blue AP substrate media recommended incubating cell supernatants with AP substrate for 30 min to 6 h. In our process development, 3 plates of RAW cells were incubated with yeast treatments for 18 h. Cell supernatants were then added to AP substrate media as previously described and AP product was determined by absorbance every 15 min on a plate reader maintained at 37°C from 30 min to 6 h. After these 3 replications ($n = 6$ wells per treatment), the 2 h time point was determined to be sufficient as all enzymatic AP curves were increasing but none had reached a plateau by this point.

Riss et al. (2004) suggested incubating resazurin with cells for 1 – 4 h before analyzing resorufin formation. In our process development, resorufin formation was measured fluorometrically 2 h and 4 h after adding resazurin to wells. The 2-h time point was initially selected to parallel the 2-h reading of AP activity and to limit time required. However, the intra-plate coefficient of variation was 8.98% for the 2 h time point and only 2.82% at 4 h. The inter-plate coefficient of variation was 17.8% at 2 h and only 5.71% at 4 h, when normalized the LPS-stimulated positive controls. Therefore, the 4 h incubation was used for statistical analysis of resorufin formation.

As another optimization step, we determined effects of treatments on cellular metabolism to represent changes in metabolism or viability that may confound measures of NFκB activation.

We also analyzed background fluorescence of treatments in the absence of RAW cells. In the absence of RAW cells, β -glucan, mannan, D-mannose, and zymosan at 1 mg/mL (final concentration in well) did not emit fluorescence when resazurin was added to wells containing yeast treatments in media. As a point of comparison, fluorescence emission was much less for zymosan without RAW cells than for unstimulated RAW cells. When zymosan was added to RAW cells, fluorescence did not change compared to unstimulated RAW cells, indicating that zymosan did not alter RAW cellular metabolism ($P > 0.05$; Figure 3.2). In contrast, live yeast cells (1 mg/mL) without RAW cells were able to convert resazurin to the fluorescent resorufin product, indicating that this live cell treatment was also metabolically active in media. In the absence of RAW cells, 1 mg/mL of the live yeast cell treatment produced significantly more resorufin than 1 mg/mL zymosan ($P < 0.05$; Figure 3.2). Interestingly, resorufin formation was similar between unstimulated RAW cells and live yeast cells at 1 mg/mL; however, the combination of RAW cells and live yeast cells resulted in decreased fluorescence emissions compared to either cell type alone. This result indicates an antagonistic, negative relationship between RAW cells and yeast cells on cellular metabolism.

Caco-2 Human Intestinal Epithelial Cells

Cell Culture Protocol

Caco-2 cells were procured from American Type Culture Collection (cat. no. HTB-37; ATCC, Manassas, VA). Caco-2 cell maintenance media consisted of: MEM with 4.5 g/L glucose (cat. no. 11095080; Thermo Fisher Scientific), 20% heat-inactivated FBS, 2mM L-glutamine (cat. no. 25030081; Thermo Fisher Scientific), 1% antibiotic-antimycotic (cat. no. 15240062; Thermo Fisher Scientific), 1 mM sodium pyruvate (cat. no. 11360070; Thermo Fisher

Scientific), and 1% non-essential amino acids (cat. no. 11140050; Thermo Fisher Scientific). Caco-2 cells were cultured in 25 cm² flasks and maintained in the same conditions as the RAW 264.7 cells. After reaching approximately 50 – 60% confluence, cells were passaged similarly to RAW cells. Caco-2 cells were plated at 1×10^5 cells per well in 96-well black-walled, clear-bottom plates for the cell viability assay. Cells were allowed to propagate in these plates until reaching 100% confluency in a monolayer to replicate intestinal epithelial cells. For qPCR, cells were plated at 4×10^5 cells per well in 24-well culture plates (cat. no. 3524; Corning).

Treatments and qPCR

Experimental protocols using Caco-2 cells were similar to those used in RAW cell analysis. Briefly, yeast compounds were added in a dose-titration progression. Treatments were 0.01, 0.1, or 1 mg/mL (final concentrations) of β -glucan, D-mannose, live yeast cells, or zymosan using the same sources as for the RAW cell treatments. One difference in protocol design for Caco-2 cells vs. RAW cells was that yeast treatments were only used at pH 7 for Caco-2 cells. Caco-2 cells were cultured in 24-well culture plates during treatments with yeast compounds.

To determine the effects of yeast treatments on immune and barrier transcripts, yeast treatments were incubated with Caco-2 cells for 18 h, and then RNA was isolated from individual wells for qPCR. Cellular metabolism was determined after the 18-h incubation with yeast treatments by resorufin formation as described with RAW cells. In addition to yeast treatments alone, Caco-2 cells were challenged with 0.03 μ g/mL of LPS for 2 h, followed by yeast treatments for 4 h. Quantitative PCR was also performed for these cells. Resazurin was

added to parallel plates at the start of the 4 h treatment incubation and resorufin formation was analyzed at the end of the 4 h.

To isolate RNA from Caco-2 cells after treatments, culture plates were first centrifuged at $500 \times g$ for 5 min at room temperature. Supernatant was discarded and 300 μL of RLT buffer (cat. no. 79216; Qiagen, Hilden, Germany) containing 2-mercaptoethanol (10 $\mu\text{L}/\text{mL}$) was added to each well to lyse cells. After a 5-min incubation at room temperature, the lysate was pipetted up and down then transferred to a microcentrifuge tube for immediate storage at -20°C until RNA isolation.

The Protein and RNA Isolation System (cat. no. AM1921; Thermo Fisher Scientific, Waltham, MA) was used to isolate RNA. Tubes were first vortexed and 300 μL of ethanol was added to the lysate. The mixture was then transferred to a Zymo-Spin IICG column (cat. no. C1006-50-G; Zymo Research, Irvine, CA) set inside a collection tube and centrifuged at $10,000 \times g$ for 30 s. After centrifugation, 400 μL of wash buffer #1 (RNA Pre-wash) was added to the column and centrifuged again at $10,000 \times g$ for 30 s. A mixture of 5 μL DNase and 75 μL DNA digestion buffer was then added directly to the column matrix, followed by 400 μL of wash buffer #1 (RNA Pre-wash). After 30 s of incubation at room temperature, the column with the collection tube was centrifuged at $10,000 \times g$ for 30 s. After centrifugation, 700 μL of wash buffer #2 (RNA Wash) was added to the column and the column was centrifuged again at $12,000 \times g$ for 2 min. To elute RNA from the column, the column was transferred to an RNase-free tube and 50 μL DNase/RNase-free water was added directly to the column. The column inside the tube was then centrifuged at $12,000 \times g$ for 1 min. The column was then discarded, and the tube containing RNA was immediately frozen at -80°C .

Quantitative PCR was performed as previously described (Yuan et al., 2013). Briefly, 2 µg of total RNA per sample was used as a template for the reverse transcriptase reaction using random primers (High-Capacity cDNA RT Kit; Applied Biosystems, Foster City, CA). Quantitative PCR was run in duplicate using 5% of the cDNA product on 96-well plates with 200 nM of gene-specific primers and SYBR green master mix (cat. no. 172_5120; BioRad Laboratories, Hercules, CA). All targets were normalized against the geometric mean of 3 reference transcripts. Reference transcripts were beta actin (*β-actin*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and 40S ribosomal protein S9 (*RPS9*). Gene-specific primers for IL-8, CLDN-1, and reference transcripts are listed in Table 3.1.

Statistical Analysis

Data were analyzed using JMP Version 13 (SAS Institute, Cary, NC). For RAW macrophages, the effects of yeast compound, yeast dose, LPS order relative to yeast treatment, LPS dose, and their interactions were evaluated, with plate as a random effect. For Caco-2 cells, all effects were evaluated except for LPS order relative to yeast treatment because only 1 LPS challenge was used with only 1 LPS dose. Values with a Studentized residual > 4 or < -4 were excluded from analysis. When a factor was found to be significant in the overall model ($P < 0.05$), treatment means were separated using Tukey's HSD test to protect against experiment-wise inflation of Type II error.

Results and Discussion

RAW 264.7 Macrophages

Yeast Treatments Alone

Both yeast compound and dose significantly affected NF κ B activation (both $P < 0.001$). To assess overall differences among yeast compounds, we separated compounds based on mean AP activity (across all doses) following 18 h of treatment with the yeast compounds alone (Figure 3.3).

Treatment with β -glucan had an intermediate effect, increasing NF κ B activation relative to untreated negative control RAW cells, whereas treatment with zymosan stimulated the greatest increase in AP activity (Figure 3.3). Overall, treatment dose also affected NF κ B activation of RAW cells ($P < 0.001$), but this reflected impacts of dose only in β -glucan and zymosan treatments, with linear responses to increasing dose for both (Figure 3.3). Zymosan is known to induce NF κ B activation in macrophages (Young et al., 2001; Gantner et al., 2003; Goodridge et al., 2009a); thus, our RAW cell results paralleled previously-published results.. Using the same transfected RAW 264.7 murine macrophage cell line as the current study, Walachowski et al. (2016) also determined that zymosan stimulated the greatest NF κ B activation, which was similar to another crude *S. cerevisiae* cell wall extract that contained only 15% β -glucan but also 33% proteins and 19% mannans, among other compounds. In contrast, the *S. cerevisiae* cell wall extracts containing 65 or 75% β -glucan, which also consisted of 3.7% and $< 1\%$ mannan, respectively, weakly induced NF κ B activation. These results contrast observations with our purified, particulate β -glucan source, as well as those reported by McCann et al. (2005) who also used RAW 264.7 murine macrophages and particulate *S. cerevisiae*-derived β -glucan. Walachowski et al. (2016) also found that TLR2 and TLR4 crosstalk with dectin-1 stimulated greater NF κ B activity with β -glucan stimulation. Similarly, Roy et al. (2011) reported that *S. cerevisiae*-derived β -glucan activated NF κ B via TLR2 and dectin-1 in macrophages isolated from murine wounds. Soluble β -glucan forms from *S. cerevisiae* can

activate NF κ B in human (Battle et al., 1998) and murine (Adams et al., 1997) macrophage cell lines, though results have not been consistent (Walachowski et al., 2016).

Recognition of β -glucan and zymosan involves dectin-1 and TLR2 (Brown et al., 2003; Taylor et al., 2007; Goodridge et al., 2009b). Though zymosan contains mannan, a polymer of mannose, the mannose receptor was not predominant in zymosan recognition by macrophages (Brown et al., 2002). RAW macrophages also required expression of myeloid differentiation primary response gene 88 (**MyD88**) to activate NF κ B in response to curdlan, a particulate and linear β -1,3-glucan derived from bacteria (Kataoka et al., 2002; Walachowski et al., 2016). Though *S. cerevisiae* β -glucans are β -1,3-1,6-glucan, a 99% pure linear β -1,3-glucan was derived from *S. cerevisiae* and similarly stimulated NF κ B activation and expression of pro-inflammatory cytokines, IL-8 and monocyte chemoattractant protein 1 (Zheng et al., 2016). This linear β -1,3-glucan interacted with TLR2 and complement receptor 3 (**CR3**). In contrast, blocking CR3 did not affect zymosan recognition by macrophages, suggesting that CR3 recognition depends on structure. However, zymosan required dectin-1, TLR2, and MyD88 to induce TNF α production in murine peritoneal and RAW 264.7 macrophages (Brown et al., 2003). Interestingly, RAW 264.7 murine macrophages were shown to express only low levels of dectin-1; greater expression of the receptor led to greater zymosan-induced TNF α production (Brown et al., 2003). However, Walachowski et al. (2016) reported that dectin-1 expression could be enhanced by incubation with β -glucans, which further increased TNF α production by this macrophage cell line.

Treatment with D-mannose, mannan, and whole yeast cells did not affect NF κ B activation of RAW cells compared to untreated negative control RAW cells ($P > 0.05$; Figure 3.3). RAW macrophages were able to recognize live *S. cerevisiae* cells, and induced TNF α production in response (Brown et al., 2003). Overexpression of dectin-1 further enhanced live *S.*

cerevisiae cell-induced TNF α production, indicating that whole *S. cerevisiae* cells may modulate similar signaling as its components. Interestingly, live *S. cerevisiae* cells induced greater macrophage binding to the yeast cells and greater TNF α production than live *C. albicans* (Brown et al., 2003), which could indicate greater NF κ B activation as well (Young et al., 2001).

Less literature is available concerning D-mannose and mannan from *S. cerevisiae* than the other compounds. However, mannan from *S. cerevisiae* was shown to induce TNF α production by human monocytes (Tada et al., 2002). This process required CD14 and TLR4, but not TLR2 (Tada et al., 2002). These results suggest that mannan from *S. cerevisiae* may have activated NF κ B, as the inflammatory transcription factor is both a target and inducer for TNF α (Li and Verma, 2002; Palić et al., 2006; Liu et al., 2017); however, this relationship was not observed in our NF κ B activation data. Mannan induced greater TNF α production than the glucan fraction of *S. cerevisiae* in one study (Tada et al., 2002), which again contrasts our observations of greater NF κ B activation with β -glucan vs. mannan (Figure 3.3). However, in the study by Tada et al. (2002), glucan from *S. cerevisiae* barely stimulated TNF α whereas glucan significantly stimulated TNF α in other studies (McCann et al., 2005; Berven et al., 2015; Walachowski et al., 2016). Caution is therefore warranted in making strong conclusions about the inflammatory responses to mannan based on this single prior study.

The mannose receptor is expressed on macrophages and can strongly bind mannose via the mannosyl / fucosyl calcium-dependent recognition pattern (Stahl and Schlesinger, 1980; Taylor et al., 1992; Fraser et al., 1998). As mannan of the *S. cerevisiae* cell wall is composed of mannose, and mannan has been considered a ligand for this pathway, *S. cerevisiae* mannan could also bind to MR on macrophages to elicit effects (Stahl and Schlesinger, 1980; Fraser et al., 1998; Gazi and Martinez-Pomares, 2009). While RAW 264.7 macrophages express MR (Jiang et

al., 2009), MR has been suggested to be more generally expressed on tissue macrophages than circulating macrophages (Fraser et al., 1998). Additionally, Taylor et al. (2002) and Brown et al. (2002) found that macrophage recognition of zymosan was not dependent on MR; however, mannan was able to inhibit dectin-1-mediated zymosan recognition (Taylor et al., 2002). Additionally, Rodriguez et al. (2003) pre-incubated head kidney leukocytes isolated from gilthead seabream with α -mannan and D-mannose, followed by addition of heat-killed *S. cerevisiae*. Pre-incubation with D-mannose, but not α -mannan, inhibited yeast phagocytosis by leukocytes. Alveolar macrophages responded to *Pneumocystis carinii* via MR-mediated NF κ B activation and subsequent IL-8 production (Zhang et al., 2005)

Even though D-mannose alone did not modulate NF κ B activity in our study, it is taken up by alveolar macrophages (Shepherd et al., 1982) and is recognized by the macrophage MR (Gazi and Martinez-Pomares, 2009). Additionally, D-mannose is of interest in intestinal health as it inhibits adherence of enteropathogenic *E. coli* to epithelial cells (Ofek et al., 1977; Firon et al., 1983), which has been implicated in the initiation of pathogenesis (Firon et al., 1983; Berkes et al., 2003), and was shown to prevent *Salmonella typhimurium* colonization of broiler chickens (Oyofe et al., 1989).

In vitro analysis is obviously limited to certain aspects of inflammatory/immune signaling which cannot replicate the many signaling interactions found *in vivo*. However, Rice et al. (2005) found that soluble *S. cerevisiae*-derived β -glucan delivered orally was eventually translocated into blood, where it would come into contact with circulating immune cells. Additionally, Hong et al. (2004) found that oral particulate *S. cerevisiae*-derived β -glucan was taken up by gastrointestinal macrophages and then transported to the spleen, lymph nodes, and bone marrow. Microfold (M) cells located in Peyer's patches of the intestinal lamina propria are

suspected to be at least partially responsible for β -glucan uptake from the intestinal lumen for further processing and transportation (Batbayar et al., 2012)

Resazurin metabolism of RAW macrophages was affected by both yeast compound and dose (both $P < 0.001$; Figure 3.4). Treatment with D-mannose and zymosan did not affect cellular metabolism compared to untreated negative control RAW cells or LPS-stimulated positive control RAW cells ($P > 0.05$; Figure 3.4). Treatment with β -glucan and mannan did not affect metabolism compared to LPS-stimulated RAW cells ($P > 0.05$; Figure 3.4). However, treatment with live yeast cells decreased RAW cell metabolism compared to both untreated and LPS-stimulated RAW cells (Figure 3.4). Dose affected cellular metabolism only for D-mannose and live yeast treatments. Treatment with D-mannose at 1 mg/mL resulted in greater RAW metabolism than D-mannose at 0.01 mg/mL ($P < 0.05$; Figure 3.4), and the 0.1 mg/mL dose was intermediate. As a simple sugar, D-mannose could have been metabolized by the RAW macrophages as shown in alveolar macrophages (Shepherd et al., 1982). Live yeast cells at 1 mg/mL resulted in the least RAW cellular metabolism by far, and the 0.1 mg/mL dose did not have apparent effects on metabolism. Macrophage metabolism and viability have not been reported alongside results of yeast component-mediated NF κ B activation previously, except for the work of Roy et al. (2011), who reported lactate dehydrogenase leakage and thus decreased cell viability of murine wound macrophages with *S. cerevisiae*-derived β -glucan at 100 μ g/mL, but not at 20 μ g/mL. In contrast, we did not observe any decreases in cellular metabolism, which may relate to viability, with β -glucan across different doses used in our study.

Though LPS has previously been reported to induce macrophage apoptosis at doses of 10, 100, and 1000 ng/mL after 48 h of incubation (Battle et al., 1998), LPS at 1 μ g/mL for 18 h did not alter RAW cell viability in our study ($P < 0.05$; Figure 3.4). In the same report, scleroglucan,

a soluble β -1,3-1,6-glucan from *Sclerotium* fungi, also did not induce apoptosis (Battle et al., 1998), consistent with results for the β -glucan treatments in our study.

Effects of Yeast Component Pre-Treatment Followed by an LPS Challenge

Activation of NF κ B was affected by yeast compound ($P < 0.001$), LPS dose ($P = 0.04$), dose of yeast compound ($P < 0.001$), and the interaction between yeast compound and LPS dose ($P = 0.009$), but not the interaction of yeast dose within LPS dose ($P = 0.74$). To assess overall differences among yeast compounds, we first separated compounds based on mean AP activity (across all compound and LPS doses) following 18 h of treatment with the yeast compounds alone and an LPS challenge at either 0.03 or 0.1 μ g/mL for 6 h (Figure 3.5). Pre-treatment with D-mannose and mannan resulted in lesser NF κ B activation than the LPS-stimulated positive control RAW cells ($P < 0.05$; Figure 3.5), suggesting that these components may attenuate effects of LPS on inflammatory signaling in macrophages. D-mannose enhanced immune tolerance of T cells by inducing production of anti-inflammatory cytokine, transforming growth factor- β (TGF- β), by CD4⁺ T cells, thus further inducing differentiation of these T cells into regulatory T cells (Zhang et al., 2017). As a polymer of mannose, mannan may have similar impacts.

In contrast, pre-treatment with β -glucan or zymosan increased NF κ B activation relative to the positive control cells ($P < 0.05$), and zymosan stimulated the greatest NF κ B activation overall ($P < 0.05$; Figure 3.5). Similarly, soluble *S. cerevisiae*-derived β -1,3-1,6-glucan and LPS synergistically induced the pro-inflammatory cytokines IL-8 and TNF α , but not IL-6 (Engstad et al., 2002). However, production of the anti-inflammatory cytokine IL-10 was also synergistically induced (Engstad et al., 2002), potentially indicating some regulatory inflammation controls as

well (Saraiva and O'Garra, 2010). Co-incubation of LPS and scleroglucan, a soluble, fungal (*Sclerotium*) β -1,3-glucan with β -1,6 branches, resulted in lesser NF κ B binding activity and nuclear translocation than LPS alone (Battle et al., 1998), suggesting a possible anti-inflammatory or antagonistic mechanism with simultaneous LPS challenge. In support of combined pro- and anti-inflammatory mechanisms, TNF α production increased while IL-12p70 production decreased when particulate β -glucans and LPS were added simultaneously to bone marrow-dendritic cells (Huang et al., 2009). The process was dectin-1-dependent but MyD88-independent (Huang et al., 2009), suggesting that particulate β -glucan-mediated activation of DC was dependent on dectin-1 but not TLR2 signaling (Batbayar et al., 2012). Macrophages and DC share common pattern recognition receptors, including dectin-1 and TLR2, that are capable of recognizing β -glucans (Batbayar et al., 2012); thus, the results from Huang et al. (2009) may also relate to macrophages from our study. Zymosan and β -glucans can signal NF κ B activation through TLR2, a similar receptor and downstream signaling pathway to TLR4 for LPS recognition (Hoshino et al., 1999). Walachowski et al. (2016) found that zymosan induced TLR4 activity in a TLR4-transfected HEK cell line similarly to LPS, but Sato et al. (2003) observed much less NF κ B activation in TLR4- compared to TLR2-transfected HEK cells in response to zymosan. Pre-treatment with live yeast cells did not alter NF κ B activation relative to LPS-stimulated positive controls ($P > 0.05$; Figure 3.5).

Activation of NF κ B was also affected by the interaction of yeast compound $\times$ LPS dose ($P = 0.009$), reflecting differences in magnitude of response to varying LPS dose across different yeast compounds (Figure 3.5). For most yeast compounds, LPS dose did not significantly alter responses, and activation of NF κ B did not differ between the two LPS doses of the positive controls, either ($P > 0.05$; Figure 3.5). The one exception was the live yeast treatment, for which

the greater LPS dose induced significantly greater NF κ B activity (0.84 vs. 0.93 ± 0.02 OD for 0.03 vs. 0.1 μ g/mL, respectively; $P < 0.05$). Furthermore, the effect of LPS dose (across all yeast compounds) was significant (1.21 and 1.26 ± 0.01 OD for 0.03 vs. 0.1 μ g/mL, respectively; $P = 0.04$).

Cellular metabolism was affected by yeast compound ($P < 0.001$). Pre-treatment with zymosan resulted in the greatest RAW metabolism after an LPS challenge, with D-mannose and mannan also increasing metabolism relative to controls ($P < 0.05$); no other treatments altered metabolism compared to the negative control RAW cells (Figure 3.6). Dose within yeast treatment also affected RAW metabolism ($P < 0.001$; Figure 3.6). Metabolic activity was affected by dose for all yeast compounds ($P < 0.05$) except for mannan. Interestingly, the live yeast cell treatment at 0.1 mg/mL resulted in much greater resazurin metabolism than at 0.01 or 1 mg/mL ($P < 0.05$). Pre-treatment with zymosan followed the same pattern but with smaller differences in metabolism across doses. Cellular metabolism was similar between 0.1 and 1 mg/mL doses of D-mannose, but the 0.01 mg/mL dose resulted in the least metabolic activity. Within the β -glucan treatment, the 0.1 mg/mL dose resulted in the greatest metabolic activity, followed by 0.01 mg/mL, and lastly 1 mg/mL.

While LPS dose and the interaction of yeast compound $\times$ LPS dose did not affect RAW cell metabolism ($P = 0.25$ and 0.39 , respectively), the interaction of yeast compound dose $\times$ LPS dose did affect the variable ($P = 0.01$). Metabolic activity differed across doses of each yeast compound within yeast compound and the LPS dose ($P < 0.05$; Figure 3.6). However, no individual yeast component doses showed a significant effect of LPS dose, suggesting that this outcome reflected subtle differences in magnitude of LPS dose effect across treatments. This result was opposite to previously observed macrophage apoptosis with LPS (Xaus et al., 2000),

but macrophages in this report were from a different source (bone marrow) than RAW macrophages. In another macrophage cell line, LPS was also reported to induce macrophage apoptosis (Battle et al., 1998). Scleroglucan, a soluble β -1,3-1,6-glucan from *Sclerotium* fungi was unable to protect macrophages from LPS-induced apoptosis (Battle et al., 1998).

Effects of Yeast Compound Treatment After an LPS Challenge

Evaluated across doses, yeast compounds affected NF κ B activation ($P < 0.001$, Figure 3.7). This result reflected lesser NF κ B activation with post-treatment of mannan and live yeast cells compared to the LPS-stimulated positive control ($P < 0.05$). These results depict mannan and *S. cerevisiae* as remedial treatments to LPS-induced inflammation. While literature is lacking on the topic, simultaneous incubation with LPS and mannan from *S. cerevisiae* did not act synergistically to induce TNF α production in human monocytes, though each compound did elicit this effect singularly (Tada et al., 2002). In further support of an inflammatory/immunoregulatory role of mannan from *S. cerevisiae*, Che et al. (2012) observed lesser TNF α production and greater IL-10 production by porcine alveolar macrophages incubated with mannan oligosaccharide. In contrast, post-treatment with β -glucan, D-mannose, and zymosan did not result in different NF κ B activation compared to the positive control (Figure 3.7). This result suggests that these yeast compounds are unable to ameliorate LPS-induced inflammatory signaling, but also do not further potentiate LPS-induced NF κ B activation post LPS-challenge.

Dose of yeast compound also affected NF κ B activation ($P < 0.001$). Activation of NF κ B was affected by dose for all yeast compounds ($P < 0.05$; Figure 3.7). Post-treatment with β -glucan at 0.01 mg/mL resulted in greater NF κ B activation than at 1 mg/mL, with 0.1 mg/mL intermediate. Post-treatment with mannan and live yeast cells at 1 mg/mL resulted in lesser

NFκB activation than at 0.01 or 0.1 mg/mL. On the other hand, treatment with zymosan at 1 mg/mL resulted in greater NFκB activation than 0.01 or 0.1 mg/mL doses.

RAW cellular metabolism was affected by yeast compound ($P < 0.001$; Figure 3.8). Post-treatment with live yeast cells resulted in the greatest metabolic activity compared to all other yeast treatments and both negative and positive controls ($P < 0.05$). This result was opposite to the two previous trials of this study (Figures 3.4 and 3.6). Thus, yeast cell incubation with RAW cells for 4 h seems to enrich the measure of cellular metabolism. While these live *S. cerevisiae* cells may induce RAW cell death or decreased metabolism when incubated with RAW cells for 18 h (Figures 3.4 and 3.6), they instead appear to increase resazurin metabolism when only incubated for 4 h (Figures 3.8). Interestingly, while cellular metabolism increased with yeast cell dose, the opposite trend was detected for NFκB activation (Figure 3.7). Thus, observed decreases in NFκB activation were most likely not due to changes in cellular metabolism or viability as the opposite relationship was observed. We speculated that the high apparent metabolism of RAW cells exposed to live yeast may have been due to metabolic activity of the yeast itself, and we indeed found that yeast placed in media in the absence of RAW cells showed substantial metabolism of resazurin (Figure 3.2).

Cellular metabolism was enhanced by D-mannose relative to controls. Post-treatment with zymosan and mannan also resulted in greater metabolic activity than the negative control but not the positive control. Metabolic activity with the β-glucan post-treatment did not differ from either positive or negative RAW cell controls. In addition, activity did not differ between the positive and negative controls. Dose of yeast compound also affected RAW cellular metabolism ($P < 0.001$), but this effect was due entirely to impacts of live yeast cell doses ($P < 0.05$; Figure 3.8), with a linear response to increasing dose.

Cellular metabolism tended to be affected by the interaction of yeast compound $\times$ LPS dose ($P = 0.05$), but no significant differences were found between LPS doses within individual yeast compounds. The interaction of yeast compound dose $\times$ LPS dose within each compound also tended to affect metabolic activity ($P = 0.08$). However, metabolism was not different between the two LPS doses overall in this LPS challenge ($P = 0.41$).

Experimental Design Effects on Viability of LPS-stimulated Macrophages

RAW cellular metabolism was also assessed across the LPS-stimulated positive controls and the negative controls from both the protection and remediation experimental designs. Lipopolysaccharide dose tended to affect metabolic activity, with the 0.1 $\mu\text{g/mL}$ dose resulting in the greatest activity, followed by untreated negative controls and the 0.03 $\mu\text{g/mL}$ LPS dose ($P = 0.08$; Figure 3.9). However, these differences between controls were most likely not biologically relevant. Metabolism of LPS-stimulated positive controls was also affected by order of the LPS challenge relative to yeast treatments, with greater metabolic activity when the LPS challenge occurred after versus before yeast treatments ($P < 0.001$; Figure 3.9). For the yeast pre-treatment experiments, cells were exposed to a 6-h LPS challenge after 18 h of yeast treatments, whereas in the yeast post-treatment experiments, the 6-h LPS challenge was followed by a 4-h yeast treatment recovery period. In the post-treatment design, RAW cells were plated and allowed to settle without stimulus for 18 h before any challenges or treatments. However, in this order of LPS challenge, media was changed after the challenge to remove the LPS stimulus. Thus, LPS stimulus was also removed from the positive controls in this challenge and replaced with normal media for the following 4 h incubation. The difference in cellular metabolism between the two approaches suggests that the media change in the during the post-treatment experiment may have

slightly decreased RAW metabolism, viability, or populations. This finding is important to consider when comparing metabolic activity values between the two LPS challenges, as the yeast post-treatment design will most likely continue to result in lesser metabolic activity than the yeast pre-treatment design.

Cellular metabolism of LPS-stimulated positive controls was also affected by the interaction of LPS dose $\times$ order of LPS challenge relative to yeast treatments ($P = 0.03$; Figure 3.9). When RAW cells were challenged with LPS after yeast treatments, metabolic activity was not affected by treatments ($P > 0.05$). When the LPS challenge occurred before yeast treatments, metabolic activity was greater for RAW cells challenged with 0.1 $\mu\text{g/mL}$ of LPS than RAW cells not challenged by LPS (0 $\mu\text{g/mL}$ dose; $P < 0.05$), but metabolic activity of RAW cells challenged with 0.03 $\mu\text{g/mL}$ was not different from 0 or 0.1 $\mu\text{g/mL}$ LPS doses (Figure 3.9). This result suggests that LPS at 0.1 $\mu\text{g/mL}$, but not the 0.03 $\mu\text{g/mL}$, increased RAW metabolism in the yeast post-treatment design compared to the negative control. Lipopolysaccharide at the greater dose may have stimulated RAW cell propagation. However, LPS doses in the yeast pre-treatment design did not affect RAW metabolism.

Caco-2 Intestinal Epithelial Cells

Innate Immune Activation: IL-8 mRNA abundance

Yeast compound did not significantly affect *IL-8* mRNA abundance in Caco-2 cells ($P = 0.11$). However, yeast compound dose did significantly affect *IL-8* mRNA abundance ($P = 0.01$). This result reflected differences across doses for β -glucan, mannan, and zymosan ($P = 0.02$, 0.003, and 0.01, respectively; Figure 3.10).

Mannan stimulated increases in *IL-8* mRNA abundance (Figure 3.10), which conflicts with those observed by , who reported no change in IL-8 secretion with mannan similarly derived from *S. cerevisiae* at 0.01, 1 or 1 mg/mL for 7 or 24 h. Literature on effects of mannan on intestinal epithelial cells is lacking, and thus the explanation for our results cannot be fully elucidated. However, increased *IL-8* expression in response to 1 mg/mL of *S. cerevisiae*-derived mannan suggests an increase in inflammatory/innate immune signaling with high doses of this compound in intestinal epithelial cells. This could result from TLR4 recognition of the compound, which has been shown to induce TNF α production in human monocytes (Tada et al., 2002).

Zymosan similarly stimulated increases in *IL-8* mRNA abundance with increasing doses in Caco-2 cells after 18 h of exposure (Figure 3.10). This result is in accordance with observations of Saegusa et al. (2004), who observed zymosan-induced IL-8 secretion by Caco-2 cells, and of Cohen-Kedar et al. (2014), who found that zymosan induced secretion of IL-8, in addition to a pro-inflammatory chemokine (C-C motif chemokine ligand 2; **CCL2**) secretion by human intestinal epithelial cell lines HT-29 and SW480. Interestingly, β -glucans in our study also affected *IL-8* mRNA abundance in Caco-2 cells, but mRNA abundance of *IL-8* decreased with escalating doses. Oppositely, *S. cerevisiae*-derived glucan induced IL-8 secretion by Caco-2 cells with increasing dose of the compound (Saegusa et al., 2004). Saegusa et al. (2004) and Cohen-Kedar et al. (2014) observed expression of the dectin-1 receptor, which recognizes β -glucans and the crude extract zymosan, on intestinal epithelial cell lines, including Caco-2 cells as used in our study. Zymosan recognition by this receptor was required to induce secretion of IL-8 and CCL2, as secretion was inhibited when dectin-1 was blocked with a soluble β -glucan (Cohen-Kedar et al., 2014). As zymosan and mannan both enhanced *IL-8* mRNA abundance by

increasing dose, and zymosan also contains mannans (Di Carlo and Fiore, 1958), whereas the β -glucans used in our study did not contain mannans and also induced opposite trends in *IL-8* mRNA abundance by dose, mannans could have contributed to differences in *IL-8* mRNA abundance with zymosan and mannan compared to β -glucan. Mannan, a polymer of mannose, could have additionally been recognized by a mannose receptor found on epithelial cells (Ofek et al., 1977).

Lipopolysaccharide challenge decreased *IL-8* mRNA abundance across yeast treatments ($P = 0.04$; Figure 3.11). In addition, the interaction of yeast compound $\times$ LPS challenge affected *IL-8* mRNA abundance ($P = 0.03$), reflecting a more dramatic suppression of *IL-8* mRNA abundance by LPS within β -glucan treatments (Figure 3.11). There was no evidence of a yeast compound dose $\times$ LPS challenge interaction for *IL-8* mRNA ($P = 0.10$). Intestinal epithelial cells, including the Caco-2 cell line, express TLRs, including TLR2, TLR4 (Cario et al., 2000; Saegusa et al., 2004), TLR6 (Saegusa et al., 2004), and TLR9 (Lee et al., 2006; Peterson and Artis, 2014). Interestingly, TLR9 present on the apical side of intestinal epithelial cells, specifically HCA-7 and Caco-2 cells, has been shown to stabilize I κ B in response to TLR ligands, thus preventing NF κ B activation, while TLR9 present on the basolateral side activates NF κ B (Lee et al., 2006). Additionally, Savidge et al. (2006) and Haller et al. (2000) found that LPS alone did not induce IL-8 production by Caco-2 cells, similar to the observations made by Savkovic et al. (1997) and Eckmann et al. (1993) in intestinal epithelial T84 cells. Demonstrating an even greater extent of tolerance, Savidge et al. (2006) observed that preincubation with LPS followed by stimulus with IL-1 β , a known stimulant of IL-8 production, resulted in lesser IL-8 secretion in an LPS-dose-dependent manner. Similarly, we observed lesser *IL-8* mRNA abundance in Caco-2 cells with the LPS challenge ($P = 0.04$; Figure 3.11); however, the extreme

increase of *IL-8* mRNA abundance with β -glucan in the absence of LPS (interaction) may have led to this observation. These observations suggest an adapted immune tolerance to microbial signals from the intestinal lumen. Though previous reports have shown that IL-8 secretion by Caco-2 cells is induced by live *S. cerevisiae* cells (Saegusa et al., 2004; Saegusa et al., 2007), the lack of IL-8 response to the live yeast cell treatment in our study may have been a result of the aforementioned immune tolerance. The discrepancy in our study compared to these previous reports could also stem from a lack of butyric acid in the medium supplied to Caco-2 cells in our study compared to these previous reports (Saegusa et al., 2004; Saegusa et al., 2007), which was shown to increase mRNA levels of receptors capable of recognizing yeast cells, namely TLR1, TLR6, and dectin-1. Additionally, previous publications have reported an ability of live *S. cerevisiae* cells to reduce enterotoxigenic *Escherichia coli*-induced pro-inflammatory cytokine mRNA abundance in intestinal cell cultures (Zanello et al., 2011; Badia et al., 2012); however, Savkovic et al. (1997) and Eckmann et al. (1993) discovered that only enteropathogenic *E. coli*, and not its purified LPS similar to that used in our study, could induce IL-8 production in T84 intestinal epithelial cells.

Tight Junction Protein: CLDN1 mRNA abundance

Yeast compounds tended to affect *CLDN1* mRNA abundance of Caco-2 cells ($P = 0.07$; Figure 3.12), but overall LS means were not significantly different across yeast compounds (Tukey test). Dose of yeast compound significantly affected *CLDN1* mRNA abundance ($P = 0.02$); however, when tested within compound, no significant dose effects were found ($P > 0.05$; Figure 3.12). Oral *S. cerevisiae*-derived β -glucan administration mitigated reductions in mRNA expression of several intestinal tight junction proteins, including *CLDN1*, induced by oral

Salmonella enterica serovar Typhimurium challenge in chickens (Shao et al., 2013). Data on effects of *S. cerevisiae* and its components on intestinal tight junction proteins *in vitro* and *in vivo* are lacking in the current literature. However, Saegusa et al. (2004) demonstrated that treatment of Caco-2 cells with live *S. cerevisiae* cells for 7 h did not alter transepithelial electrical resistance as a measure of barrier function. Live *S. cerevisiae* cells did decrease transepithelial electrical resistance after 24 h of incubation, but not nearly to the same extent as *Candida albicans*, another yeast species.

Intestinal epithelial cells encounter LPS from Gram negative bacteria within the luminal contents, and LPS can damage the intestinal barrier (Czerucka et al., 2000; Guo et al., 2013). We therefore additionally subjected Caco-2 cells to an LPS challenge after treatment with yeast compounds to determine potential protective effects. While *Saccharomyces boulardii* was shown to prevent enteropathogenic *E. coli*-induced suppression of zonula occludens-1 in intestinal epithelial T84 cells (Czerucka et al., 2000), abundance of *CLDN1* mRNA was not significantly affected by LPS challenge at 0.03 µg/mL for 2 h (Figure 3.13), nor interaction with yeast compound or dose in our study ($P = 0.36, 0.68, \text{ and } 0.65$, respectively). In contrast, basolateral LPS at 1 ng/mL induced Caco-2 tight junction permeability as demonstrated by time-dependent decreases in transepithelial electrical resistance, without inducing cell death; however, this reduction was not observed until d 4 of LPS treatment (Guo et al., 2013). Therefore, our LPS challenge may not have been long enough to affect the intestinal barrier, but a 4-d incubation would not be conducive to the high-throughput objective of our screening system.

Caco-2 Cellular Metabolism

When Caco-2 cells were treated with yeast alone for 18 h, yeast compound significantly affected cellular metabolism ($P < 0.001$). The live yeast cell treatment resulted in the greatest metabolic activity among the yeast compounds ($P < 0.05$; Figure 3.14), which could have resulted from their ability to convert resorufin to fluorescent resazurin on their own (Figure 3.2). Treatment with zymosan resulted in greater metabolic activity than with D-mannose, but was not different from any other yeast compound or the LPS-stimulated positive control, and thus was most likely not biologically relevant. Dose of yeast compound also significantly affected Caco-2 metabolism ($P < 0.001$), which was driven by differences across doses of live yeast cells and zymosan. Live yeast cells at 0.1 mg/mL resulted in lesser metabolic activity than either the 0.01 or 1 mg/mL dose ($P < 0.05$). Zymosan, on the other hand, resulted in greater activity at 1 mg/mL compared to both 0.1 and 0.01 mg/mL doses (Figure 3.14). Caco-2 cellular metabolism viability did not differ between untreated negative controls and any yeast compound; therefore, metabolic activity most likely did not contribute to differences observed in *IL-8* or *CLDN-1* mRNA abundance.

When Caco-2 cells were treated with yeast compounds then subjected to an LPS challenge, yeast compound also affected cellular metabolism ($P < 0.001$; Figure 3.15). Similar to metabolic activity with yeast treatments alone, live yeast cells resulted in the greatest metabolic activity among the yeast compounds ($P < 0.05$). Treatment with zymosan resulted in greater Caco-2 metabolic activity than with D-mannose but was not different from any other yeast compound or the LPS-stimulated positive control. Furthermore, cellular metabolism did not differ between untreated negative controls and any yeast compound ($P > 0.05$; Figure 3.15). Dose of yeast compound also significantly affected Caco-2 cellular metabolism ($P < 0.001$; Figure 3.15), which was reflected by differences across doses only for live yeast cells and

zymosan. Live yeast cells at 0.01 mg/mL resulted in greater metabolic activity than at 0.1 or 1 mg/mL ($P < 0.05$; Figure 3.15). On the other hand, zymosan at 1 mg/mL resulted in greater activity than at 0.01 mg/mL ($P < 0.05$; Figure 3.15), but the 0.1 mg/mL dose was not different from either 1 or 0.01 mg/mL ($P > 0.05$; Figure 3.15). However, cellular metabolism with the LPS challenge at 0.03 μ g/mL overall was not significantly different from the untreated negative control ($P = 1.0$).

Limitations

This RAW macrophage screening system is based on one simple measurement *in vitro*, and thus has limitations on what information can be collected. Most obviously, only one immune cell type was used. *Saccharomyces cerevisiae* and its components also impact other innate immune cells, including dendritic cells and neutrophils (Goodridge et al., 2009a; Sonck et al., 2010; Qi et al., 2011). These cell types also contain NF κ B and pattern recognition receptors that respond to *S. cerevisiae* components; thus, stimulus with *S. cerevisiae* components generally results in similar outcomes as macrophages. However, a main action of both neutrophils and macrophages is oxidative burst, which is affected by *S. cerevisiae* components but was not measured in this system (Sonck et al., 2010); this function is related to NF κ B activation though (Morgan and Liu, 2011). Additionally, adaptive immune cells, such as B and T lymphocytes, and their interactions with antigen-presenting innate immune cells were not evaluated in this screening system.

Though effects of components on an innate immune cell type and a parenchymal cell type were evaluated separately in this system, interactions between immune cells and parenchymal cells were not evaluated. For example, intestinal epithelial cells may help present or prevent interactions with their underlying lamina propria immune cells (Volman et al., 2008; Batbayar et

al., 2012). In this simple cell screening system, potential changes in structure of components through the digestive process is also lacking, which could affect how immune cells or intestinal cells interact with the various components. The availability of these components to immune cells, such as circulating macrophages, also remains unknown; thus, concentrations of components used in this screening system may not be accurate to what would be available to immune cells *in vivo*.

Conclusions

The *in vitro* cell screening system using RAW 264.7 murine macrophages was an accurate, quick, sensitive model for determining effects of *Saccharomyces cerevisiae* components on innate immunity. Assessing cellular metabolic activity also helped to determine if changes in NF κ B activation due to treatments were related to changes in amount of viable, metabolically-active RAW macrophages. For example, we could have erroneously interpreted decreased NF κ B activity induced by the high dose of live yeast to reflect an anti-inflammatory effect (Figure 3.5), rather than a simple decrease in cellular metabolism, viability, or population (Figure 3.6). Screening broad classes of candidate feed additives will likely include some with toxic effects, making the metabolic assay an important component of the system.

Several key steps in protocol optimization were achieved in the process of completing these objectives, including plate-to-plate normalization with LPS-stimulated positive controls, checking background AP activity of FBS sources, and analyzing resorufin formation for cell viability measurements at 4 h instead of 2 h. When RAW cells were treated with various yeast compounds alone, zymosan and β -glucan stimulated the greatest NF κ B activation, consistent with many previous reports in various cell types. Live yeast cells at the greatest dose (1 mg/mL)

appeared to decrease RAW cellular metabolism, which may be due to competition between cell types. Therefore, this RAW cell screening model may not be optimal for assessing effects of “live” treatments, such as by probiotics, on immune cells. The use of LPS challenges before or after yeast treatments was also able to detect differences between treatments and treatment doses to determine potential remedial or protective effects of treatments with a challenge. Treating with LPS at either 0.03 or 0.1 µg/mL increased cellular metabolism compared to the negative control, but LPS challenge before yeast treatments resulted in lesser metabolic activity compared to challenge after treatments, most likely due to different protocol steps between challenges. Nevertheless, the most dramatic differences between yeast components appeared when they were tested as a “recovery” agent after endotoxin challenge, suggesting that this may be a fruitful screening strategy.

The intestinal immunity screening model using mRNA abundance from Caco-2 cells was able to detect differences by treatment and treatment dose with and without an LPS challenge for the innate immune marker *IL-8*, but less so for transcript abundance of the tight junction protein *CLND1*. Further development using a more functional test for Caco-2 intestinal barrier function, such as fluorescein isothiocyanate-dextran permeability assays as described by Oppong et al. (2013), would improve throughput for this step. Overall, this 2-step *in vitro* cell screening system was able to detect differences between treatments and treatment doses on immune cells and intestinal cells.

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Tables and Figures

Table 3.1: *Homo sapiens* primers for quantitative PCR of human Caco-2 cells

Trancript ¹	Primer	Primer Sequence (5' to 3')	Accession Number ²
Inflammatory marker			
IL-8	Forward	TGTCTGGACCCCAAGGAAAAC	NM_000584.3
	Reverse	TGGCATCTTCACTGATTCTTGG	
Tight junction protein (intestinal barrier function)			
CLDN1	Forward	CTGTCATTGGGGGTGCGATA	NM_021101.4
	Reverse	CTGGCATTGACTGGGGTCAT	
Reference genes			
β-actin	Forward	GGCGCCCTATAAAACCCAGC	NM_001101.4
	Reverse	GCGCGGCGATATCATCATCC	
GAPDH	Forward	GAAAGCCTGCCGGTGACTAA	NM_001256799.2
	Reverse	AGGAAAAGCATCACCCGGAG	
RPS9	Forward	TGAAGCTGATCGGCGAGTATG	NM_001013.3
	Reverse	CCTTCTCATCAAGCGTCAGCA	

¹*IL-8* = interleukin-8; *CLDN1I* = claudin-1; *β-actin* = beta actin; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *RPS9* = 40S ribosomal protein S9

²From NCBI Nucleotide Database (<https://www.ncbi.nlm.nih.gov/nucleotide/>)

Figure 3.1: Fetal bovine serum sources vary in alkaline phosphatase activity. To test background AP activity of heat-inactivated FBS without RAW cells, FBS was added to Minimum Essentials Media to replicate 10% of FBS in RAW maintenance media. This mixture was then added to AP substrate media, similarly to the process using RAW cell supernatants, and incubated for 2 h. Without the presence of RAW cells, different FBS sources produced AP product on their own, measured in optical density. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly ($P < 0.05$, Tukey's HSD).

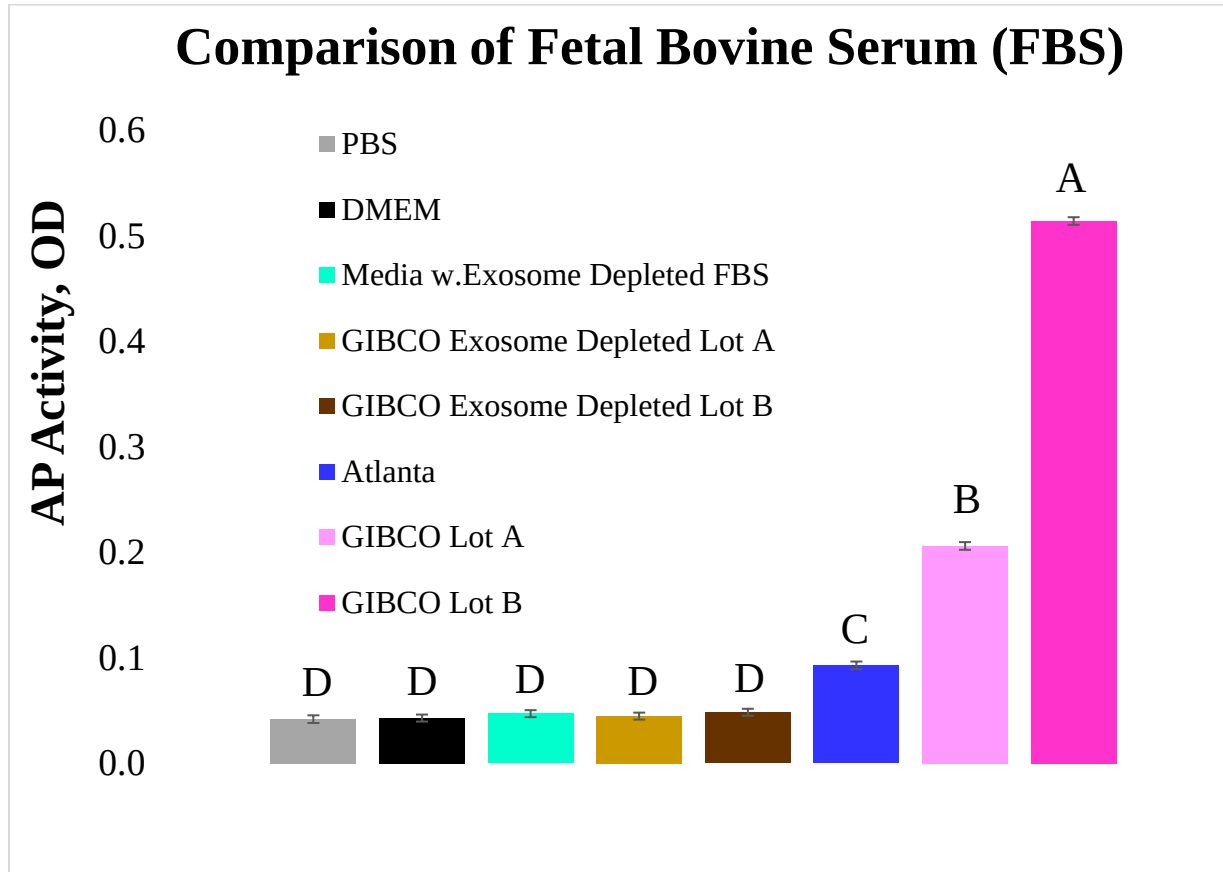


Figure 3.2: Metabolic activity of 1 mg/mL of live yeast cells or zymosan with or without the presence of RAW 264.7 murine macrophages after 18 h of incubation in media. Metabolic activity was assessed by metabolism of resazurin to the fluorescent product resorufin. Untreated RAW cells represent the negative control. Values are LS means $\pm$ SEM, and means of treatments in the absence of RAW cells that do not share a letter differ significantly ($P < 0.05$, Tukey's HSD). Significant differences between means of treatments in the presence of RAW cells are signified: $*P < 0.05$. Zymosan did not emit considerable fluorescence in the absence of RAW cells and did not alter metabolic activity of RAW cells in the presence of RAW cells. Live yeast cells emitted similar fluorescence in the absence of RAW cells as the untreated control RAW cells, but the combination of live yeast cells and RAW cells resulted in decreased fluorescence compared to both untreated RAW cells and live yeast cells without RAW cells.

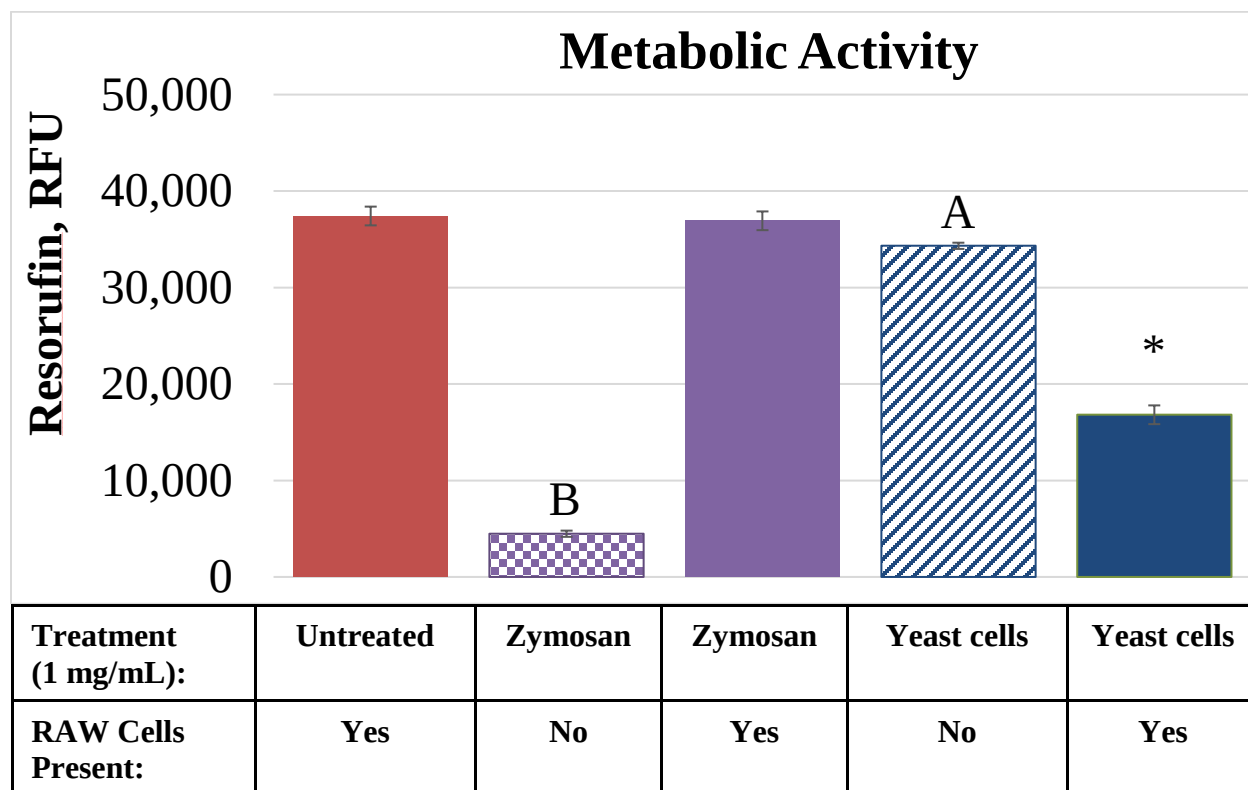


Figure 3.3: Nuclear factor κ B (NF κ B) activation of RAW 264.7 murine macrophages after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL. Activity of NF κ B was assessed with a reporter plasmid driving expression of alkaline phosphatase (AP). Untreated RAW cells represent the negative control, and RAW cells stimulated with 1 μ g/mL LPS served as the positive control. Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly ($P < 0.05$, Tukey's HSD). Significant dose effects within treatment compound are signified: * $P < 0.05$.

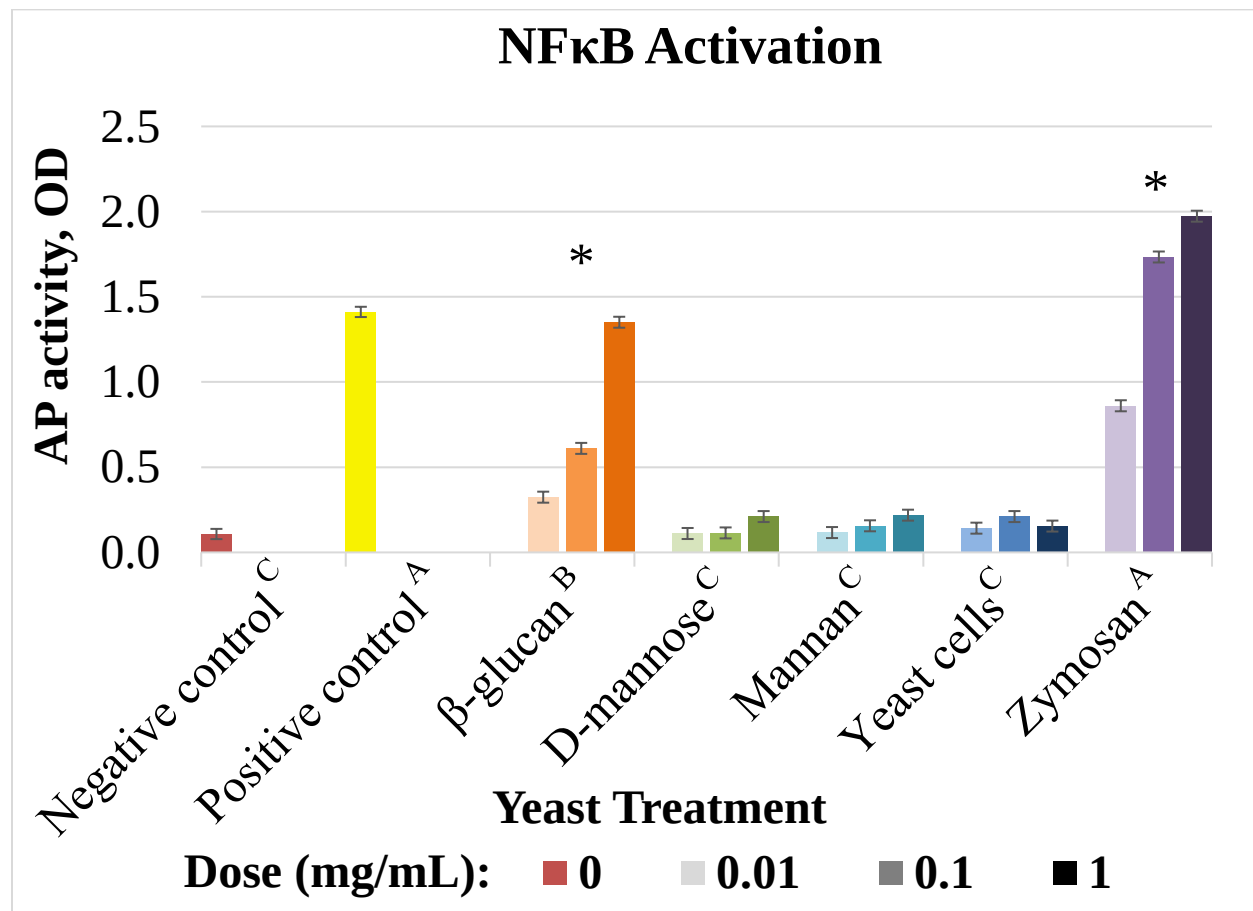


Figure 3.4: Metabolic activity of RAW 264.7 murine macrophages after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL. Metabolic activity was assessed by metabolism of resazurin to the fluorescent product resorufin. Untreated RAW cells represent the negative control, and RAW cells stimulated with 1 μ g/mL LPS served as the positive control. Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly ($P < 0.05$, Tukey's HSD). Significant dose effects within treatment compound are signified: * $P < 0.05$.

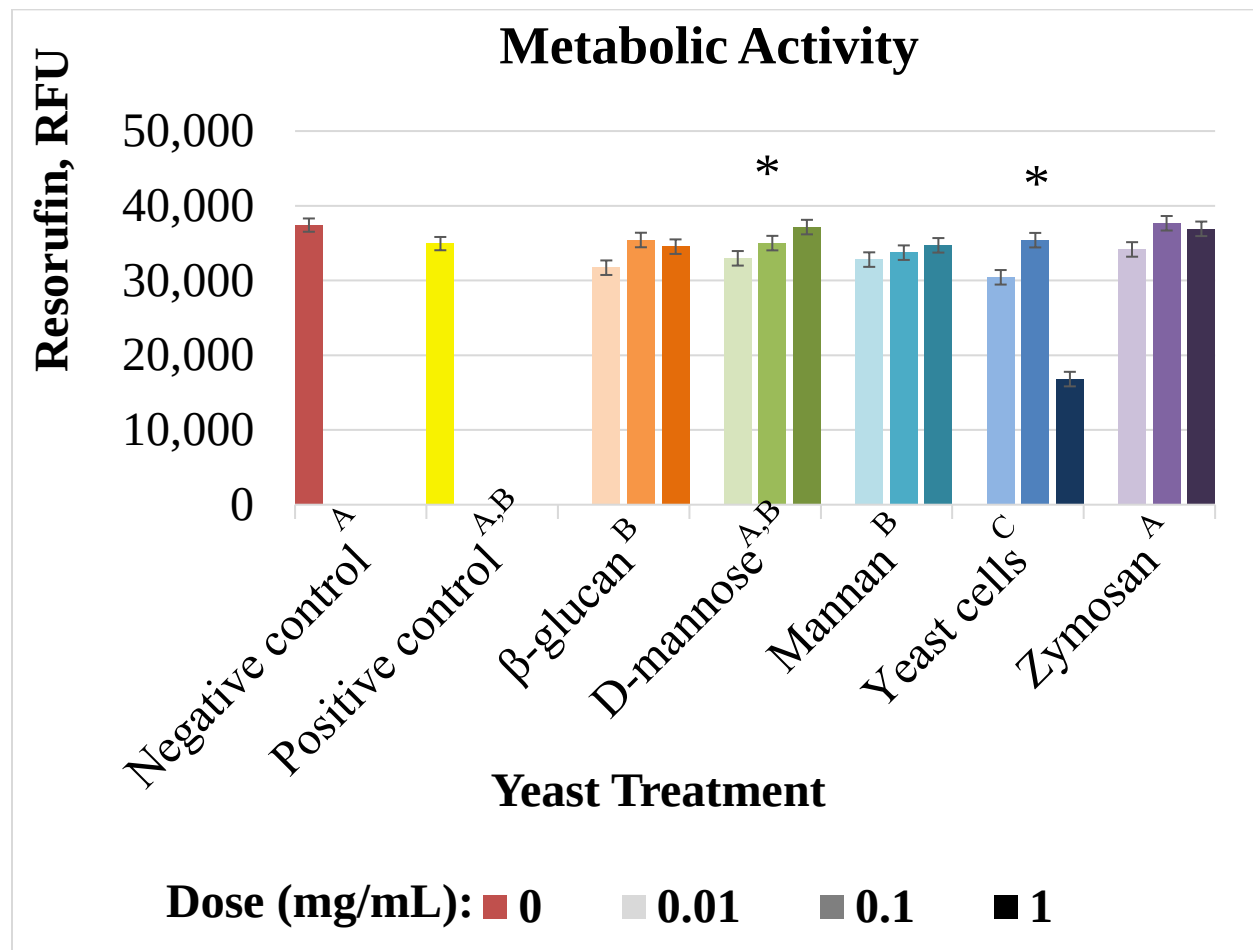


Figure 3.5: Nuclear factor κ B (NF κ B) activation of RAW 264.7 murine macrophages after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL followed by an LPS challenge at either 0.03 or 0.1 μ g/mL for 6 h. Activity of NF κ B was assessed with a reporter plasmid driving expression of alkaline phosphatase (AP). Untreated RAW cells represent the negative control (Neg. con.), and RAW cells stimulated with only 0.03 or 0.1 μ g/mL of LPS represent the positive control (Pos. con.). Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly by yeast compound ($P < 0.05$, Tukey's HSD).

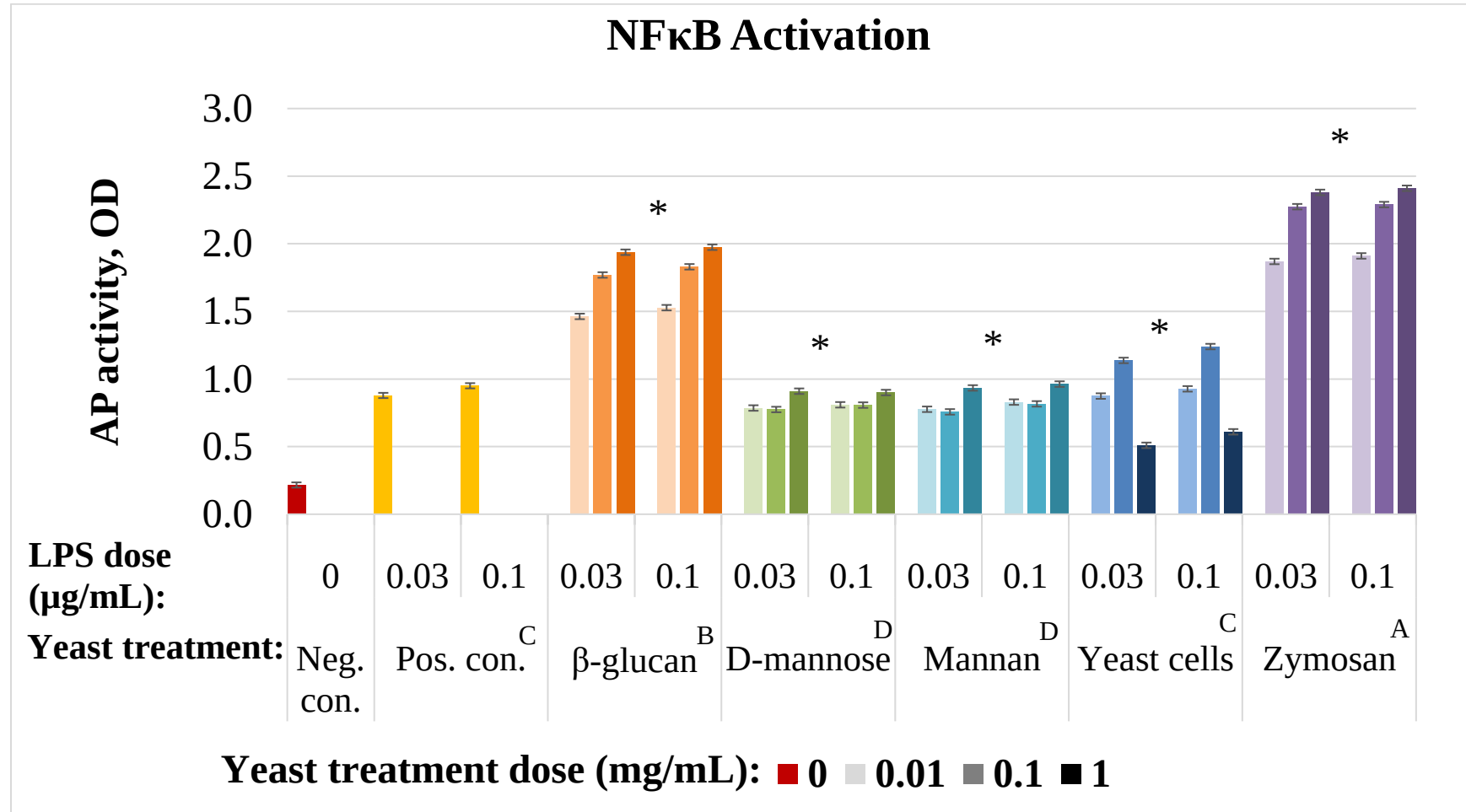


Figure 3.6: Metabolic activity of RAW 264.7 murine macrophages after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL followed by an LPS challenge at either 0.03 or 0.1 µg/mL for 6 h. Metabolic activity was assessed by metabolism of resazurin to the fluorescent product resorufin. Untreated RAW cells represent the negative control (Neg. con.), and RAW cells stimulated with only 0.03 or 0.1 µg/mL of LPS represent the positive control (Pos. con.). Increasing doses are signified by increasing color intensity within treatment compound. Overall, cellular metabolism was not affecting by LPS dose ($P = 0.39$). Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly by yeast compound ($P < 0.05$, Tukey's HSD). Significant dose effects within yeast compound are signified: $*P < 0.05$.

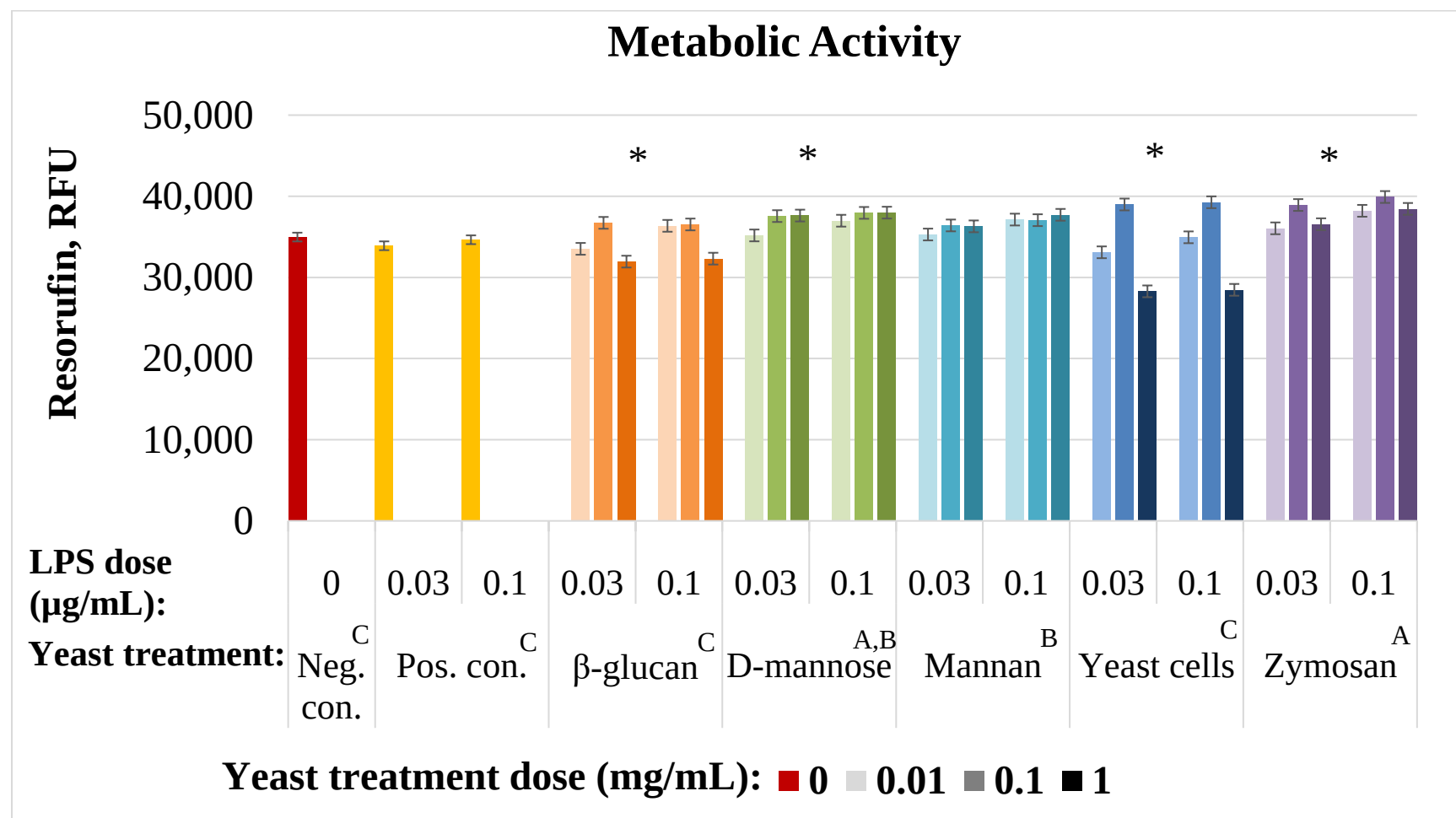


Figure 3.7: Nuclear factor κ B (NF κ B) activation of RAW 264.7 murine macrophages after an LPS challenge at either 0.03 or 0.1 μ g/mL for 6 h followed by 4 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL. Activity of NF κ B was assessed with a reporter plasmid driving expression of alkaline phosphatase (AP). Untreated RAW cells represent the negative control (Neg. con.), and RAW cells stimulated with only 0.03 or 0.1 μ g/mL of LPS represent the positive control (Pos. con.). Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly by yeast compound ($P < 0.05$, Tukey's HSD). Significant dose effects within treatment compound are signified: * $P < 0.05$. Differences between doses of LPS ($P < 0.05$) for each yeast component are indicated by †.

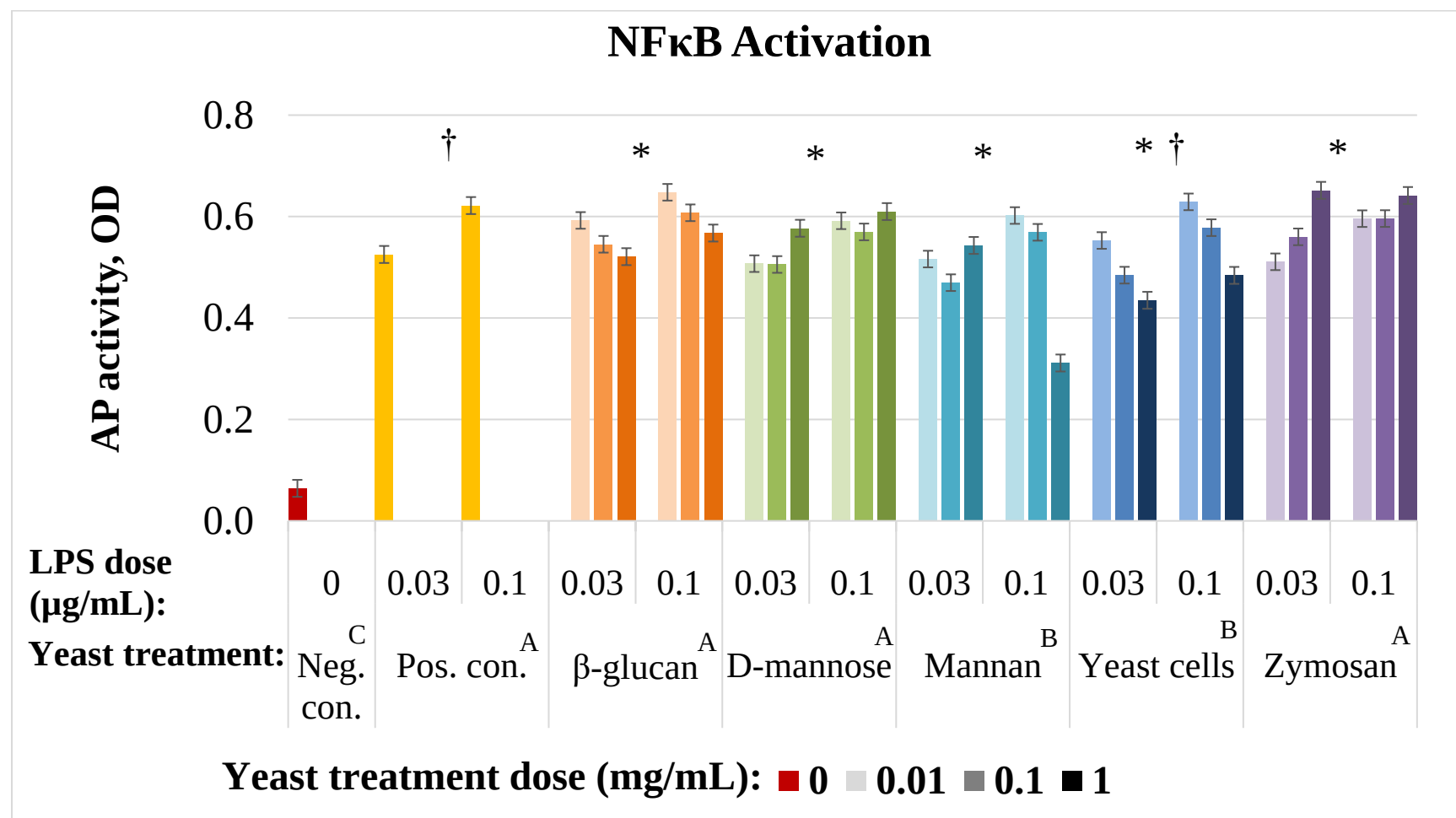


Figure 3.8: Metabolic activity of RAW 264.7 murine macrophages after an LPS challenge at either 0.03 or 0.1 $\mu\text{g/mL}$ for 6 h followed by 4 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL . Metabolic activity was assessed by metabolism of resazurin to the fluorescent product resorufin. Untreated RAW cells represent the negative control (Neg. con.), and RAW cells stimulated with only 0.03 or 0.1 $\mu\text{g/mL}$ of LPS represent the positive control (Pos. con.). Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly by yeast compound ($P < 0.05$, Tukey's HSD). Significant dose effects within treatment compound are signified: * $P < 0.05$. Differences between doses of LPS for each overall yeast treatment are indicated by †($P < 0.05$).

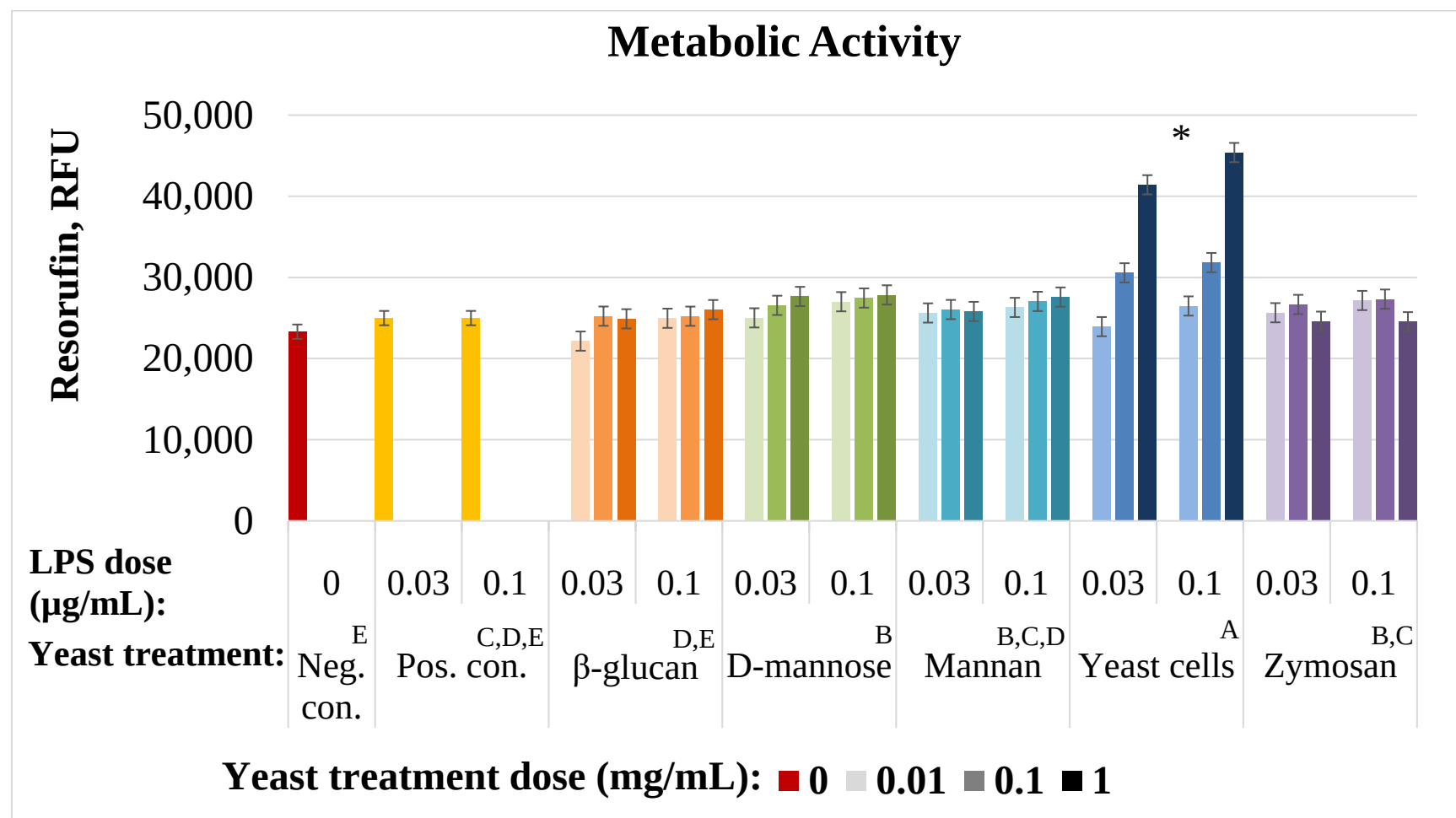


Figure 3.9: Metabolic activity of negative and positive controls of RAW 264.7 murine macrophages between experimental approaches to LPS challenge. Metabolic activity was assessed by metabolism of resazurin to the fluorescent product resorufin. Untreated RAW cells represent the negative control (Neg. con.), and RAW cells stimulated with 0.03 or 0.1 $\mu\text{g/mL}$ of LPS represent the positive controls (Pos. con.). Order 1 represents LPS challenge first followed by yeast post-treatment, while order 2 represents yeast pre-treatment followed by LPS challenge, though data from yeast treatments were not included in this analysis. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly ($P < 0.05$, Tukey's HSD).

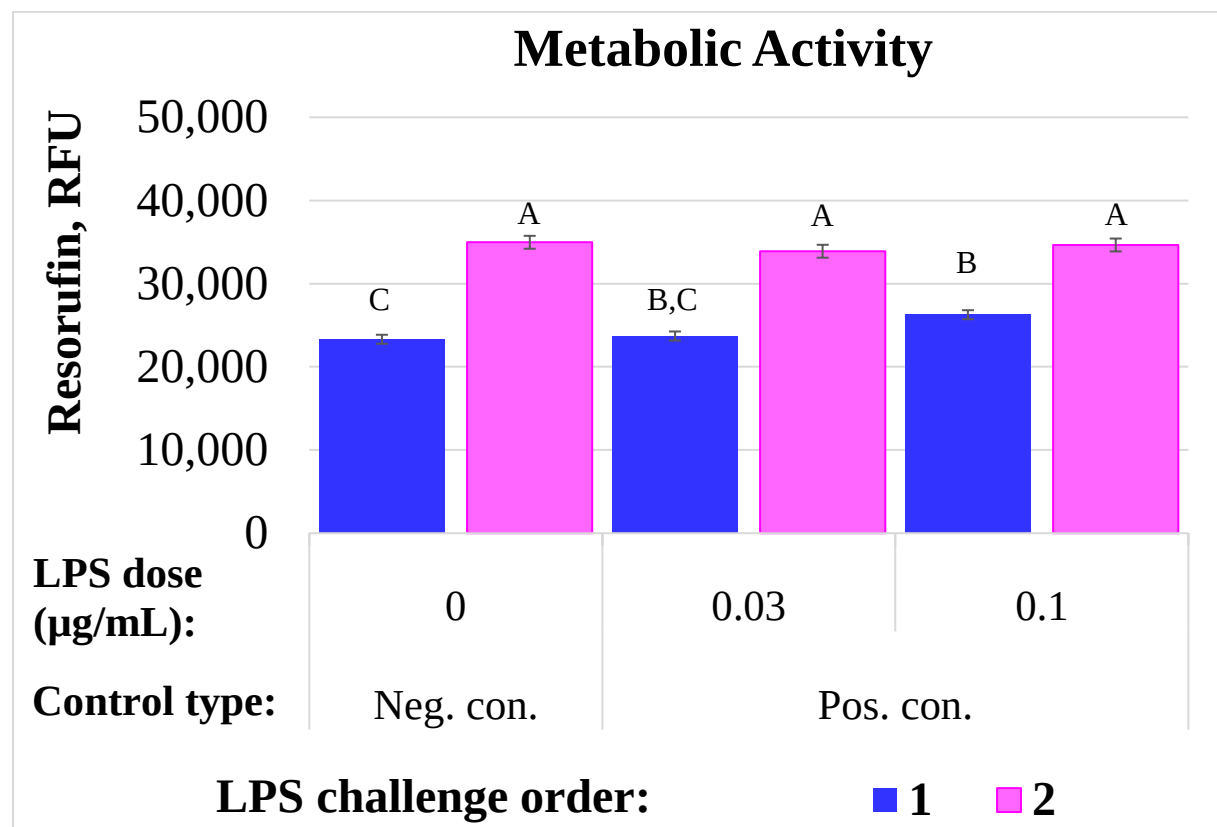


Figure 3.10: Interleukin-8 (*IL-8*) mRNA abundance of Caco-2 cells after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL alone or followed by an LPS challenge, displayed by dose of yeast compound. Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM. Yeast compound did not affect *IL-8* mRNA abundance ($P = 0.11$). Values are LS means $\pm$ SEM. Significant linear dose effects within treatment compound are signified: $*P < 0.05$.

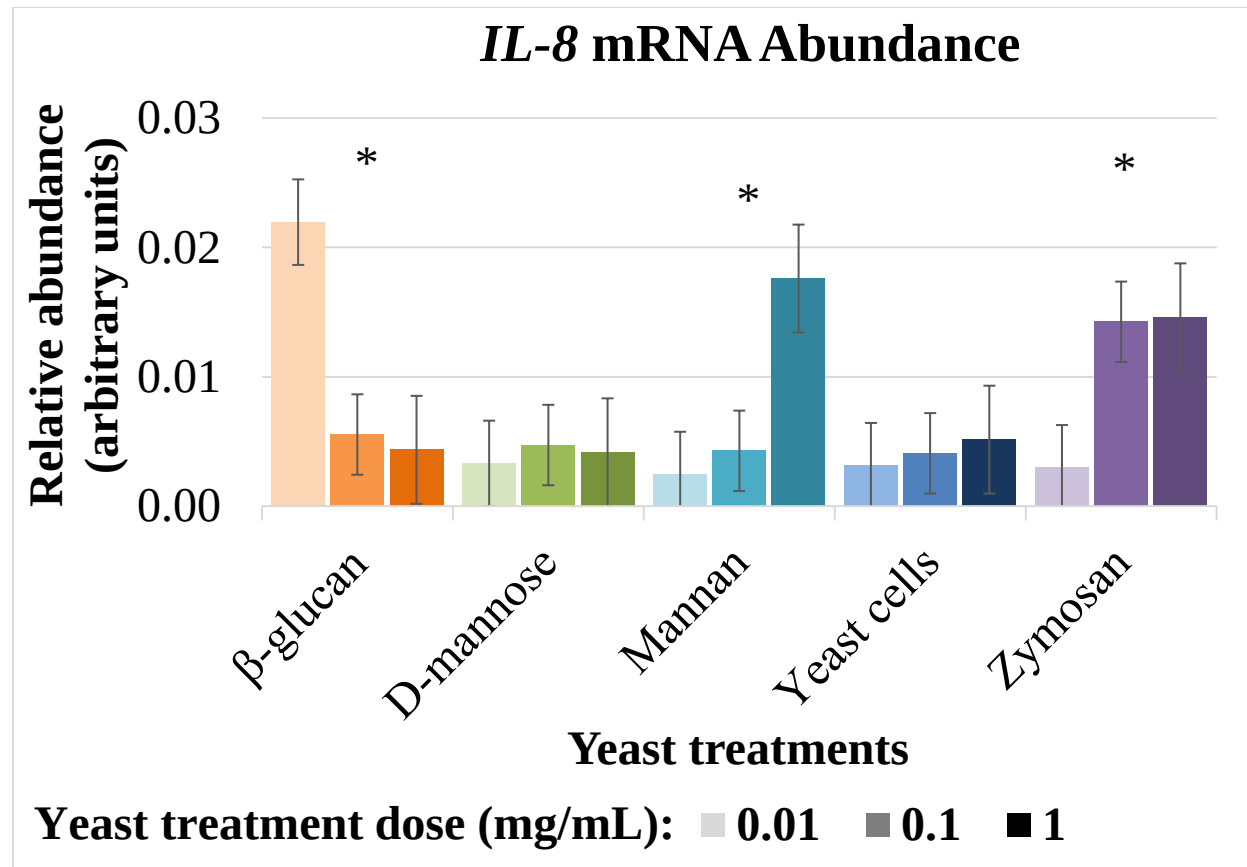


Figure 3.11: Interleukin-8 (*IL-8*) mRNA abundance of Caco-2 cells after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL alone or followed by an LPS challenge, displayed by LPS dose. Increasing LPS dose is signified by increasing color intensity within LPS dose. Values are LS means $\pm$ SEM. Yeast compound did not affect *IL-8* mRNA abundance ($P = 0.11$). Significant dose effects between LPS doses are signified: $*P < 0.05$.

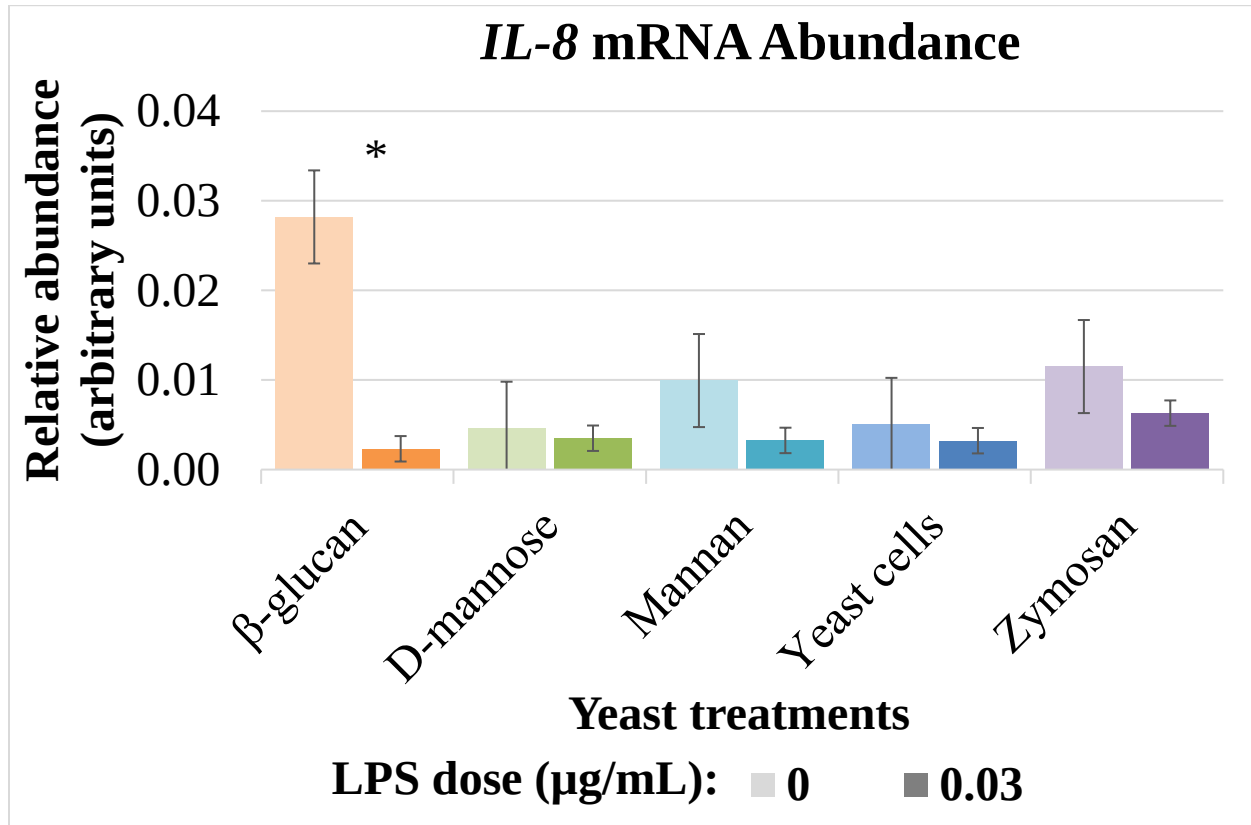


Figure 3.12: Claudin-1 (*CLDN1*) mRNA abundance of Caco-2 cells after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL alone or followed by an LPS challenge, displayed by dose of yeast compound. Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM. Yeast compound tended to affect *CLDN1* mRNA abundance ($P = 0.07$). Doses within each yeast treatment were not significantly different ($P > 0.05$).

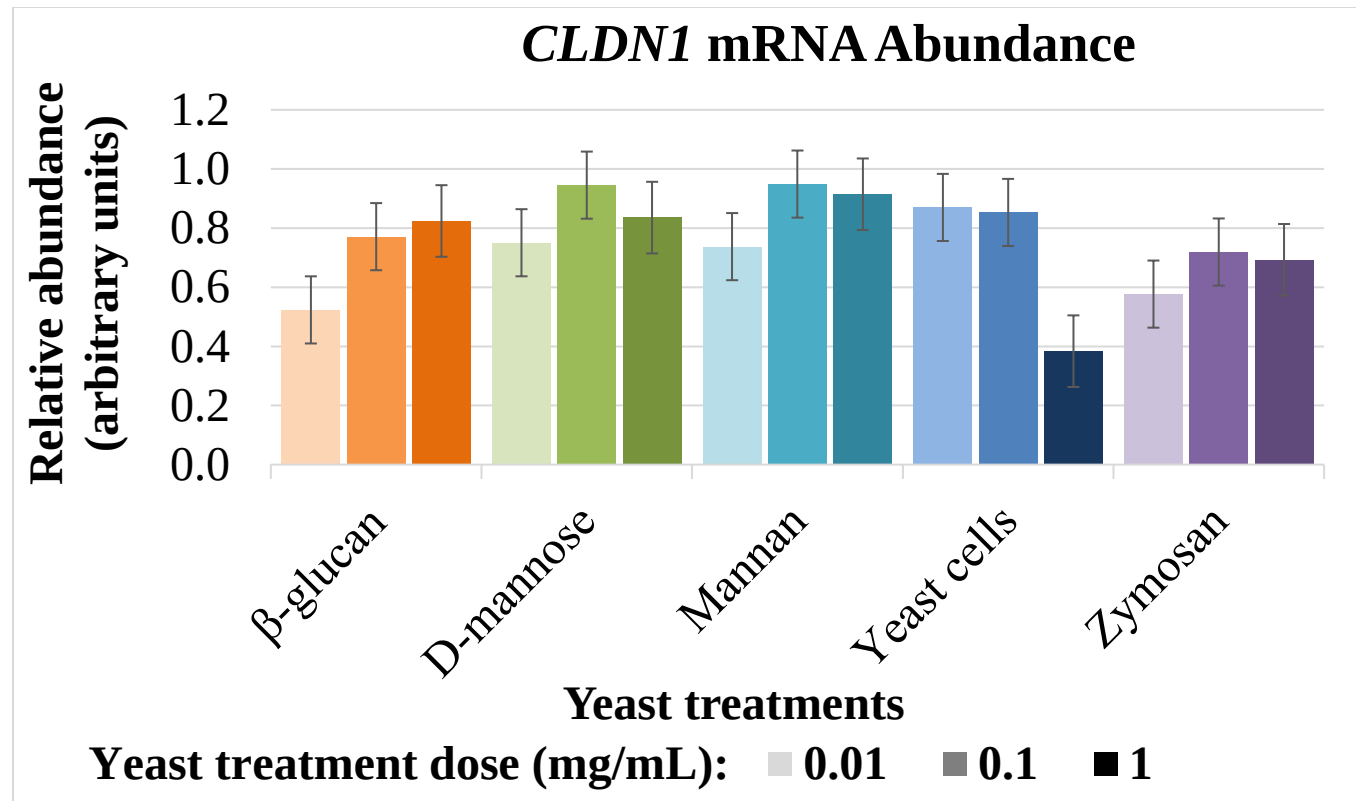


Figure 3.13: Claudin-1 (*CLDN1*) mRNA abundance of Caco-2 cells after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL alone or followed by an LPS challenge, displayed by LPS dose. Increasing LPS dose is signified by increasing color intensity within LPS dose. Values are LS means $\pm$ SEM. Values are LS means $\pm$ SEM. Yeast compound tended to affect *CLDN1* mRNA abundance ($P = 0.07$), but *CLDN1* mRNA abundance was not different between LPS doses.

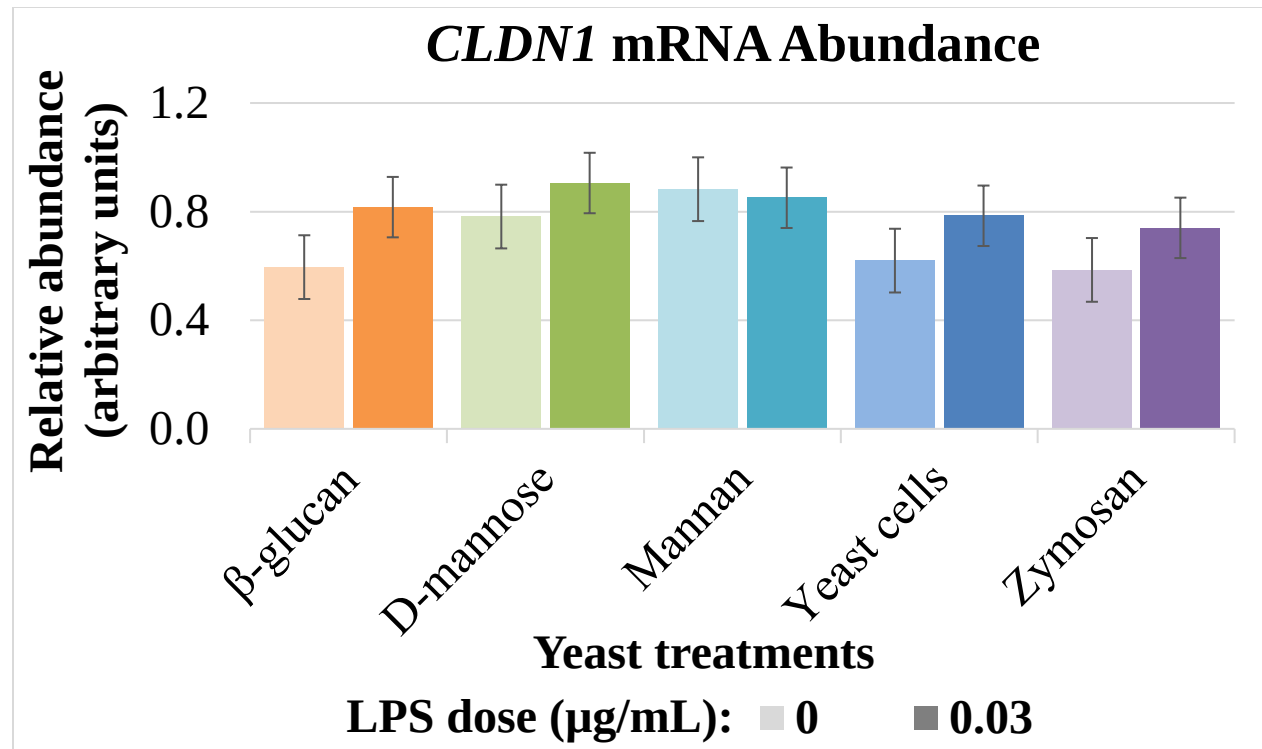


Figure 3.14: Metabolic activity of Caco-2 cells after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL for 18 h. Metabolic activity was assessed by metabolism of resazurin to the fluorescent product resorufin. Untreated RAW cells represent the negative control (Neg. con.), and RAW cells stimulated with only 0.03 µg/mL of LPS represent the positive control (Pos. con.). Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly by yeast compound ($P < 0.05$, Tukey's HSD). Significant dose effects within treatment compound are signified: * $P < 0.05$.

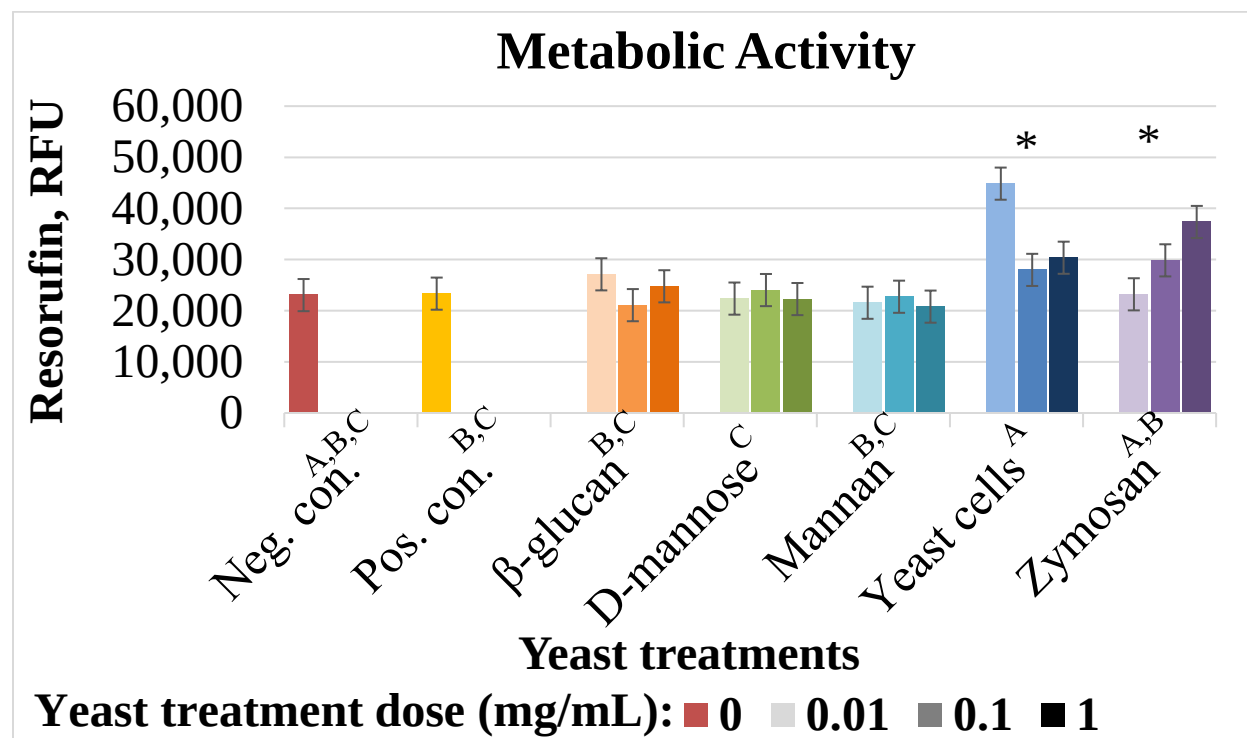


Figure 3.15: Metabolic activity of Caco-2 cells after 18 h of treatment with components of *Saccharomyces cerevisiae* at doses of 0.01, 0.1, or 1 mg/mL for 18 h followed by an LPS challenge at 0.03 µg/mL for 2 h. Metabolic activity was assessed by metabolism of resazurin to the fluorescent product resorufin. Untreated RAW cells represent the negative control (Neg. con.), and RAW cells stimulated with only 0.03 µg/mL of LPS represent the positive control (Pos. con.). Increasing doses are signified by increasing color intensity within treatment compound. Values are LS means $\pm$ SEM, and means that do not share a letter differ significantly by yeast compound ($P < 0.05$, Tukey's HSD). Significant dose effects within treatment compound are signified: * $P < 0.05$.

