Investigating the potential to source novel postbiotics with anti-microbial or immune-modulatory activity from distillery waste

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DECLARATION

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, with the following exceptions:

LPS-induced model of sepsis mouse trial was performed by Dr. Sinéad Corr and Dr. Elaine Dempsey. All other live mouse handling was performed by Dr. Elaine Dempsey, Sarah Stiegeler and Kate Sheehan.

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Chapter 1

Introduction

Summary

The purpose of this project is to find a novel source for antimicrobial and immunomodulatory compounds. Here we investigate the antimicrobial and immunomodulatory potential of postbiotics sourced from whisky distillation. With the increasing threat of antimicrobial resistance (AMR) and rising incidence of chronic inflammatory conditions such as Inflammatory Bowel Disease (IBD) there is an urgent and pressing need to identify new sources of antimicrobial and immune-modulators which can either directly target bacteria or modify the immune response. Indeed, every year the incidents of deaths attributed to multidrug resistance MDR strains of bacteria increases, and the critical need for novel antimicrobials rises. Chronic conditions like IBD can stem from a dysregulation of the intricate relationship between the gut microbiome and host immune system, with overactivation of the immune system being linked to an altered gut bacterial microbiome, in particular an increase in pathogenic strains. Central to this inflammatory response are the resident macrophages, the first responders of the immune system. Thus, this study investigated the antimicrobial potential of postbiotics sourced from whisky distillation waste and furthermore, their ability to modulate the macrophage inflammatory response. Results revealed significant antimicrobial properties, both inhibitory and bactericidal. Additionally, the postbiotics demonstrated immunomodulatory effects by enhancing cytokine activity, particularly inflammatory and anti-inflammatory responses, and significantly increasing macrophage phagocytosis. These findings suggest potential immune training activity by novel postbiotic samples, highlighting their relevance and encouraging further research for potential human health benefits.

1. Introduction

There are many crises facing human health today, including the rise in AMR and inflammatory diseases. The misuse of antibiotic treatments and lack of novel antibiotic discoveries has led to the AMR crisis. AMR threatens to be one of the leading causes of death in the next few decades as cases of MDR strains rise each year[1]. The discovery of antibiotics is one of the greatest medical advancements in history, and its effectiveness is now in jeopardy. The situation becomes more dire each year as our pool of effective antimicrobials dwindles against the growing number of resistant bacterial strains. Inflammatory conditions such as IBD are also on the rise. In Europe alone, an estimated 2.5-3 million people are affected by IBD [2]. Inflammatory bowel disease (IBD) includes both Ulcerative Colitis (UC) and Crohn's Disease (CD), both are incurable and chronic conditions that pose heavy burden on the health care system. IBD are complex diseases with many risk factors both genetic and environmental of which most can be linked with the gut microbiome.

Both AMR and inflammatory diseases have incredible health and economic implications. This causes a pressing need for new strategies to tackle infections; either direct targeting of bacteria or by enhancing the immune response to infections. Similarly in the case of inflammatory conditions strategies which dampen inflammatory responses are also required.

There has been increasing interest in utilising gut microbiome-derived components, either whole bacteria or their by-products. Included in this group are prebiotics, probiotics and postbiotics. Prebiotics are essentially dietary fibre, formulated to feed the healthy commensal bacteria in the gut microbiome and restore diversity. Probiotics are live commensal bacteria which confer a health benefit and help restore a healthy diverse gut microbiome. Probiotics have been found to have immune-modulatory potential and can have antimicrobial activity.

One of the newer options being examined for immunomodulatory, anti-microbial and anti-inflammatory effects in the gut are postbiotics. A step beyond prebiotics and probiotics.

Postbiotics, which can be either the secreted bacterial metabolites or bacterial cell components and whole inactivated cells, have also demonstrated immune and antimicrobial activity. These microbiome-based interventions represent a new age of health interventions targeting the relationship between the intestinal microbiome and the host immune system.

1.1 Intestinal microbiome and host immune system

Gut health has become an increasingly critical area of research as the incidences of gastrointestinal disorders including Inflammatory bowel disease (IBD) are increasing globally over recent decades. The gut microbiome and its host immune system have complicated relationship where balance is key. When this balance functions as it should, the gut epithelial barrier absorbs the proper nutrients for the body and maintains a tolerance to the commensal bacteria that are crucial to gut function, while also keeping harmful pathogens at bay. The immune system present in the intestinal barrier environment must maintain a healthy tolerance and resistance to unnecessary inflammatory responses. Dysregulation and inflammation of this carefully regulated environment can have catastrophic consequences for the host, including predisposition to intestinal infection and inflammatory bowel diseases[3]. Inflammatory bowel diseases (IBD) are becoming more prevalent globally and with that, the need for innovative therapeutics is growing. Especially as the AMR crisis worsens, making antibiotic treatments less desirable for both infections and IBD symptoms.

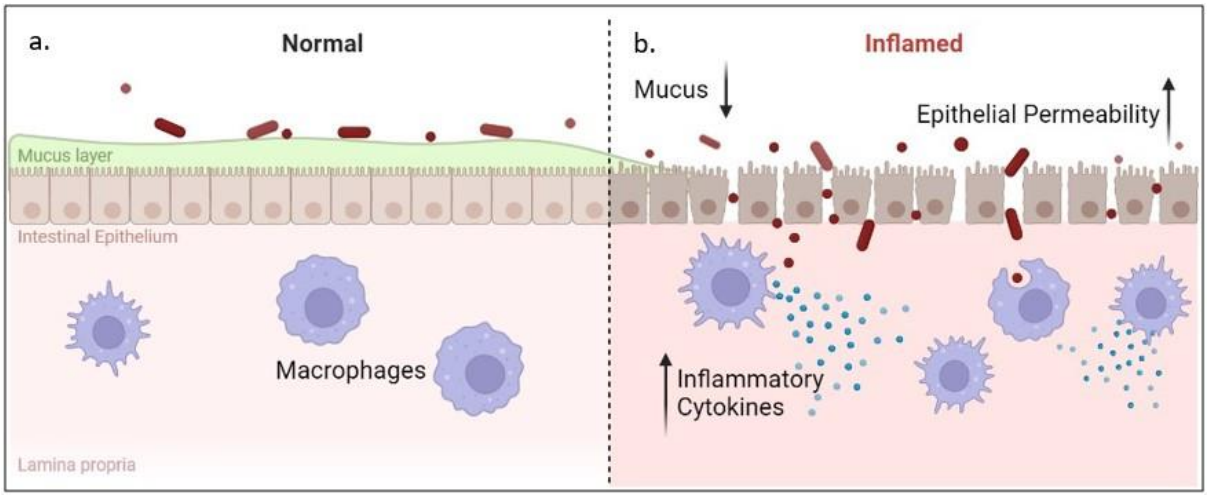
1.2 Gut Barrier Integrity

With respect to host-microbiome communication, the intestinal epithelial cells (IECs) play an especially important role as the barrier between the contents of the gut lumen, containing metabolites, nutrients, and microbes both pathogenic and not. IECs form a semi-selective

barrier for the contents of the gut lumen. This is essential to innate intestinal immunity. It can allow the absorption of water, electrolytes, select metabolites, and other nutrients, while also blocking pathogens or toxins entry [4]. The very first physical layer of immune defence is the mucosal layer to protect the epithelial cells, providing nutrients to commensal bacteria as well as an extra barrier to keep pathogenic bacteria away from the rest of the body[5]. The GI tract mucosa is the largest mucosal surface present in mammals. This mucosal layer is produced and regulated by goblet cells, located in the epithelial monolayer, and their excretion of mucins. It is made up of proteins, carbohydrates, lipids, and high levels of water, as one of its purposes is lubrication and hydration[6]. The next layer of defence is the epithelial monolayer, made up of many cell types including enterocytes, Paneth cells, goblet cells and M cells. All held tightly together by anchoring junctions. These anchoring junctions are tightly regulated by cytokines to hold barrier integrity. Dysregulation of these regulators is associated with IBD (Fig 1.1). The most abundant cell type in the epithelial monolayer being the enterocyte, specialised for absorption. The apical surface of these enterocytes is lined with microvilli as added surface area for absorption of nutrients[7]. The surface of the epithelial layer in the small intestine is highly increased compared to that of the colon because of the presence of villi and microvilli[6]. It is even speculated that enterocytes can act as antigen presenting cells and communicate with T cells[8].After this, in the lamina propria, reside the resident immune cells tasked with engulfing any invaders that may cross the epithelial barrier as well as tissue repair and immunoregulation.

When the integrity of this barrier is compromised, mucus layers are depleted, the epithelial cells no longer maintain a tight formation, and the lamina propria is exposed to pathogens causing the immune response to promote greater inflammation. As epithelial permeability increases, contents of the intestinal lumen including pathogenic bacteria make it through the IEC barrier, the resident macrophages are one of many immune cells exposed. Immune cells

then react to invading pathogens causing compound inflammatory responses. This is known as "leaky gut" and is associated with inflammatory diseases such as IBD[9,10]. While loss of barrier function alone cannot be responsible for pathogenesis of IBD, it may be enough to start a cascade of inflammation that combined with other risk factors induces disease.



.**Figure 1.1 Normal Vs. Inflammed state of intestinal barier.** A. Basic breakdown at main of a balanced gut, healthy amount of protective mucus, tightly joined epithilial cells and macrophages in the lamina propria. B. View of the imflammed intestinal barrier, the mucus layer has been depleated, exposing the epithilial cells to the contents of the intestinal lumen, including pathogenic bacteria, causing more epithilial permiability. Activated macrophages work to kill bacteria while releasing inflammatory cytokines that are harmful to the epithilial cells. This figure was made using BioRender.

1.3 Macrophages

Macrophages are an important effector cell with a key role in the innate immune response. Derived from the Greek word for "big eaters", macrophages are responsible for engulfing any potential pathogen or threat to the host. Usually, macrophages are the first responders to any invading cells, and therefore set the precedent for the rest of the immune response. Phagocytosis, the engulfing and ingestion of bacteria or other pathogens, is key job of the macrophage. Receptors on the cell surface of the macrophage, called pattern recognition receptors (PRRs) detect pathogen-associated molecular patters (PAMPs) and damage-associated molecular patterns (DAMPs). Macrophage receptors can then connect with certain bacterial surface antigens, creating a bridge, the macrophage membrane then surrounds the bacteria, and it is absorbed into what is known as a phagosome[11]. Once inside the phagosome

the cell can elicit a number or sterilization techniques to kill the pathogens, including production of oxygen radicals and free fatty acids as well as lowering of the pH[12–14]. After neutralizing the initial threat of the invading pathogen, the macrophage then communicates with the rest of the immune system by release of different cytokines that can elicit an inflammatory response and by antigen presenting to other immune cells.

In the past macrophages have been misunderstood, for a long time it did not make sense that these cells could have the ability to inhibit growth and kill, as well as promote healing and repair. The classification of macrophages into M1 and M2, accounts for these different immunological responses seen[15](Fig 1.2). Important precursory research to this M1/M2 paradigm includes Nathan et al., which demonstrated that IFN-γ activates the antimicrobial activity of the human macrophage[16]. Then in 1992, Stein et al. showed stimulation with IL-4, promotes murine macrophage mannose receptor activity, which indicates an alternative immunomodulatory macrophage activation[17]. Later, Mills et al. demonstrated that M1 or M2 phenotypic macrophages and their responses can influence the inflammatory immune response that follows, including Th1/Th2 pathways[18]. Today many other types of macrophage phenotype are recognized as reviewed in Olleros et al.[19]. Now, many are revaluating this classification of macrophages, dubbing it an oversimplification, as reviewed in Martinez et al.[20]. However, macrophages being in a general M1 or M2 state can still be indicative of the environment surrounding them and levels of inflammation present.

M1, or the classically activated macrophages, are highly anti-microbial and are associated with the inflammatory immune response[21]. M1 macrophages while having lower levels of phagocytosis, are extremely effective at killing any engulfed pathogens. The M1 phenotype can be induced by different stimuli including lipopolysaccharide (LPS) and INF- γ. M1 macrophages are responsible for the release of many pro-inflammatory cytokines, including TNF and IL-1B, which when released, attract more unpolarized macrophages to the M1

phenotype. This can be done through different molecular pathways, including activation of the transcription factor NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells). NF-κB is a master regulator of the immune response in macrophages. Activation of NF-κB induces the inflammatory immune response and promotes the release of the corresponding cytokines[22]. While the stimulation of M1 macrophages is necessary for clearance of pathogens, overstimulation can be detrimental. These specialised and enhanced anti-microbial properties can also be associated with host tissue damage. Overproduction of certain pro-inflammatory cytokines, such as TNF-a, can have direct associations with compromised intestinal epithelial barrier integrity[23].

M2 macrophages, or alternatively activated macrophages have an anti-inflammatory effect. These macrophages are associated with tissue repair and remodelling. Their activation is also essential in response to parasites and allergens. M2 macrophages have a higher capability for phagocytosis of pathogens however, they are less effective at killing engulfed pathogens. The main signalling pathway for M2 polarization is controlled by transcription factor STAT6[24]. This can be directly activated by IL-4 and IL-13 and also indirectly by other anti-inflammatory cytokines such as IL-10. M2 polarized macrophages have been broken down into further classified phenotypes, M2a, M2b, M2c, and M2d. These cell types have high plasticity and can change between phenotype easily. M2a cells are essential to parasite and allergy immune response. M2b has more of an immunoregulatory role. M2c has immunoregulatory and tissue repair abilities. M2d macrophages are associated with tumour progression[25].

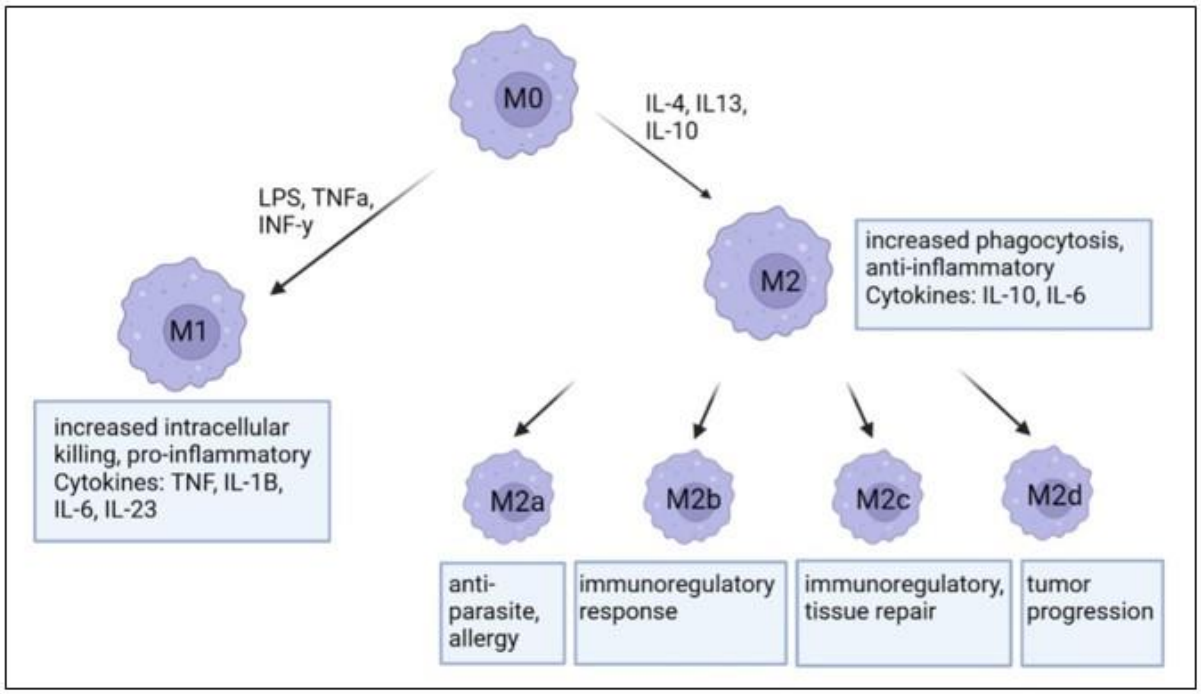


Figure 1.2 Macrophage Polarization Shematic. Un-polarized macrophages, due to different cellular signals such as cytokines, differentiate into either M1 or M2 phenotype which induce the inflammatory or anti-inflammatory immune responses respectivly. M2 macrophages are further broken down into M2a, b, c, and d. This figure was made using BioRender.

Intestinal macrophages, present in the lamina propria, represent the largest type of macrophages in the body. Intestinal macrophages are constantly replenished by circulating monocytes to keep up with the high demand in the gut[26].These macrophages have the unique responsibility of maintaining a balance between reacting to pathogenic bacteria while maintaining a healthy tolerance to the commensal bacteria present in the gut as well as food-related antigens. Intestinal macrophages have adapted well to these unique conditions and even help maintain and protect the epithelial layer in the gut[27]. A study by Mazzini et al. showed that oral tolerance can be obtained via macrophages feeding antigens through epithelial gap junctions to dendritic cells[28]. Intestinal macrophages can illicit higher levels of phagocytic activity without initiating a full inflammatory response. This is due changes in molecular make up, intestinal macrophages have lower levels of innate immune response receptors including CD14, which detects LPS[29]. In murine intestinal macrophages, increased

production of anti-inflammatory cytokine IL-10 was seen in response to contact with commensal bacteria[30]. This tolerance is crucial, as the digestive tract is exposed daily to antigens and microbes of all kinds, overreaction to this leads to destructive outcomes for the host. The many important functions of intestinal macrophages and their promise for disease prevention and control are reviewed in Wang. Et al.[31].

1.4 Therapeutics: tackling chronic inflammation and infectious disease

Therapeutics for inflammatory diseases such as IBD have highly variable effects from patient to patient and some have substantial side effects. Disease activity is related to different factors such as intestinal epithelial barrier permeability, active and inflammatory immune response, and dysregulated gut microbiome. Therapeutic options often attempt to bring back balance to the intestinal environment. Current treatment for IBD patients consists of stepwise options starting with simple anti-inflammatory drugs and then moving up to more complex immunosuppressant therapeutic options. Individualized treatment plans where the risk-to-benefit ratio is considered are key here as many treatments have significant side effects [32].

Immunosuppressant drugs are one option of treatment for chronic inflammatory diseases patients. One example is the immunosuppressant humanized monoclonal antibody, Natalizumab, approved for use in moderate to severe CD patients. It works by blocking the a4 integrin on lymphocytes[33]. However, it does not come without its risks, it can cause reinfection of John Cunningham virus (JCV) leading to deadly nervous system complications[34]. All immunosuppressant drugs come with a risk of an impaired immune response when a serious infection is acquired.

As inflammation in relation to the microbiome and immune response in the gut becomes more understood, research on microbiome-based interventions for a balanced gut has become popular. Antibiotics have shown to be effective in some cases for IBD treatment[35]. However,

they have also displayed harmful deregulatory effects on the gut microbiome[36]. Faecal microbiota transplant (FMT) is also an option. This is where the microbiome of a healthy individual is transplanted into the intestine of an IBD patient in attempt to reintroduce a diverse and balanced microbiome. This is commonly used in treatment of C. difficile infection but has promise in IBD therapy. Again, this drastic manipulation of the gut microbiome does not come without risks of further inflammation[37]. Other possible interventions to promote a healthy and diverse microbiome include prebiotics, probiotics and postbiotics. As the anti-inflammatory effect from prebiotics/probiotics has been conflicting in many studies, more research on the pathways involved are needed[38].

Treating infectious disease is becoming increasingly difficult as AMR rises across the globe. Numbers of drug resistant bacteria are rising each year and beginning to make our pool of once effective antibiotic drugs, obsolete[39]. In recent decades the discovery of new antibiotics has slowed. This, along with overuse of known antibiotics has led to higher rates of resistance to antimicrobials. Multi-drug resistant bacteria are hard to treat, making them deadly in many cases. This is why there is a dire need for novel antimicrobial compounds[40]. Combating infectious disease can be done by compounds with a direct antimicrobial effect, or compounds which fortify the host immune system.

1.5 Therapeutic strategies to enhance mucosal immunity or combat intestinal infection: Probiotics-Prebiotics

Probiotics are defined as live microorganisms administered to the digestive tract designed to elicit health benefit for the host. The introduction of beneficial commensal bacteria into the gut microbiome, promotes stability and diversity[41]. Research shows a diet with a healthy number of probiotics (live bacteria) is important to keep the gut microbiome diverse, and levels of beneficial SCFAs high. As far as probiotics for clinical treatment of disease the

results are mixed. However, in cases probiotics have shown benefits in treatment of diarrheal disease and IBD activity. Treatment with probiotics can reduce the risk of antibiotic associated diarrhoea[42]. In a clinical trial including 20 patients with active ulcerative colitis, treatment with bifidobacterial-fermented milk showed less disease activity compared to placebo group[43]. There is little concrete evidence that probiotic treatment will have a noticeable effect on inflammation in the gut.

While there is some evidence to support the beneficial properties of probiotics, there are real risks involved. The administration of live bacteria poses the potential for infection and inflammation. Infection risk is a very important subject for concern. The risk is very low for healthy individuals, however the immunocompromised are more susceptible. When probiotic bacteria translocate from the gut in healthy individuals, the immune system will detect and kill the cells, however this ability may be impaired in immunocompromised individuals. *Lactobacillus* strains can prove to be opportunistic pathogens in immunocompromised patients[46–48]. While rare, there have been cases of *lactobacillus* endocarditis in immunocompromised individuals, speculated to be due to ingestion of probiotics[49,50]. In a study done with patients with severe acute pancreatis, patients who received the probiotic treatment had an increased rate of mortality[51]. While probiotics are praised for their anti-inflammatory effects, other studies demonstrate the pro-inflammatory effects of probiotics[48,52,53]. Those who are immunosuppressed are often advised to not take probiotics.

Another area for concern is that the way some probiotics are designed, possibly selecting for more virulent strains. Some postbiotics are modified to have stronger adhesion ability, possibly increasing virulence, and inviting risk of infection[54]. Horizontal gene transfer has also been an area of concern for scientists, the potential for virulence factors to be transferred to or from probiotics in the gut. However, there is not enough research on this phenomenon.

Another problem with probiotic supplements is that it is hard to establish that the bacteria will reach the gut alive. Only certain encapsulation methods for delivering supplemental probiotics are proven to keep the bacteria alive through the harsh digestive system environments[55]. Some commercialised probiotic supplement brands do not guarantee the probiotics reach the gut alive.

The risks and concerns do not discredit all the well-researched positive effects of probiotics in healthy people. The evidence that supports probiotic use substantial, however in recent years, it has been noted that not all these positive effects require the microbial cells to be alive [56]. This, plus the very real risk involved in administering live bacteria, leads us to the next area of research, postbiotics (Fig 1.3).

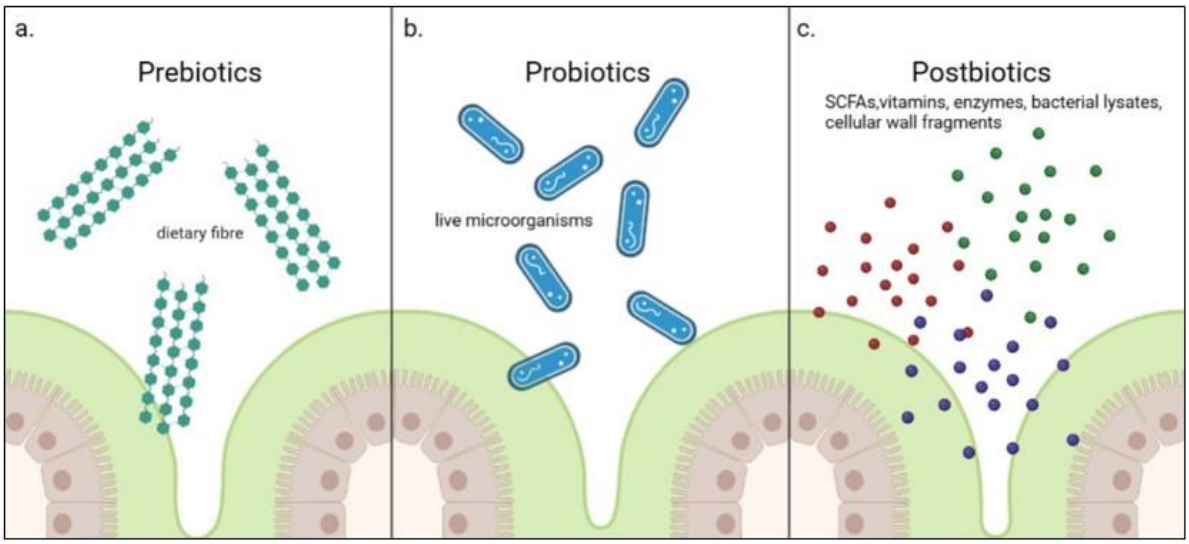


Figure 1.3 Prebiotics vs Probiotics vs Postbiotics. (A.) Prebiotics are dietary fibres that fuel the bennificial microbes present in the gut, these microbes are refered to as (B.) Probiotics, live microorgaisms admistoerd to diversify and stablize gut microflora. (C.) Postbiotics are the excreted by-products from said probiotics, incluing SCFAs, vitamins, enzymes, bacterial lysates, and cellular wall fragments. This figure was made using BioRender.

1.6 Postbiotics: A step beyond probiotics

This opens a new avenue for therapeutic interventions because of a deeper understanding of the gut microbiota and its role in human diseases. The term postbiotics has been defined by

The International Scientific Association of Probiotics and Prebiotics (ISAPP) as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [57]. Postbiotics are naturally derived compounds with the potential to be used as an entirely new class of therapeutics. Postbiotics can contain a few different categories of bioactive material. They may contain inanimate microorganisms, bacterial cell fragments or structures such as cell wall fragments or anchored proteins as well as the end-products or metabolites from bacterial fermentation[44]. Bacterial cell fragments have shown to be anti-inflammatory and anti-microbial[58]. Bacterial fragments can work as an immune priming agent, altering and readying the immune response to future infections[59]. The intermediate products of bacterial metabolism, or metabolites, encompass a variety of bioactive material. Many studies have shown the targeted effects of different metabolites in the gut, including that of metabolites ability to modulate immune responses in macrophages[60]. Postbiotics are essentially the fermented supernatant, which can be either cell containing (CCS) or cell free supernatant (CFS). Cell containing or not, postbiotics encompass a range of bioactive materials that can alter the gut microbiome as well as have potential anti-microbial, immunomodulatory, and anti-inflammatory effects. Many reviews outline beneficial properties of postbiotics; however, they remain a very new topic[61–63].

The use of postbiotics over probiotics has many benefits, including less risk, with no live microbes being administered, better absorption and distribution, as well as production and storage being made easier. They may even prove to be more effective. According to a study done using the mouse colitis model, significantly stronger gut microbiome modulatory effects were seen in mice treated with postbiotics compared to probiotics[64].

1.7 Antimicrobial effects of postbiotics

As the antimicrobial resistance (AMR) crisis continues to worsen, novel alternative antimicrobial products are in high demand. If over and misuse of antibiotics continues, by 2050 the leading cause of death for humans will be antimicrobial resistant infections at an estimated 10 million deaths per year[40]. The rise of AMR in healthcare systems across the globe is proving more serious as resistant bacteria become more common. AMR is undermining effective prevention of infectious diseases, and making the treatment more complex, increasing the risk of spread, severe disease, and death[40]. Steps need to be taken in both human and agricultural medicinal practices, and novel antimicrobials must be developed. This is especially pertinent for the agricultural industry as per a study done in the US, over 70% of medically relevant antibiotics sold are for animal livestock[65]. Animals are receiving more antibiotics than ever, and these antibiotics find their way into our food as well as the soil and water systems surrounding these animals. There is still a place for carefully administered antibiotic treatment in veterinary medicine, however other antimicrobial treatments are urgently required to replace mass antibiotic treatments[40].

Postbiotics are one of the many avenues being researched as replacements for antibiotics in livestock feed. Postbiotics from *lactobacillus sakei* isolated from cow milk, have shown antibacterial activity against mastitis causing pathogens[66]. Mastitis is a commonly acquired infection in cows and is commonly treated with antibiotics. In a study done on supplementation of diet with postbiotics, the postbiotic group showed significantly altered gut microbiota, promoting growth and non-specific immunity in the zebrafish model[67].Postbiotic supplemented diet for broiler chicks displayed significant changes in the gut microbiome, showing lower Enterobacteriaceae and *E. coli* counts while maintaining a healthy Bifidobacterium population[68]. This selective anti-microbial effect is very promising for further research. A clinical trial done in children has shown supplementation with milk

fermented with *lactobacillus paracasei* prevents infectious diseases[69]. Other clinical trials have had similar findings[70].

As well as postbiotics alone inducing a range of antimicrobial effects, they can also work synergistically with prebiotics and probiotics to deliver results. A study done in 2022 showed the functionality of a probiotic edible coating on salmon fillets was significantly increased with the addition of postbiotics, inhibiting growth of food borne pathogens ($p < 0.05$) and increasing shelf life[71]. This idea of prebiotics, probiotics and/or postbiotics administered together to enhance beneficial effects on the host is referred to as 'synbiotics' and is another growing area of research.

1.8 Immunomodulatory effects of postbiotics

As well as direct antimicrobial effects, postbiotics are also known to elicit immunomodulation effects on the host. Often referred to in older publications as probiotic cell-free supernatant, is shown to have anti-inflammatory effects in both epithelial cells and macrophages stimulated with LPS[72]. A study done in macrophages shows the ability of intestinal metabolites to increase phagocytosis and clearance of bacterial infections, suggesting modulation of the macrophage immune response[60]. An increase in plasma immunoglobulins M and G (IgM, IgG) was seen in response to postbiotic diet supplementation in birds[68]. Postbiotic treatment can help balance the Th1 and Th2 immune response to regulate and optimize immune function in the gut. It does this by enhancing barrier function as well as stimulating the anti-inflammatory immune response. Regulation of this inflammatory innate immune response is promising for therapeutic research. A study on postbiotic treatment with heat-treated *Bifidobacterium* shows anti-inflammatory effects as well as gut barrier protection[73].

1.9 New sources for postbiotics: whisky distillery waste & sustainability

The whisky distillation process creates multiple forms of waste that end up both economically and environmentally detrimental. The process begins with the preparation of the raw materials (ex. barley), or malting, breaking down the grains to free the starch. The next phase is referred to as mashing, here the starch is converted to sugar, mixed with water, and cooked into what is called mash. This stop creates a by-product called draff, or brewers spent grain (BSG). Fermentation is next, whereby the mash is inoculated with yeast that converts the sugars into alcohol. In the final step, distillation, the fact that alcohol boils at a lower temperature than water is used to separate and concentrate the alcohol content. This is done through heating the liquid and then condensing it. The two distillation steps create the waste products, pot ale and spent lees. From here the whisky can be aged in wooden casks for different amounts of time before being bottled and sold (Fig 1.4).

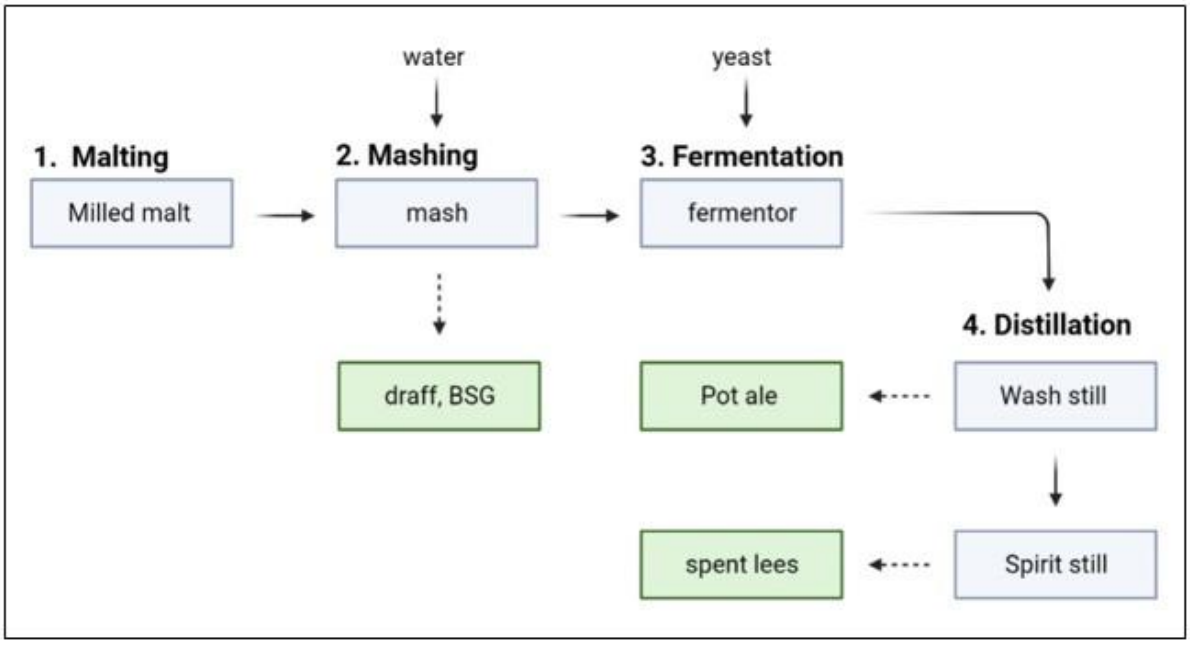


Figure 1.4 Whisky Distillation. (1.) Malting. The grains are broken down to release the starch. (2.). Mashing. Water is added and the mash is cooked, turning the starch into sugars and creating the draff byproduct. (3.) Fermentation. Yeast is added to the mash, converting sugars to alcohol (4.) Distillation. The whisky is clarified by two steps of heating and condensing to concentrate alcohol content. These distilation steps create the pot ale and spent lees waste products. This figure was made using BioRender.

In a 2015 report by the green alliance Every year over 2.7 billion litres of pot ale are produced as a co-product of the Scottish malt whisky distillery process. Some of the pot ale is evaporated and some is used in animal feed, however, the majority is disposed of by anaerobic digestion or land/sea disposal, at great expense to both the brewer and the environment. The pot ale consists of water, dead yeast, barley proteins, carbohydrates, and <0.1% alcohol. Its ingredients make it a possible nutrient dense supernatant for postbiotic production [74]. Here we will screen distillation waste as a medium for growth of lactic acid bacteria, and aspergillus, known producers of antimicrobial and anti-inflammatory factors. The addition of these microbes not only adds beneficial bioactive metabolites, but it also eases the economic waste burden by reducing the biomass of the pot ale. Similar to the anaerobic digestion method used currently to reduce waste material in the whisky industry, and many other waste products. This product aims to find a use for a waste product that can relieve a great deal of environmental and economic burden.

The other major waste product of this process, BSG, will also be considered for postbiotic effects in this research. While this waste product is not considered a postbiotic, as it has not been inoculated with any microorganisms, the contents are still of interest because they are also present in the pot ale. The BSG by-product is rich in soluble dietary fibre (SDF), which can have many beneficial effects in the gut.

As the climate crisis becomes more serious and natural resources are depleting, a switch from a linear economy to a circular economy is imperative. A circular economy refers to a system where products are used and reused, and waste products are made useful in other ways. If the global economy continues with its current over consumption and waste pattern, it is going to cause irreversible climate change[75]. To increase the circularity of a system, the environmental, social, and economic sustainability must be considered[76]. Waste material management is a major opportunity to create circularity, by better directing waste and

promoting the reuse of products, the environmental and financial burden can be lessened. As forementioned, the waste disposal of pot ale from Scottish whisky production is a costly process. Waste management also accounts for a large portion of greenhouse gas emissions[77], so its proper disposal should be prioritised.

In the past five years, the circularity of the world's economy has shrunk from 9.1% to 7.2% circularity. This is due to both failing to create more circular pathways and an increase in material use. Without novel ideas to create more circularity and waste management, this index is going to continue to fall. If a circular economy could be implemented fully in just four sectors of the economy, food, goods and consumables, transport, and building, the overall use of new material could be reduced by 34%[78]. This has the potential to limit the global temperature rise to just 2-degrees[79].

As these sustainability issues continue to be at the forefront of discussion globally, more consumers now expect sustainable products and solutions. Companies that can market products to customers with 'sustainably made' as a selling point will financially benefit. The possibility to repurpose pot ale from whisky distillation as postbiotic medium is a financially and environmentally friendly application.

To summarize, while global health crises such as AMR and the rise of inflammatory diseases threaten human health, new strategies are being investigated. AMR rises, new strategies to tackle these resistant infections, either by directly targeting bacteria or by enhancing the immune response to infection are in high demand. Similarly, in the case of inflammatory conditions such as IBD, treatments that can dampen the inflammatory response are being sought out. As the gut microbiome and its relationship with the host immune response has become more understood, microbiome-based treatments have been investigated including probiotics/postbiotics. These supplements can promote gut barrier function, stimulate the anti-inflammatory immune response, and stabilize/diversify the gut microbiome. Here we investigate a novel source of postbiotics, the step beyond probiotics. Postbiotics have promise in direct anti-microbial effects as well as in immunomodulation, helping maintain balance and anti-inflammatory states in the gut. This novel source of postbiotics, fermentation of whisky pot ale, could help relieve the economic and environmental burden of this waste product. This is especially important as waste management is a major contributing factor to detrimental climate change. Alleviation of this waste product will help promote a circular economy.

1.10 Overall aims of thesis

The main goal of this research is to investigate the by-products of the whisky distilling process as a potential novel source of postbiotics. The project will address this question in two specific aims:

1. Determine if whisky by-products are a new source of antimicrobial compounds.
2. Determine if whisky by-products are new source of postbiotics with immunomodulatory activity in macrophages.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Postbiotics

Raw pot ale was obtained from Lochranza Distilleries, Ilse of Arran, Scotland. Postbiotic samples were prepared by Marigot Ltd. As described in 2.2.1.

2.1.2 Bacterial strains

The following bacterial strains were sourced from the Moyne Institute.

Table 2.1: Bacterial strains

Strain	**Provided by:**
Salmonella Typhimurium (S. typhimurium) UK1	Dr. Sinéad C. Corr
Escherichia coli (E. coli) NCTC12900 (Shiga toxin negative O157:H7)	Dr. Marta Martins
Klebsiella pneumoniae (K. pnuemoniae) ATCC700603	Dr. Marta Martins
Pseudomonas aeruginosa (P. aeruginosa) PAO1	Dr. Marta Martins
Staph aureus (S. aureus) ATCC25923	Dr. Marta Martins
Acinetobacter baumannii (A. Baumanaii) 19606	Dr. Marta Martins
Listeria monocytogenes (L. monocytogenes) EGDe	Dr. Sinéad C. Corr

2.1.3 Bacterial growth media

Growth media Luria-Bertani (LB) and Mueller Hinton broth (MH2) were prepared according to the manufacturer's instructions by the Moyne Institute of Preventative Medicine's prep room and purchased from Oxoid. Bacterial cultures were grown in glass labware.

2.1.4 Cell culture reagents/materials

Table 2.2: Cell culture reagents

Reagent	**Manufacturer**
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco Biosciences
Foetal Calf Serum (FCS)	Gibco Biosciences

Red Blood Cell (RBC) lysis buffer	Sigma-Aldrich
Gentamicin (50mg/ml)	Sigma-Aldrich
Penicillin/Streptomycin (PS) cocktail	Sigma-Aldrich
Normacin	InvivoGen
Zeocin	InvivoGen
HEK-Selection	InvivoGen
PBS	InvitroGen
LPS	Enzo Life Sciences
TNF-a	Proteintech
PAM3CSK4	InvivoGen

Other cell culture supplies including cell culture plates (12 and 24 well plates) and other plastics were purchased from Corning Costar. Cell culture flasks (T75 and T25) and 96 well cell culture plates were purchased from Starstedt. Syringes, needles and cell filters were purchased from Becton Dickinson. Cell scrapers were manufactured by Fisher Scientific.

2.1.5 Cell lines

Caco-2 cells and L929 were purchased from ATCC Cell Biology collection. RAW-Blue, HEK-TLR4, and HEK-TLR2 cells bought from Invivogen, were provided by Dr. Fred Sheedy's lab.

2.1.6 Cell viability reagents

The alamarBlue indicator dye (00-025) was purchased from ThermoFisher Scientific.

2.1.7 Quanti blue assay reagents

Quanti-Blue Assay kit was purchased from Invivogen, containing the Quanti-Blue reagent and the Quanti-Blue buffer.

2.1.8 RNA extraction

RNA was extracted using the PureLink RNA mini kit purchased from Invitrogen. This kit contains RNA extraction columns and collection tubes along with the following reagents, RNase free water, wash buffer I, wash buffer II and RNA lysis buffer.

2.1.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Table 2.3: qRT-PCR materials

Kit	Manufacturer
cDNA synthesis	Applied Biosystems
PowerUp SYBR green	Applied Biosystems

All qRT-PCRs were carried out on StepOne Plus PCR machine from Applied Biosystems.

2.1.10 Primers

For mRNA targets, used with SYBR green, the primers were sourced from Eurofins MWG Operons. A list of said primer sequences is seen below in figure 2.5.

Table 2.4: SYBR Primer sequences

Primer	Species	Forward (5'-3-)	Reverse (3'-5')
GAPDH	Murine	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
Nos2	Murine	CAACAGGGAGAAAGCGCAAA	GGGATTCTGGAACATTCTGTGC
IL-10	Murine	TTGAATTCCCTGGGTGAGAAG	TCCACTGCCTTGCTCTTATTT
TNF-a	Murine	TGGAACTGGCAGAAGAGGCACT	GAGATAGCAAATCGGCTGACGG
IL-1B	Murine	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC

2.1.11 Enzyme-linked immunosorbent assay (ELISA) reagents

Mouse IL-6, IL-10, IL-1B and TNF-a ELISA kits were obtained from Invitrogen/ThermoFisher. ELISA wash buffer is made with 10X PBS purchased from BioSciences and Tween-20 was purchased from ThermoFisher. ELISAs were carried out on 96 well plates from Thermo Scientific.

2.1.12 Animals

Animal studies were performed with C57/BL6 OlaHsd mice at the Comparative Medicine Unit (CMU) at the Trinity Biomedical Sciences Institute (TBSI) at Trinity College Dublin (TCD). The Animals were maintained in ventilated cages at 21 ± 1 °C, humidity 50 ± 10% with a 12h-light/12h-dark light cycle The facility operates with specific pathogen-free conditions and in line with Irish and European Union rules and regulations…

2.1.13 Miscellaneous reagents

Ethanol was obtained from the Hazardous Materials Facility (HMF) and TCD. Molecular grade water was sourced from Cytiva.

2.2 Methods

2.2.1 Postbiotic Preparation

Postbiotic samples were manufactured by Marigot Ltd., using raw pot ale. Pot ale, a nutrient rich waste product from whisky distillation was collected from Lochranza Distilleries. This pot ale was then incubated at 80°C for 1 hour and then mixed thoroughly (PB1). Then the pot ale was centrifuged at 4000 rpm for 30 minutes and the clear supernatant was collected (PB3). This supernatant was then used as growth media for other microbes. For PB9, the pot ale was fermented with *L. casei* at 37°C with agitation (50 rpm) for 24 hours. The sample was then incubated at 80°C for 1 hour and then mixed thoroughly (PB9). Then the pot ale was centrifuged at 4000 rpm for 30 minutes and the clear supernatant was collected as the cell free supernatant (PB14). For the final postbiotic of interest, *A. oryzae* was fermented in the pot ale at 30°C with agitation (125 rpm) for 24 hours. The sample was then incubated at 80°C for 1 hour and then mixed thoroughly, and then the pot ale was centrifuged at 4000 rpm

for 30 minutes and the clear supernatant was collected as the cell free supernatant (PB25). These postbiotic samples were prepared and sent to Trinity College Dublin by Marigot Ltd..

Table 2.5: Postbiotic samples

Postbiotic Sample	Description
PB 1	Pot ale containing yeast cells
PB 3	Cell-free pot ale
PB 9	Pot ale fermented with *L. casei*
PB 14	Cell-free *L. casei* fermented pot ale
PB 25	Cell-free *A. oryzae* fermented pot ale

2.2.2 Bacterial cultures

Cultures of the bacterial strains were prepared in LB (phagocytosis assays) or MH2 (Well diffusion/MIC/MBC experiments) media and grown overnight at 37°C at 200 RPM. On the morning of experiments, overnights of bacteria were used at an OD600nm = 1.

2.2.3 Well diffusion assay

For the well diffusion assay the following protocol was observed. Before this experiment, cultures of the bacteria of interest were grown in Mueller Hinton 2 broth were grown overnight in 37°C. Then Mueller Hinton agar was inoculated with bacteria of interest, before pouring into petri dishes. The plates were poured and let to harden. Next, small evenly spaced holes were punched from the media. The compounds of interest were then added to their respective wells/holes in the agar. The plates are then incubated overnight at 37°C and examined for zones of clearance in the agar.

2.2.4 Minimum inhibitory concentration and Minimum bactericidal concentration

Overnight cultures of bacterial strains of interest are incubated at 37°C. The bacterial cultures were made up to the McFarland standard. For the MIC, 96 well plates are prepared with 100 µl of Mueller Hinton II broth. Postbiotic samples were then added to the first column and serially diluted across the plate. Each well is then inoculated with the bacterial strains,

including bacterial growth control wells. The plates are then incubated for 18-20 hours at 37°C. The plate absorbance is read at 600nm. For the MBC, new 96 well plates are prepared with 100 µl of Mueller Hinton II broth per well. Using a replicator under sterile conditions, the MBC plates are inoculated with the corresponding MIC plates. The plates were then incubated for 18-20 hours at 37°C.

2.2.5 Cell Culture

2.2.5.1 L929 culture

Using an L929 fibroblast cell line, L929 conditioned media was generated for use in the differentiation of bone marrow into bone-marrow derived macrophages (BMDMs). L929s were maintained in DMEM (10% FBS) at 37°C, 5% CO2 and 95% relative humidity. Conditioned media was harvested when the cells reached 80% confluence between passages 7-20. The media was then filtered through a 0.45µm pore vacuum filter and stored at -20°C until use.

2.2.5.2 Bone marrow-derived Macrophages (BMDMs) culture

Bone marrow was isolated from the femur and tibias of mice (age and sex matched) using DMEM cell culture media. After isolation from the bone, the bone marrow was resuspended in 3ml red blood cell lysis buffer for 3 minutes. 30ml DMEM (10% FBS, 100 U/ml-100ug/ml P/S) was added which was followed by centrifugation for 5 minutes at 1500rpm. If there was any red colouring in the remaining pellet, this RBC lysis step was repeated until the pellet was a white colour. Next, the bone marrow was resuspended in 3ml complete DMEM (10% FBS, 100 U/ml-100ug/ml P/S) and passed through a 100µM cell strainer to collect any remaining debris. The bone marrow was then added to three 10mm untreated petri dishes containing 1ml bone marrow suspension and 9ml DMEM (10% FBS, 100 U/ml-100ug/ml P/S) with 20% L929-CM each, to differentiate. On day 3, the media was replaced. On day 6

of differentiation, the BMDMs were removed from the petri dishes by cell scraping and seeded into 12 well plates at 5 x 10^5 cells/well, in DMEM (10% FBS) supplemented with 20% L929-CM. The cells settled overnight in the plates before being used in experiments. The cells were maintained at 37°C, 5% CO2 and 95% relative humidity. BMDMs are considered M0 macrophages, in the resting, unpolarized state.

2.2.5.3 RAW Blue culture

A Raw-Blue murine macrophage cell line was used to model macrophages. The cells were maintained at 37°C, 5% CO2 and 95% relative humidity. The cells were maintained in growth media, complete DMEM, 10% heat-inactivated FBS (30 min at 56°C), Normacin (100µg/ml), Pen-Strep (100 U/ml- 100 ug/ml). This media was further supplemented by an addition of Zeocin (200µg/ml) at every other passage. These cells were utilised at 15-25 passages. For experiments, Raw-Blue macrophages were removed from maintenance flasks by cell scraping and then seeded into 24-well plates at 5 x 10^5 cells/well and allowed to settle overnight before experiments were carried out. All experiments with Raw-Blue macrophages were carried out in the test medium containing complete DMEM, 10% heat-inactivated FBS, Pen-Strep (100 U/ml- 100 ug/ml). All RAW-Blue cells were used between passage 15-25.

2.2.6 Cell Viability measurement using alamarBlue Dye

The manufacturer's protocol was followed. Cells were seeded at 5 x 10^4 cells/ml into 96 well plates and were left overnight to settle in 37°C, 5% CO2. The cells were then treated with the adjusted concentrations of postbiotic sample for 24 hours. Then 1/10^{th} volume of the cell viability reagent was added directly into the wells, incubated for 4 hours at 37°C (protected from direct light) and then the absorbance was measured using a fluorescence excitation wavelength of 570 nm using 600 nm as a reference wavelength.

2.2.7 Quanti-Blue analysis

The protocol was performed according to the manufacturer's instructions. Firstly, the QUANTI-Blue solution was prepared using the amounts displayed in table 2.13 and then was mixed well and incubated at room temperature for 10 minutes before use. The reaction is carried out in a 96 well plate, 180 µl of the QUANTI-blue solution and 20 µl of cell supernatant (from SEAP-expressing cells) are added to each well or the negative control (cell culture medium). The plate was then incubated at 37°C for 15 min to 6 hr and OD was then read at 620-655 nm using a microplate reader.

Table 2.6: Quanti-Blue solution

Reagent	**ml**
QB reagent	1
QB buffer	1
Sterile H2O	98

2.2.8 RNA analysis

2.2.8.1 RNA extraction

RNA was extracted from cells using a PureLink RNA mini kit as described by the manufacture's manual. Cell monolayers were washed x3 with PBS. The cells were then lysed in RNA lysis buffer and scraped off the wells using the pipette tip. Next was the steps of the PureLink RNA mini kit, where the RNA was run through columns, and washed with 70% ethanol along with the mini kit's wash buffers I and II. The RNA was eluted using RNase-free water and quantified using the nanodrop to normalize further steps. Next, the samples were treated with DNase to ensure that all genomic DNA was removed. DNase treatment was completed at the following specifications.

Table 2.7: DNase Treatment

Reagent	**1X Master Mix (µls)**
RNA	10
RNase free H2O	7
DNase I	1

10X Reaction buffer	2

Samples are then placed in the thermocycler at 37°C for 30 minutes. Then 2 µl of EDTA was added to each sample tube and they were placed in the thermocycler at 65°C for 10 minutes. RNA was then stored at -20°C for later use.

2.2.8.2 cDNA synthesis

cDNA synthesis reaction master mix was prepared for qRT-PCR using the specifications displayed in table 2.7.

Table 2.8: cDNA synthesis

Reagent	**1X Master Mix (µls)**
Reaction Buffer	2
dNTPs	0.8
Random Primers	2
Reverse Transcriptase	0.5
RNase Inhibitor	0.2
RNase Free water	4.5

To run cDNA synthesis 10 µls of the prepared DNase treated RNA was added to 10 µls of master mix to give a final volume of 20 µls in each reaction tube. cDNA synthesis was then carried out using a thermocycler with the following specifications.

Table 2.9: Thermocycler specifications cDNA synthesis

Step	**Temperature °C**	**Duration**
1	25	10 minutes
2	37	120 minutes
3	85	5 minutes
4	4	∞

Following the cDNA synthesis, the cDNA is then diluted for qPCR use, by addition of 60 µls of RNase free water.

2.2.8.3 qRT-PCR

qRT-PCRs were completed in 96-well PCR plates. Results were then analysed by the comparative Ct method (2-(ΔΔCt)). The expression of mRNA targets was analysed using

PowerUp SYBR green master mix and relative expression was then compared against a housekeeping gene. Each SYBR reaction was set up according to table 2.9. Then 6 µl of the master mix was placed into each reaction well, followed by 2 µl of cDNA. The qPCR plate was then placed into a qPCR machine and the cycling parameters for the reaction are laid out in table 2.10.

Table 2.10: SYBR qRT-PCR

Reagent	**1X Master Mix µls**
PowerUp SYBR	4
Forward Primer	0.8
Reverse Primer	0.8
H2O	3.4

Table 2.11: SYBR qRT-PCR cycling parameters

Step	**Temperature °C**	**Duration**
Hold	50	2 minutes
Hold	95	2 minutes
Cycle (x40)		
Denature	95	3 seconds
Anneal/Extend	60	30 seconds

2.2.9 Enzyme-linked immunosorbent assay (ELISA)

According to the Invitrogen (ThermoFisher) ELISA kit instructions, the following protocol was executed. The day before the ELISA assay, the plates were coated with the capture antibody, diluted in coating buffer. The plates were then incubated in the cold room on a shaker overnight. The plates were then emptied and washed using wash buffer (1X PBS, 0.05% Tween-20). The wells are then blocked using ELISA diluent (1X) and incubated on a rocker at room temperature for 1 hour, followed by washing with wash buffer. The standards are next prepared and added to the plate. Next the supernatant samples were added to the ELISA plates, with diluent added for the blank wells. The plates are then incubated on a rocker at room temperature for 2 hours, followed by washing with wash buffer. Next, plates were emptied and washed before adding the detection antibody. Incubated for 1 hour on

rocker at room temperature. Then, the Streptavidin/Avidin-HRP in was added to the plate followed by a 30-minute incubation. After washing thoroughly, the TMB solution is added to the wells, and the plate placed on the rocker for 15 minutes. Then the stop solution (1M H2SO4) is added to the wells. Lastly the plate is read at 450 and 570 nm. The values are then standardized using the standard curve and cytokine levels represented as picograms per millilitre (pg/ml).

2.2.10 Bacterial phagocytosis assays

Macrophage cells (RAW-Blue, or BMDM) were grown in cell culture, maintained at 37°C, 5% CO2 and 95% relative humidity. The cells are seeded into 24 well pates at 5 x 10^5 cells/ml, in antibiotic free DMEM (10% FBS), and left to settle overnight. Cells were then treated with postbiotic compounds for 24 hours. A culture of *E. coli* NCTC12900 was prepared in LB and grown overnight at 37°C. After 24 hours of treatment with postbiotics, macrophages were treated at multiplicity of infection (MOI) 20:1, followed by centrifugation at 300rpm for 2 minutes and then incubated at 37°C, 5% CO2. Following 30 minutes of incubation, the media was removed and replaced with media containing 100ug/ml gentamicin to kill off extracellular bacteria. After 15 minutes in the gentamicin media, the supernatant is removed, monolayers washed with PBS, and lysed with ice cold sterile water. This is also done at 3- and 6-hours post-infection. After 1 hour in 100ug/ml gentamicin media, it is replaced with 10ug/ml gentamicin. Next, to quantify the number of intracellular bacteria, the cell lysates are serially diluted and plated onto L agar at 37°C overnight. Colonies were then counted and represented as log colony forming units (LogCFU/ml).

2.2.11 Animals

Mouse breeding and maintenance was performed by the CMU staff at TBSI TCD. Animal studies were performed in age- and sex-matched C57BL/6J mice. Mice

were maintained in ventilated cages at 21 ± 1 °C, humidity 50 ± 10%, with a 12 h light/12 h dark light cycle under specific pathogen-free conditions, in line with Irish and European Union regulations. Food and water were monitored and available ad libitum throughout the experiments. All experiments were subject to ethical approval by Trinity College Dublin's Animal Research Ethics Committee and were carried out in accordance with the Irish Health Products Regulatory Authority, the competent authority responsible for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes in accordance with the requirements of the S.I No 543 of 2012.

2.2.12 LPS-induced model of sepsis

Male and female C57BL/6Ntac mice, (aged 14-15 weeks) were randomly assigned to experimental groups. Briefly, PB9 was administered by oral gavage to mice 24h and 2h prior to intraperitoneal injection with LPS (15 mg/kg). Four hours later mice were euthanised in a CO2 chamber. Peritoneal epithelial cells (PECs) were harvested by peritoneal lavage using 5 mL sterile phosphate-buffered saline (PBS) (pH 7.2). After centrifugation at 120 × g for 5 min, supernatants were collected for analysis and the cell pellets were resuspended in RNA lysis buffer and processed using the RNA isolation kit. Whole blood samples were also harvested, and serum was collected by centrifugation at 1,000 x g for 12 min.

Chapter 3

Novel postbiotics derived from pot-ale display antimicrobial potential

3.1 Introduction

As the Antimicrobial Resistance (AMR) crisis continues to threaten public health on a global scale, finding novel antimicrobial compounds is a priority. WHO has stated AMR as one of the top 10 global public health threats, needing urgent action. Drivers of AMR include clinical misuse and overuse, public perception and behaviour surrounding antimicrobials, agricultural applications, vaccination reluctance and commercial pressures[80]. The two main contributors being overuse of antibiotics in both a human clinical setting as well as widespread and reckless use in the agricultural world. If the clinical world continues the current trajectory of over and misuse of antibiotics, by 2050 the leading cause of human deaths will be antimicrobial resistant infections[40]. The urgency is similar in the agricultural industry as mass antibiotic treatments being the norm are entirely unsustainable and only compound the issue[40].

As laid out in the WHO Global Action plan on AMR, the control of antimicrobial misuse in clinical settings needs to be managed through proper prevention of infections as well as alternative treatment solutions. Antimicrobial resistance education and stewardship can certainly aid in efforts to conquer AMR[81], but this is not enough. Alternative antimicrobials with minimal side effects are becoming more and more essential as levels of multidrug resistant strains raise each year. According to a study from 2019, total number of deaths attributed to AMR that year was estimated at 1.27 million[82]. Resistant strains are becoming more common and harder to treat as our pool of effective antimicrobial dugs dwindles.

Possible new avenues for mining antimicrobials are currently being investigated. Among these are postbiotics, commonly thought of as a step beyond pre- and probiotics. Postbiotics are defined as “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” by the International Scientific Association of Probiotics

and Prebiotics (ISAPP)[83]. Postbiotics have been shown to have antimicrobial activity against infectious diseases [66,84,85]. Postbiotic supplementation has also displayed ability to help diversify and promote non-specific immunity within the gut microbiome [67,86,87]. Postbiotics are also being researched in tandem with probiotic supplements, it has been seen that a synergistic approach also has beneficial antimicrobial outcomes[84.] This idea of prebiotics, probiotics and/or postbiotics administered together to enhance beneficial effects on the host is referred to as 'synbiotic' and is another growing area of research.

Amongst the growing need for novel antimicrobial compounds, this project aims to explore the antimicrobial capabilities of novel postbiotics. The postbiotics studied in this project have been supplied by Marigot. Ltd., a company hoping to find new purpose for whisky distillery waste. The waste in question, pot ale, is currently a burden, both economically and environmentally to dispose of for hundreds of distilleries. The pot ale, which is itself a yeast postbiotic, is also inoculated with other strains such as *L. casei* to produce different forms of postbiotics.

The AMR crisis needs action now, this portion of our research aims to begin the assessment of these postbiotics samples as antimicrobial agents.

3.2 Results

3.2.1 Postbiotics do not display ability to inhibit bacterial growth through diffusion

To investigate the antimicrobial ability of the postbiotic samples, we first performed a well diffusion assay. As some of the leading pathogens associated with MDR are *E. coli, S. aureus, K. pneumoniae, A. baumannii, and P. aeruginosa*[82], these species along with *S. typhimurium and L. monocytogenes* were chosen for this experiment. MH agar inoculated with each bacterial strain was exposed to each of the following postbiotic samples; PB 1, PB 3, PB 9, PB 14, and PB 25. No zones of clearance were observed in the inoculated agar plates as displayed in table 3.1.

Zones of clearance (0cm)					
	PB 1	PB 3	PB 9	PB 14	PB 25
E. coli	0	0	0	0	0
S. typhimurium	0	0	0	0	0
P. aeruginosa	0	0	0	0	0
K. pneumoniae	0	0	0	0	0
S. aureus	0	0	0	0	0
A. baumannii	0	0	0	0	0
L. monocytogenes	0	0	0	0	0

Table 3.1 Well diffusion zones of clearance Zero zones of clearance seen for each postbiotic sample. Values are representative of three individual experiments, consisting of two replicates each.

3.2.2 Postbiotics display ability to inhibit growth of different bacterial pathogens

To investigate the ability of the postbiotic samples to inhibit growth of bacteria, a minimum inhibitory concentration (MIC) was performed. In the previous experiment 3.2.1, there was one concentration of each postbiotic strain present, measuring its ability to diffuse across inoculated agar. In this experiment multiple concentrations of postbiotic are mixed into the broth, which is then inoculated with various bacterial strains. Measuring the ability of the postbiotic to inhibit growth in the broth. Each postbiotic, at concentrations between 50-1.562%, was tested against the following bacterial strains; *E. coli* NCTC 129000, *S. Typhimurium* UK1, *K. pnuemoniae* ATCC700603, *P. aeruginosa* PAO1, *S. aureus* ATCC25923. After inoculation, plates were incubated for 18hrs at 37°C and then absorbances read at OD 600. PB 1 shows a significant reduction in bacterial growth of *S. typhimurium, P. aeruginosa, K. pnuemoniae and S. aureus* at a 25% PB concentration and 12.5% PB 1 concentration inhibits growth in *E. coli* [Figure 3.2]. PB 3 significantly inhibits growth of all tested strains, *E.coli, S. typhimurium, P. aeruginosa, K. pnuemoniae and S. aureus*, at a concentration of 25% PB [Figure 3.3]. PB 9 is less effective with inhibition only seen at 50% PB concentration for all strains except *P. aeruginosa*, where inhibition can be significantly seen at 25% PB concentration [figure 3.4]. PB 14 showed the least amount of antimicrobial activity with the only significant inhibition being seen at a concentration of 50% PB with *E. coli and S. typhimurium* [figure 3.5]. The postbiotic that shows the greatest antimicrobial promise is PB 1.

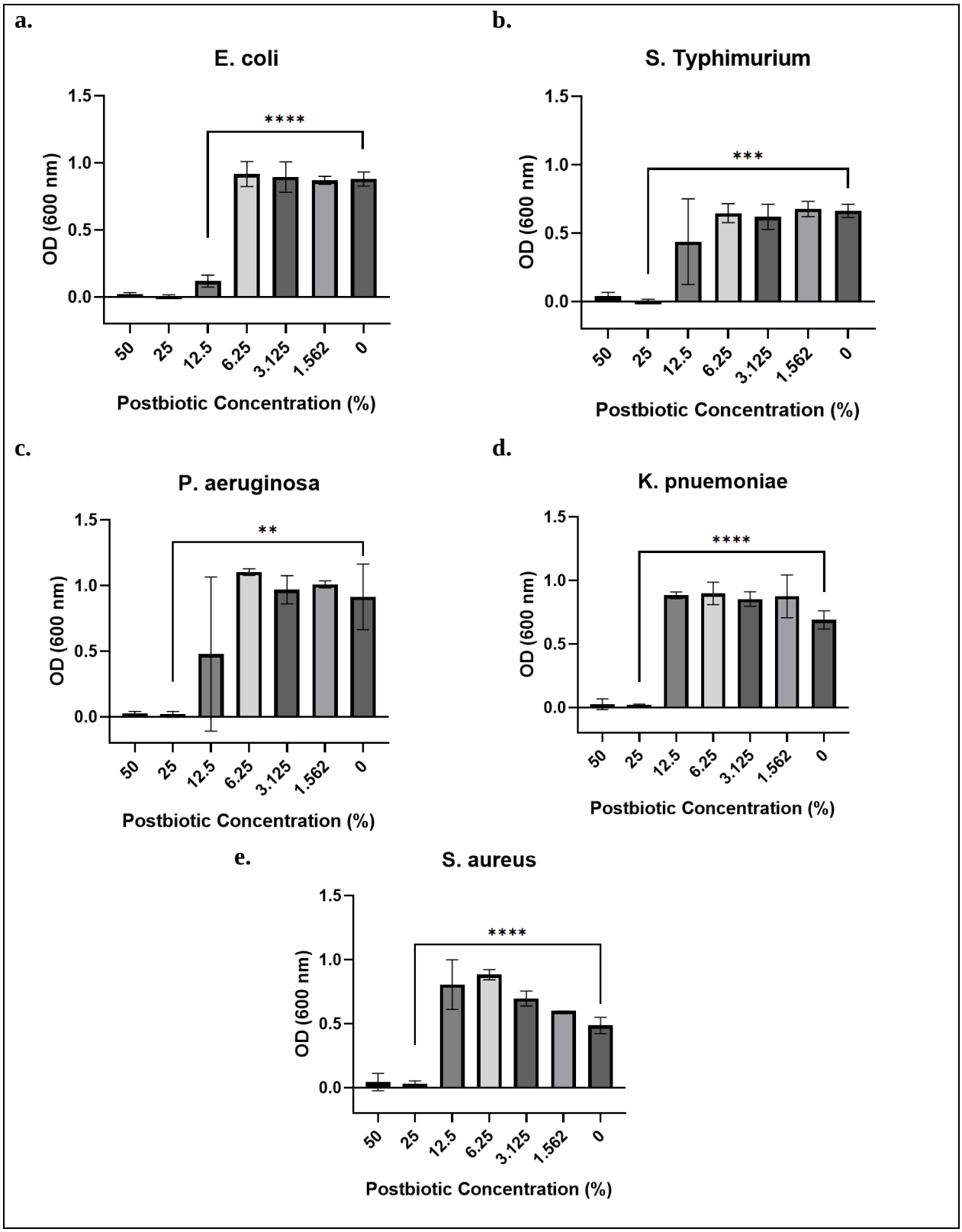


Figure 3.2 MIC – Postbiotic 1 displays significant bacterial growth inhibition MH II broth containing a range of PB concentrations were inoculated with the following bacterial strains: *E. coli* NCTC 129000, *S. Typhimurium* UK1, *K. pnuemoniae* ATCC700603, *P. aeruginosa* PAO1, *S. aureus* ATCC2592. Results were read 18 hrs after inoculation. Results are representative of three separate experiments with two replicates each. An ordinary one-way ANOVA was performed, and significance is indicated as follows, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

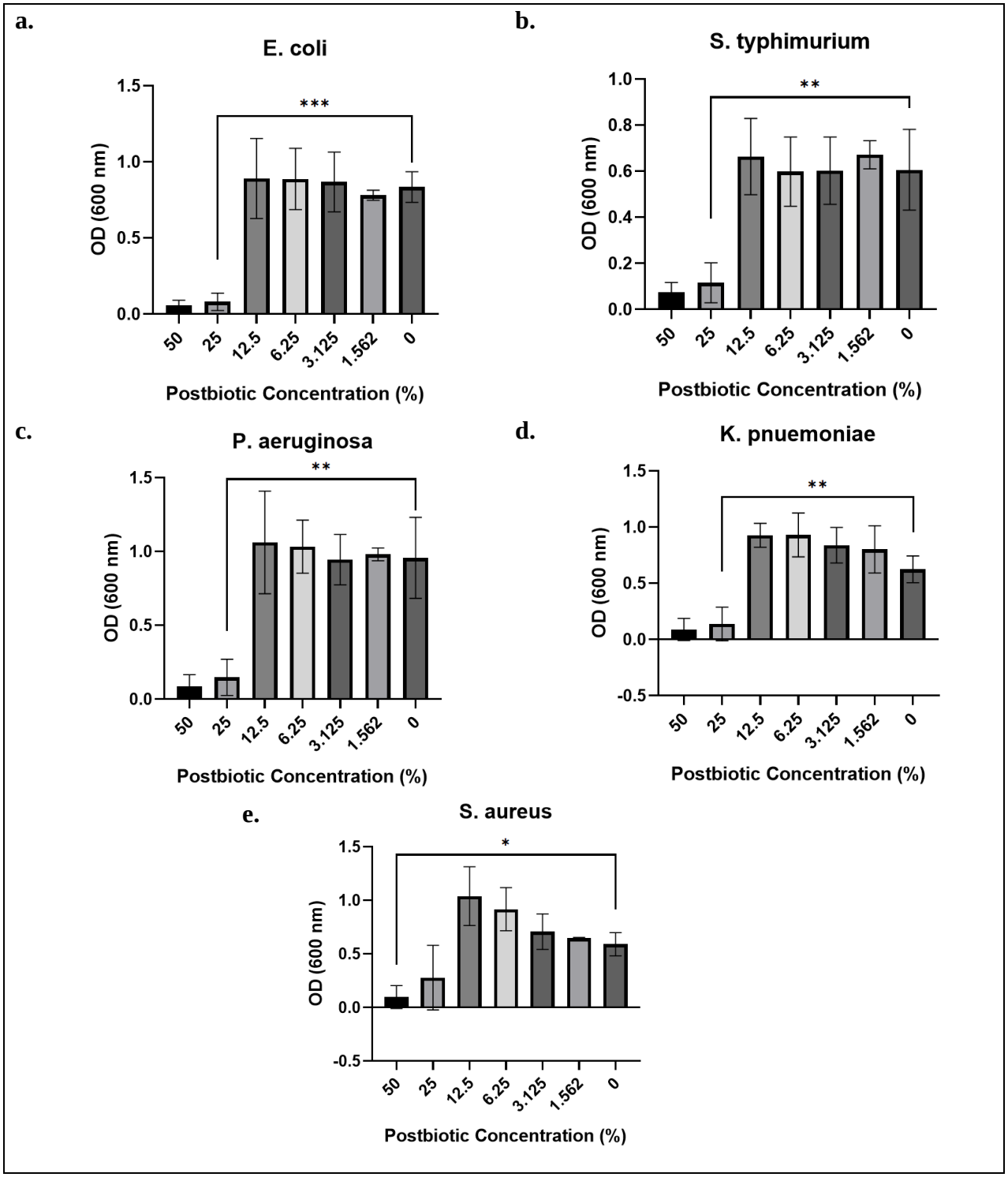


Figure 3.3 MIC – Postbiotic 3 displays significant bacterial growth inhibition MH II broth containing a range of PB concentrations were inoculated with the following bacterial strains: *E. coli* NCTC 129000, *S. Typhimurium* UK1, *K. pnuemoniae* ATCC700603, *P. aeruginosa* PAO1, *S. aureus* ATCC2592. Results were read 18 hrs after inoculation. Results are representative of three separate experiments with two replicates each. An ordinary one-way ANOVA was performed, and significance is indicated as follows, $p < 0.05$ = *, $p \leq 0.01$ = ** and $p \leq 0.001$ = ***.

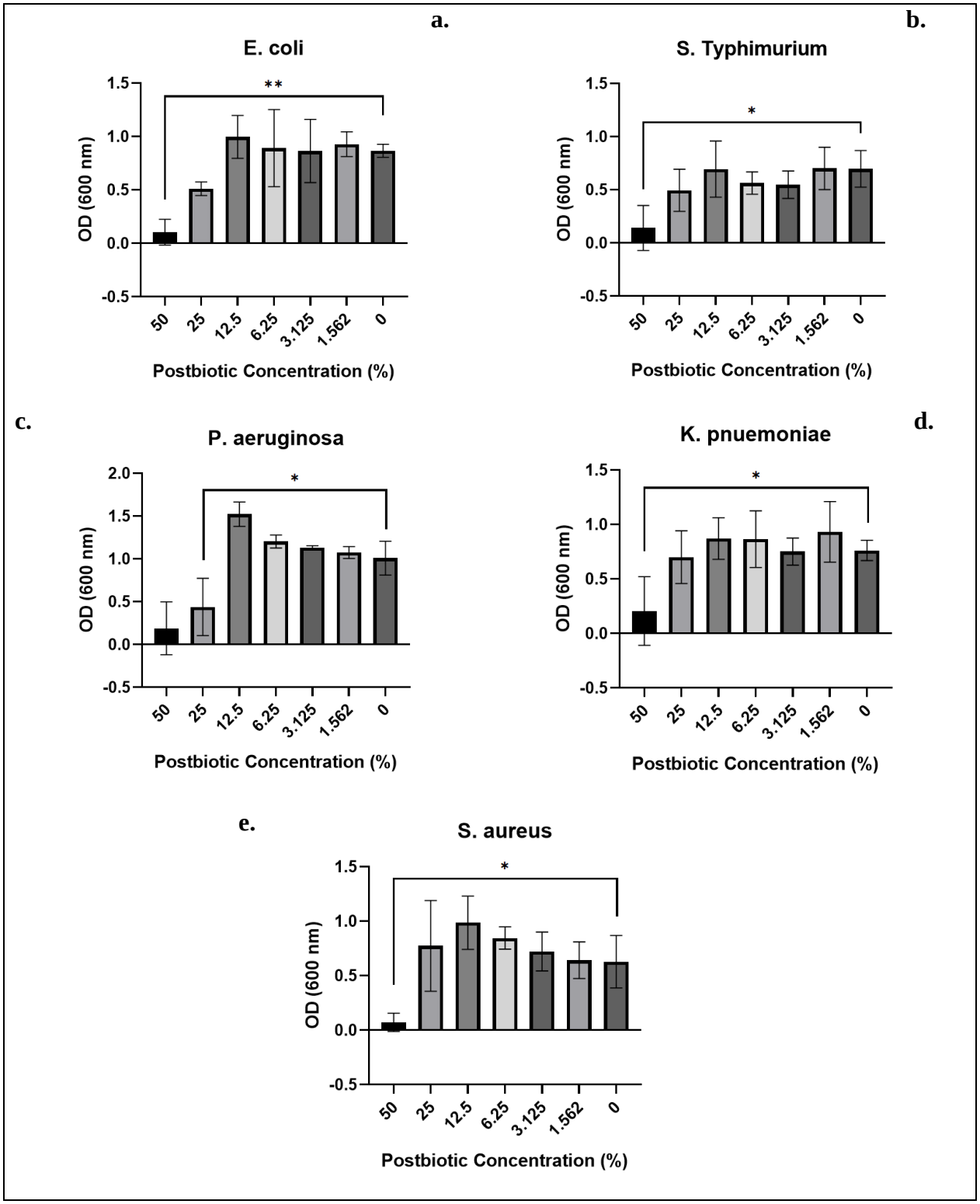


Figure 3.4 MIC – Postbiotic 9 displays slight inhibition of bacterial growth MH II broth containing a range of PB concentrations were inoculated with the following bacterial strains: *E. coli* NCTC 129000, *S. Typhimurium* UK1, *K. pnuemoniae* ATCC700603, *P. aeruginosa* PAO1, *S. aureus* ATCC2592. Results were read 18 hrs after inoculation. Results are representative of three separate experiments with two replicates each. An ordinary one-way ANOVA was performed, and significance is indicated as follows, $p < 0.05$ = * and $p \leq 0.01$ = **.

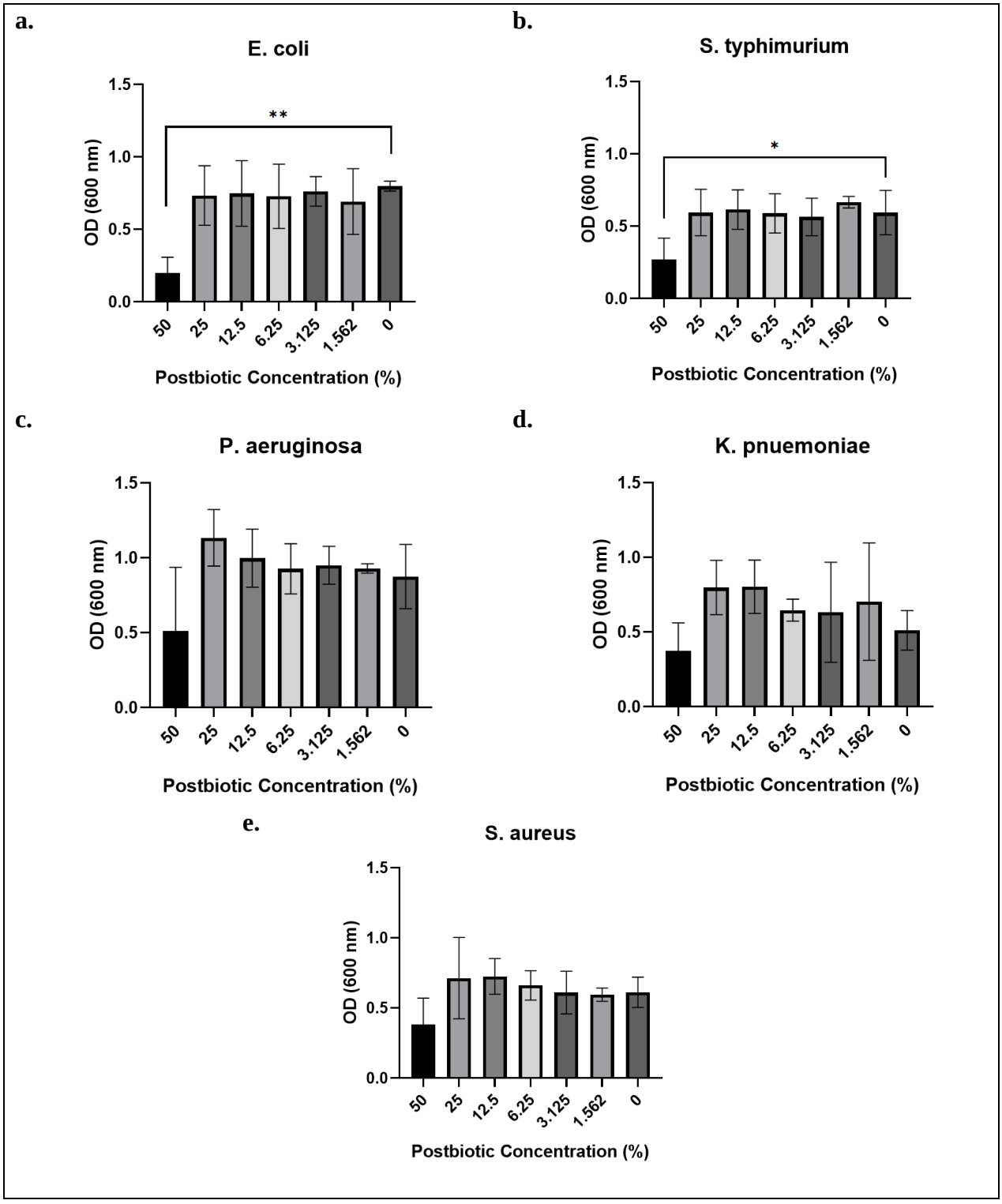


Figure 3.5 MIC – Postbiotic 14 displays little significant inhibition of bacterial growth MH II broth containing a range of PB concentrations were inoculated with the following bacterial strains: *E. coli* NCTC 129000, *S. Typhimurium* UK1, *K. pnuemoniae* ATCC700603, *P. aeruginosa* PAO1, *S. aureus* ATCC2592. Results were read 18 hrs after inoculation. Results are representative of three separate experiments with two replicates each. An ordinary one-way ANOVA was performed, and significance is indicated as follows, $p < 0.05$ = * and $p \leq 0.01$ = **.

3.2.3 Postbiotics display bactericidal activity

Next we evaluated the bactericidal ability of the postbiotics to inhibitory ability, with a minimum bactericidal concentration (MBC). Using a replicator under sterile conditions, the MBC wells were inoculated with the corresponding MIC wells. PB 1 displays bactericidal ability at 25% concentration to all tested strains excluding *S. aureus*. PB 3 displays similar ability at 25% PB concentration, bactericidal against all tested strains except *S. aureus* and *K. pneumoniae*. PB 9 shows little bactericidal ability, only at 50% against all strains except *S. typhimurium*. PB 14 displays no bactericidal ability against any strain at any concentration. Here, PB 1 Shows the strongest antimicrobial activity, and PB 14 the weakest.

		PB Concentrations (%)					
Postbiotic	Test Organism	50	25	12.5	6.25	3.125	1.5625
PB 1	*E. coli*	-	-	+	+	+	+
	S. typhimurium	-	-	+	+	+	+
	P. aeruginosa	-	-	+	+	+	+
	K. pneumoniae	-	-	+	+	+	+
	S. aureus	-	+	+	+	+	+
PB 3	*E. coli*	-	-	+	+	+	+
	S. typhimurium	-	-	+	+	+	+
	P. aeruginosa	-	-	+	+	+	+
	K. pneumoniae	-	+	+	+	+	+
	S. aureus	-	+	+	+	+	+
PB 9	*E. coli*	-	+	+	+	+	+
	S. typhimurium	+	+	+	+	+	+
	P. aeruginosa	-	+	+	+	+	+
	K. pneumoniae	-	+	+	+	+	+
	S. aureus	-	+	+	+	+	+
PB 14	*E. coli*	+	+	+	+	+	+
	S. typhimurium	+	+	+	+	+	+
	P. aeruginosa	+	+	+	+	+	+
	K. pneumoniae	+	+	+	+	+	+
	S. aureus	+	+	+	+	+	+

Table 3.2 Postbiotics display some significant bactericidal effects Multiple concentrations of each Postbiotic are tested against the following strains: *E. coli* NCTC 129000, *S. Typhimurium* UK1, *K. pnuemoniae* ATCC700603, *P. aeruginosa* PAO1, S. *aureus* ATCC2592. MBC plates were incubated for 18 hours before having bacterial growth read. (+) indicates the presence of growth. Results are representative of three separate experiments, each containing two replicates.

3.3 Discussion

As the of mining novel antimicrobials is essential in combating the rise of the AMR crisis and its rising death toll, this chapter of the project is focused on beginning to uncover any antimicrobial activity in novel postbiotics. Novel and sustainable sources for health intervention such as postbiotics are scarce. This leaves a gap in research that this project aims to address. Strains for these experiments were chosen specifically as strains likely to develop resistance, including *E. coli* NCTC 129000, *K. pnuemoniae* ATCC700603, *P. aeruginosa* PAO1, *S. aureus* ATCC2592, and A. *baumannii*[39,88]. *S. Typhimurium* UK1 and *L. monocytogenes* were also chosen as they are common pathogens that can cause gastrointestinal infections. A postbiotic that can inhibit the growth of any of these stains would be a tool for preventing the presence of a MDR bacteria finding a home in the gut microbiome and a tool for preventing infection from a pathogenic strain which would then cut out the need for antibiotic treatment.

Current research on postbiotics demonstrates their anti-microbial ability, with potential applications ranging from food packaging to dietary supplementation[61,66,89,90]. Our research aims to investigate if this novel source of postbiotics can maintain this anti-microbial ability held by other postbiotic samples. For this novel source of postbiotics to be deemed a valid postbiotic it must confer a benefit to the host[83]. Anti-microbial activity towards pathogenic bacteria is one hugely beneficial effect for the host.

The four main postbiotics in this study can be split into two distinct groups as stated before. PB 1 and PB 3 are yeast postbiotics, cell-containing and cell-free respectively; PB 9 and PB 14 are *L. casei* postbiotics cell-containing and cell-free respectively. In this study in terms of antimicrobial ability, the yeast postbiotics are more effective than the *L. casei* postbiotics. Supernatants from lactic acid bacteria (LAB) like *L. casei*, have been shown to have

antimicrobial effects, especially in the preservation of food products[91,92]. Although some of these effects are due to the acids generated by metabolites of LAB[91], which in this case would be hindered because our postbiotic samples are pH neutralised. However, the postbiotic samples PB 1 and PB 3, the yeast postbiotics or plain pot-ale, demonstrated the strongest antimicrobial potential here, which is interesting given past research on the benefits of *L. casei* supernatants.

Interestingly in the results from the MIC/MBC the cell-containing postbiotic samples (PB 1, PB 9) displayed more antimicrobial activity than their cell-free counterparts (PB 3, PB 14). This suggests that the presence of the whole inactivated cell aids in inhibiting bacterial growth. This fits well within current research available in the field as, in terms of gut health, it is known dead cells being present provides more protection as it boosts innate immunity[93]. More research is needed here to better understand he mechanism by which these postbiotics inhibit bacterial growth, as there are many potential pathways. There are several potential pathogen-associated molecular patterns (PAMPs) present on inactivated/dead bacterial cells, some of these include cell wall components, peptidoglycans, and bacterial DNA. These PAMPs are recognised by host defences by pattern recognition receptors (PRRs) on immune cells. Examples of these PRRs include toll-like receptors (TLRs) and NOD-like receptors (NLRs), which both illicit different pro-inflammatory responses. While the presence of inactivated bacterial cells can activate the innate immune system, it can also aid the adaptive immune system. The bacterial cells/their parts can be picked up by antigen presenting cells (APCs) and presented to T-cells to prime the adaptive immune system [93]. These are just a few possible general pathways, by which whole inactivated bacterial cells can prime the immune system. Enzyme-linked immunosorbent assay (ELISA) can be performed to detect cytokines from specific inflammatory pathways, and western blotting can be performed to detect signalling proteins downstream of PRRs activation. Another option would be a luciferase

reporter assay, to study cellular signalling pathways by introducing a genetically engineered luciferase gene that controls a promoter region of interest. Then the activation of the luciferase enzyme is measured by the bioluminescence emitted, indicating the specific signalling pathway that is active.

In the MIC experiments, the percentage of postbiotic required to see inhibitory or bactericidal effects is relatively high. PB 1 significantly inhibits growth at 12.5% for *E. coli* and 25% for *K. pnuemoniae, P. aeruginosa, S. aureus, and S. Typhimurium*. While inhibiting growth at a concentration of 25% could potentially be attributed to the significant discrepancy in amount of growth agar, this is not the case here because we clearly see the same strains having no growth inhibition all the way up to 50% PB 14 concentration. These results display valid and significant inhibition of growth, and the MBC results support the pattern. However, to get a better understanding exact MIC, more concentrations should be tested here. Incorporating other MDR strains in experiments would be beneficial as well.

In this chapter we examined the potential antimicrobial effects of these novel postbiotic samples. Previously researched postbiotics maintain anti-microbial ability in some cases, so those abilities were tested on our novel postbiotic samples. PB 1, PB 3, and PB 9 showed promising antimicrobial abilities against pathogens that are at high risk for MDR. This makes them important in the fight against AMR. While postbiotics having antimicrobial effects against pathogenic bacteria is key for AMR research, the other side of the infection story is also important. Can these novel postbiotics modulate the hosts immune system, therefore strengthening the host immunity and decreasing need for harmful current antibiotic interventions. In the next chapter, the immunomodulatory effects of these postbiotics will be tested.

Chapter 4

Novel pot-ale derived postbiotics display immunomodulatory effects on macrophages

4.1 Introduction

Over recent decades, the prevalence of IBD has grown tremendously[2]. IBD includes Ulcerative Colitis and Crohn's Disease (CD), chronic and incurable diseases, causing severe pain and discomfort. Many of the current treatment options, ranging from antibiotic treatments to faecal microbiota transplant (FMT) have significant side effects and are costly to both patients and healthcare systems[94].IBD are highly variable and complex diseases with many risk factors. These include genetics, geography, lifestyle, gut microbiome balance and immune function[95]. Two of these key causes of IBD are uniquely linked. The relationship between the gut microbiota and the host immune system is complex and not fully characterized at this time. However, dysregulation of this partnership promotes inflammation in the gut which can lead to several health issues including IBD [96].

Immune system function is central to the pathology of IBD. The immune system has a complicated job in the gut, maintaining the gut barrier and fighting off pathogenic bacteria while also maintaining a healthy tolerance to commensal bacterial communities. Macrophages are considered the "first responders" of the immune system, responsible for engulfing any foreign intruders. They are especially important in the gut, as the lamina propria is home to the highest concentration of macrophages in the body[97,98]. They are also essential for eliciting inflammatory responses. As mentioned before, the immune system in the gut needs to fight pathogens and maintain tolerance simultaneously. Macrophages aid in this mission by having two main polarized states known as M1 and M2. While there is speculation that this is an oversimplified model, it is still useful when trying to gauge the level of inflammation present in the environment[99,100].

M1 or classically activated macrophages are responsible for the inflammatory response. They are highly antimicrobial, as they have higher levels of intracellular killing, and lower levels of

phagocytosis. A shift to M1 polarization can be induced by many things including bacterial infection, or stimulation with endotoxins such as LPS. M1 macrophages are responsible for the inflammatory response, so they release inflammatory cytokines. This can be done through multiple pathways, one of the main pathways being the activation of the NF-κB transcription factor. NF-κB is a hallmark of inflammatory diseases. NF-κB is a master regulator of the inflammatory immune response, and its activation leads to the release of pro-inflammatory cytokines such as TNFa, IL-1B, and IL6[101–103]. These cytokines have different roles, but most importantly, they alert surrounding cells to initiate inflammation[99]. The release of nitric oxide synthase 2 (NOS2) is also associated with M1 macrophages, as it is highly antimicrobial. NOS2 synthesises nitric oxide (NO) which is defensive against bacterial invasions[104,105]. In certain situations, like bacterial infections, this inflammatory response is useful and essential to protect the host, however, incorrect overstimulation of this pathway can lead to long term inflammation that is detrimental to the host[106]. For example, the overproduction of TNFa during a prolonged inflammatory response can lead to compromised intestinal epithelial barrier integrity[107].

This is where the M2 macrophage polarization comes in. M2 macrophages are associated with anti-inflammatory pathways and responsibilities include immunoregulation and tissue repair. M2 macrophages have higher phagocytic ability but inhibited intracellular killing ability. The M2 polarization is mostly controlled by STAT6, a transcription factor[108]. M2 macrophages release cytokines such as IL10, which signals to nearby cells to initiate anti-inflammatory and immunoregulatory pathways[109]. Both M1 and M2 polarization states are essential to the homeostasis of the immune system.

As previously discussed, the macrophages in the gut have the very complicated job of maintaining tolerance to commensal bacteria while also reacting to pathogenic invaders. For

this reason, intestinal macrophages have adapted specific skills for the unique environment of the gut. Intestinal macrophages can help maintain and protect the epithelial cell layer. Intestinal macrophages as tolerate higher levels of phagocytic activity without causing a full inflammatory response. These special macrophages have lower levels of innate immune response receptors for stressors such as LPS[110]. It has also been shown that macrophage cells help build tolerance by feeding antigens through the epithelial barrier to resident dendritic cells[28]. As the intestine is exposed to high levels of antigens and microbes daily, this tolerance is essential. An overreaction by the immune system can lead to unwanted inflammation.

As balance between the gut microbiome and host immune system has become key to understanding the pathology of IBD, microbiome-based anti-inflammatory treatment options are being explored. Supplementing with interventions such as prebiotics, probiotics, or postbiotics can help promote a diverse and healthy microbiome. Prebiotics, defined as a product that are selectively provided to feed specific microorganisms, which then confers a health benefit. Dietary fibres make up most of the prebiotic supplemtents[44]. Dietary fibre helps to maintain a healthy and diverse microbiome, as it is a food source for commensal bacteria in the gut[45]. Another way of supporting the beneficial commensal bacterial communities in the gut is supplementing with live bacteria, or probiotics. This also promotes a diverse and healthy microbiota[41]. Probiotic supplementation many benefits including protection from antibiotic induced diarrheal disease activity[42], and in some studies, reduce the disease activity of patients with active UC compared to placebo groups[43]. However, there are also real risks involved in administering live bacteria, including infection and inflammatory effects. While the risk is minimal for healthy individuals, immunocompromised people are susceptible as some probiotic stains can be opportunistic pathogens[46–48]. While probiotics are

praised for their anti-inflammatory effects, other studies demonstrate the pro-inflammatory effects of probiotics[48,52,53].

As the concern for administration of live bacteria rises, the subject of postbiotics has become more popular. Defined by The International Scientific Association of Probiotics and Prebiotics (ISAPP) as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [83]. Essentially, postbiotics are fermented supernatant. They can be cell containing (CCS) or cell free supernatant (CFS), both having potential anti-microbial, immunomodulatory, and anti-inflammatory effects. There is a lot of bioactive potential as postbiotics contain inanimate microorganisms, bacterial cell fragments/structures and metabolites /end-products of bacterial fermentation[83]. Bacterial cell fragments can be antimicrobial and anti-inflammatory[111,112], this can be done through training the immune response, readying immune cells for infections[113]. Postbiotics can also have restorative effects of gut dysbiosis in TNBS-induced colitis as shown in Zhou et al.[114]. Some studies even show that postbiotic effects can be even stronger than that of probiotics, confirming that live bacteria are not central to the benefits of these supplements [64]. While they remain a novel area of research, many reviews outline beneficial properties of postbiotics[61–63].

A study done recently demonstrated the ability of individual bacterial metabolites to modulate the intestinal environment. The metabolites displayed the ability to protect against gut barrier dysfunction and permeability as well as influence the phagocytic ability of resident macrophage cells[60]. Similarly, the goal of this project is to uncover the effects of novel postbiotic samples on macrophage immune function. This will be done through a series of experiments investigating the postbiotic samples effect on macrophage function, by monitoring inflammatory cytokines and phagocytic activity.

4.2 Results

4.2.1 Postbiotics have no significant effect on cell viability

Firstly, cytotoxicity of the postbiotic samples to our *in vitro* macrophage cell lines was investigated. This was done using the Alamar blue assay, an assay that measures levels of metabolic activity. Figure 4.1 displays the cell viability of RAW-Blue macrophages after 24-hour treatments with distinct concentrations of each postbiotic (PB 1, PB 3, PB 9, PB 14, and PB 25). While there is some drop off in cell viability at the 10% postbiotic concentration, there is no significant reduction of cell viability. Figure 4.2 displays cell viability of bone marrow derived macrophage (BMDM) cells after 24-hour treatments with distinct concentrations of each postbiotic. While there is some increase of cell viability at the 10% postbiotic concentration, there is no significant change in cell viability.

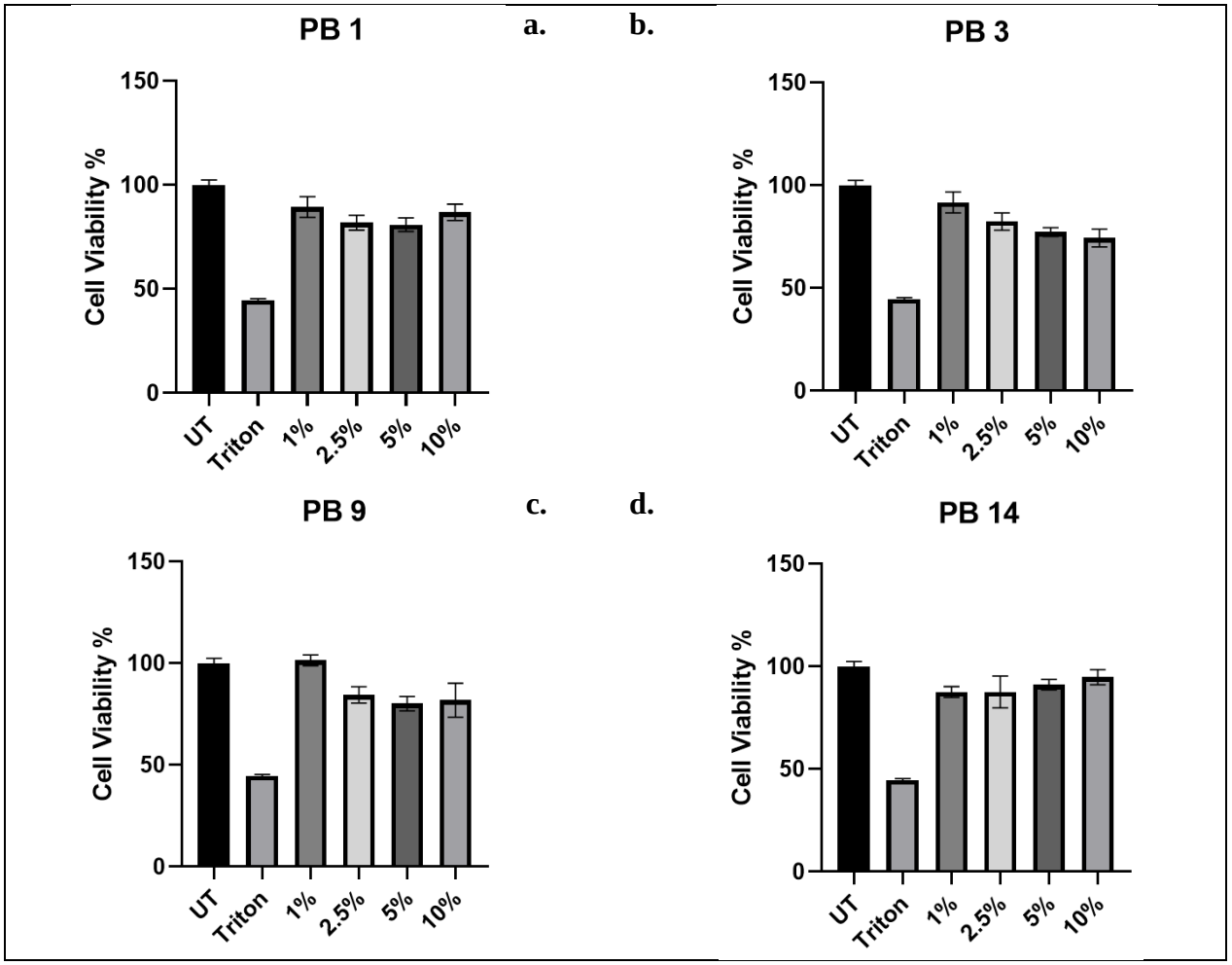


Figure 4.1 Postbiotics do not affect cell viability RAW-Blue macrophages were treated for 24-hours with specific concentrations between 1% and 10% of [A] PB 1, [B] PB 3, [C] PB 9, [D] PB 14. Data is representative of three separate experiments with three replicates each. A student's t-test was performed, and no significant change was measured.

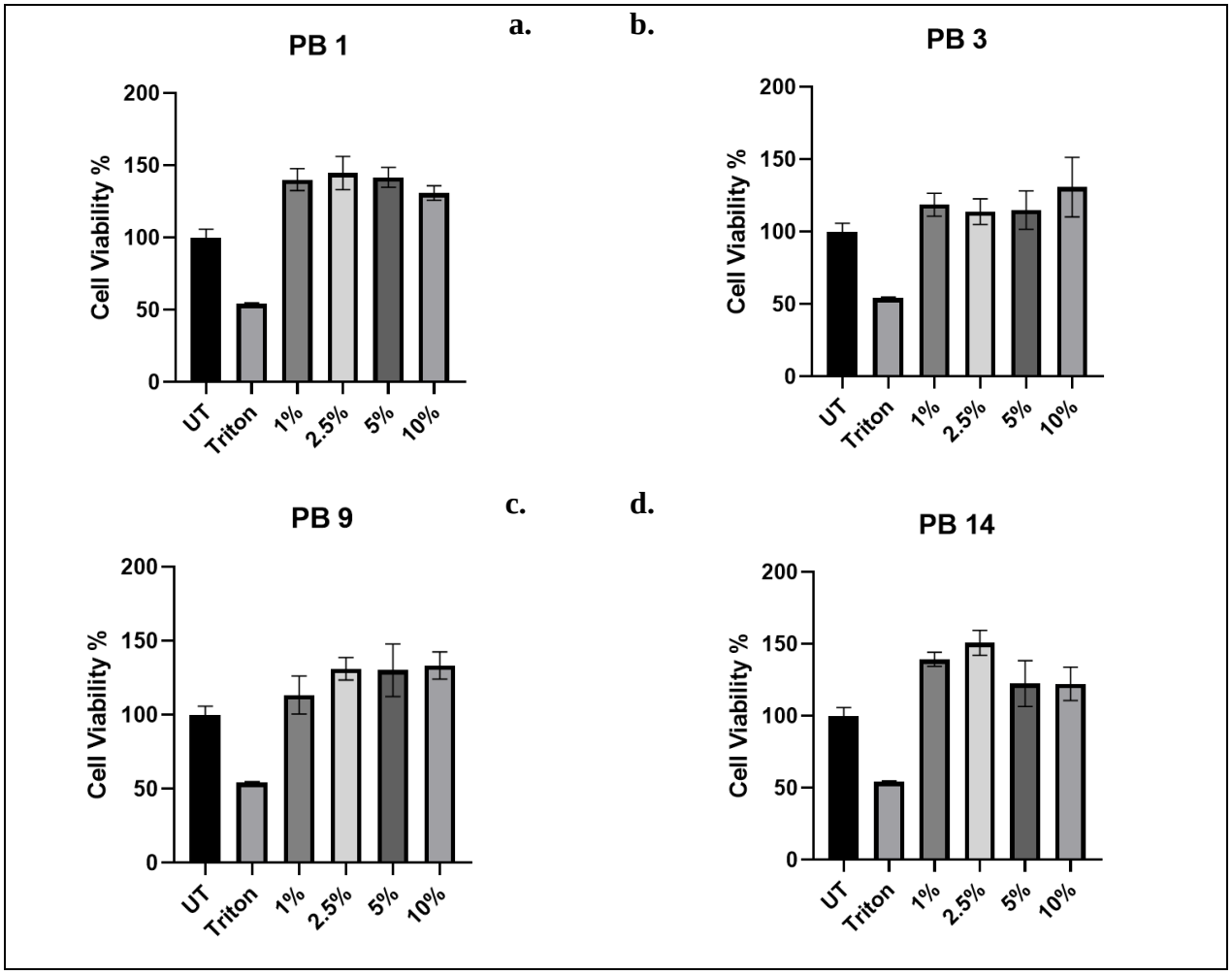


Figure 4.2 Postbiotics do not affect cell viability BMDMs macrophages were treated for 24-hours with specific concentrations between 1% and 10% of [A] PB 1, [B] PB 3, [C] PB 9, [D] PB 14. Data is representative of three separate experiments with three replicates each. A student's t-test was performed, and no significant change was measured.

4.2.2 Postbiotic treatment modulates NF-κB activity on macrophages

To quantitatively measure the level of NF-κB expression, the Quanti-Blue colorimetric enzyme assay. NF-κB is a key immunomodulatory transcription factor in macrophage cells. The Quanti-Blue assay determines the alkaline phosphatase activity in cell supernatant and the presence of this secreted embryonic alkaline phosphatase (SEAP) is indicative of NF-κB activity inside the cell. RAW-Blue macrophages were pre-treated with postbiotic samples, and then challenged with either LPS [figure 4.3], TNFa [figure 4.4], or Pam3CSK4 [figure 4.5] for 24hr. After incubation the cell supernatant was collected and added to Quanti-Blue solution for 4 hours and then absorbance was read in the plate reader. Figure 4.3 displays the NF-κB levels of macrophages treated with postbiotic samples and subsequently challenged with an LPS stimulation. While there are no significant differences in the results, the general trend shows that basally postbiotic treatment slightly increases NF-κB activity compared to untreated, and in response to an LPS stimulation, postbiotic treatment slightly decreases NF-κB activity compared to untreated. In figure 4.4, postbiotics are shown to slightly stimulate NF-κB activity in response to a TNF challenge, although not significantly. TNF stimulation alone did not significantly stimulate SEAP activity, TNF activation of SEAP would have been expected here. Generally, there is a slight increase in NF-κB in postbiotic treated macrophages compared to untreated, basally. In response to a TNF stimulation postbiotics are seen to slightly increase NF-κB activity compared to untreated, excluding postbiotic 3 which shows no change at all. It is worth noting here that an increase in SEAP would be expected in response to just TNF treatment, as TNF is a known to activate NF-κB. It is not clear as to why a clear increase in SEAP is not observed here. Lasty, in figure 4.5, pre-treatment with postbiotic samples is shown to slightly increase NF-κB activity basally and in response to a PAM3CSK4 challenge. Although these results are not statistically significant.

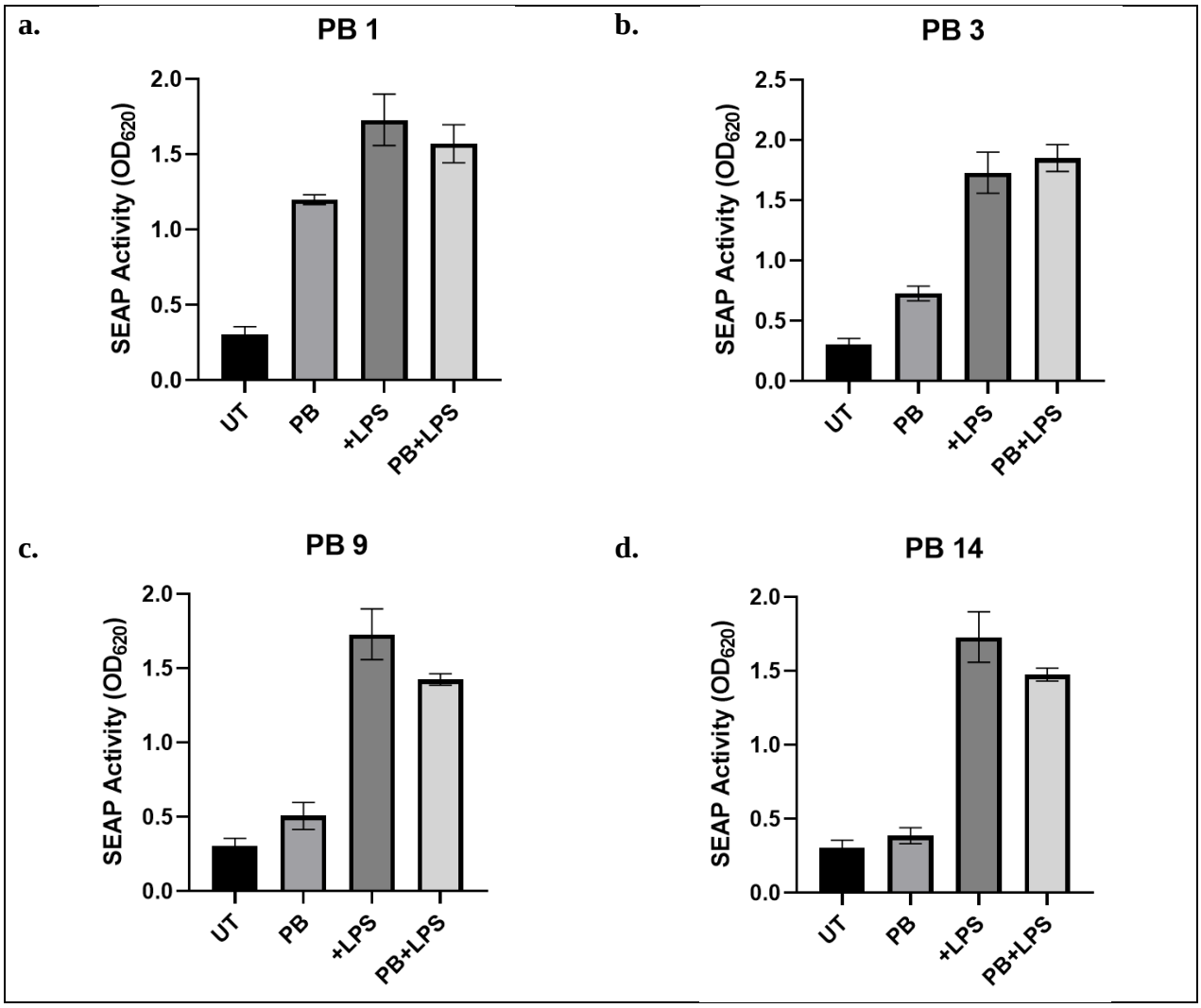


Figure 4.3 Postbiotics stimulate NF-κB activity basally and in response to LPS stimulation RAW-Blue macrophages were untreated or pre-treated with postbiotic at a concentration of 2.5% for 2 hours and then stimulated with a 10ng/ml LPS stimulation. Data is representative of three separate experiments with three replicates each. A student's t-test was performed, and no significant change was measured.

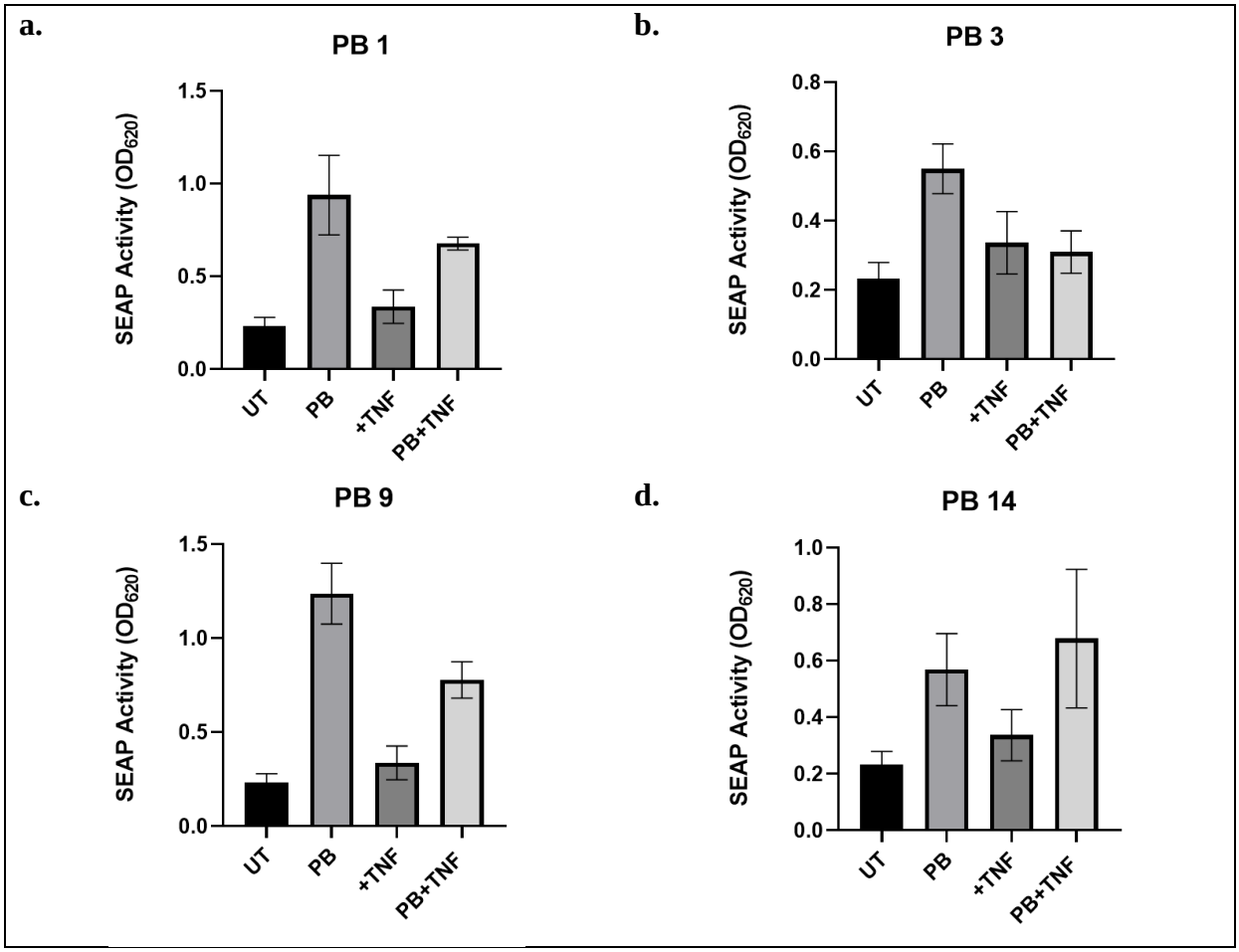


Figure 4.4 Postbiotics stimulate NF-κB activity basally and in response to TNF stimulation RAW-Blue macrophages were untreated or pre-treated with postbiotic at a concentration of 2.5% for 2 hours and then stimulated with a 100ng/ml TNF stimulation. Data is representative of three separate experiments with three replicates each. A student's t-test was performed, and no significant change was measured.

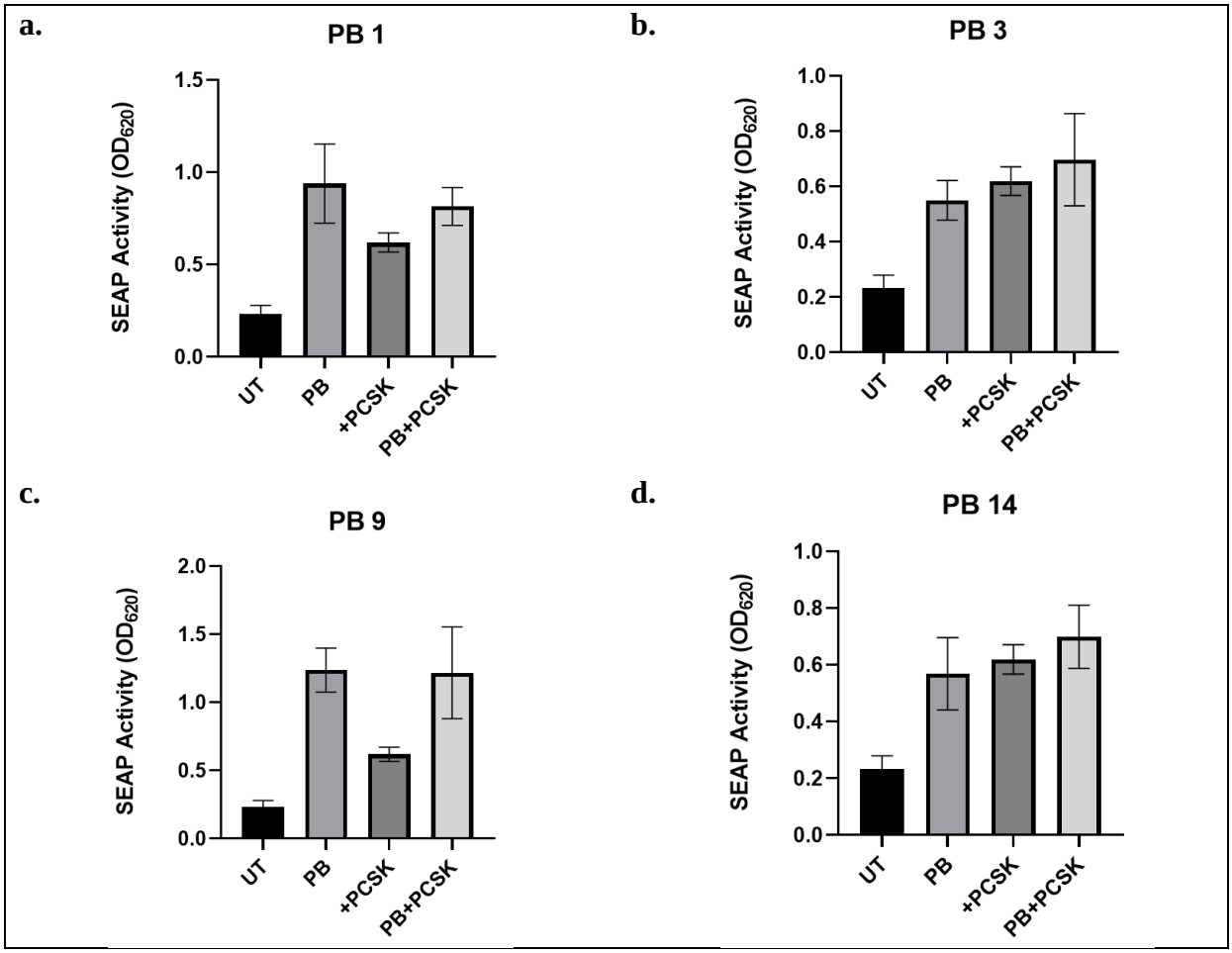


Figure 4.5 Postbiotics stimulate NF-κB activity basally and in response to Pam3Csk4 stimulation RAW-Blue macrophages were untreated or pre-treated with postbiotic at a concentration of 2.5% for 2 hours and then stimulated with a 100ug/ml Pam3CSK4 stimulation. Data is representative of three separate experiments with three replicates each. A student's t-test was performed, and no significant change was measured.

4.2.3 Postbiotic treatment +/- LPS treatment modulates inflammatory response biomarkers

4.2.3.1 Postbiotic treatment +/-LPS treatment modulates mRNA expression of inflammatory biomarkers

For this section of the experiment, RAW-Blue macrophages are pre-treated with media containing 2.5% postbiotic and were then given an LPS stimulation to simulate an infection. The macrophage RNA is then harvested and analysed using qPCR, to quantify amounts of biomarkers [A] IL-10, [B] TNFa, [C] IL1B, and [D] NOS2. Figure 4.6 displays the effect of PB 1 on mRNA expression of inflammatory biomarkers. PB 1 slightly increases levels of IL-10, although not statistically significant [Figure 4.6 A]. PB 1 significantly increases TNFa levels basally and elicits no significant change when treated with LPS [Figure 2.6 B]. PB 1 seems to decrease levels of IL1B basally and in response to LPS, although not significantly [Figure 4.6 C]. Finally, PB 1 increases levels of NOS2 expression [Figure 4.6 D]. Figure 4.7 displays effect of PB 3 on mRNA expression of the same biomarkers. PB 3 increases levels of IL-10 in response to LPS stimulus [Figure 4.7 A]. PB 3 significantly increases TNFa levels basally and slightly increases expression when treated with LPS [Figure 4.7 B]. Levels of IL1B show non-significant variable results [Figure 4.7 C]. PB 3 increases levels of NOS2 expression, although not significantly [Figure 4.7 D]. Figure 4.8 shows the effect of PB 9. PB 9 slightly and non-significantly increases levels of IL-10 basally and in response to LPS stimulus [Figure 4.8 A]. PB 9 significantly increases TNFa levels basally and slightly increases expression when treated with LPS [Figure 4.8 B]. Levels of IL1B treated with PB 9 show non-significant variable results [Figure 4.8 C]. PB 9 significantly increases levels of NOS2 expression basally [Figure 4.8 D]. PB 14 and its effect on mRNA expression of inflammatory biomarkers is displayed in figure 4.9. PB 14 shows no significant effect on expression of IL10 or TNF, either basally or in response to LPS [Figure 4.9 A, B]. A slight trend of decreased IL1B expression is seen in response to PB 14 pre-treatment [figure 4.9 C].

PB 14 pre-treatment significantly increases levels of NOS2 both basally and in response to an LPS challenge [Figure 4.9 D].

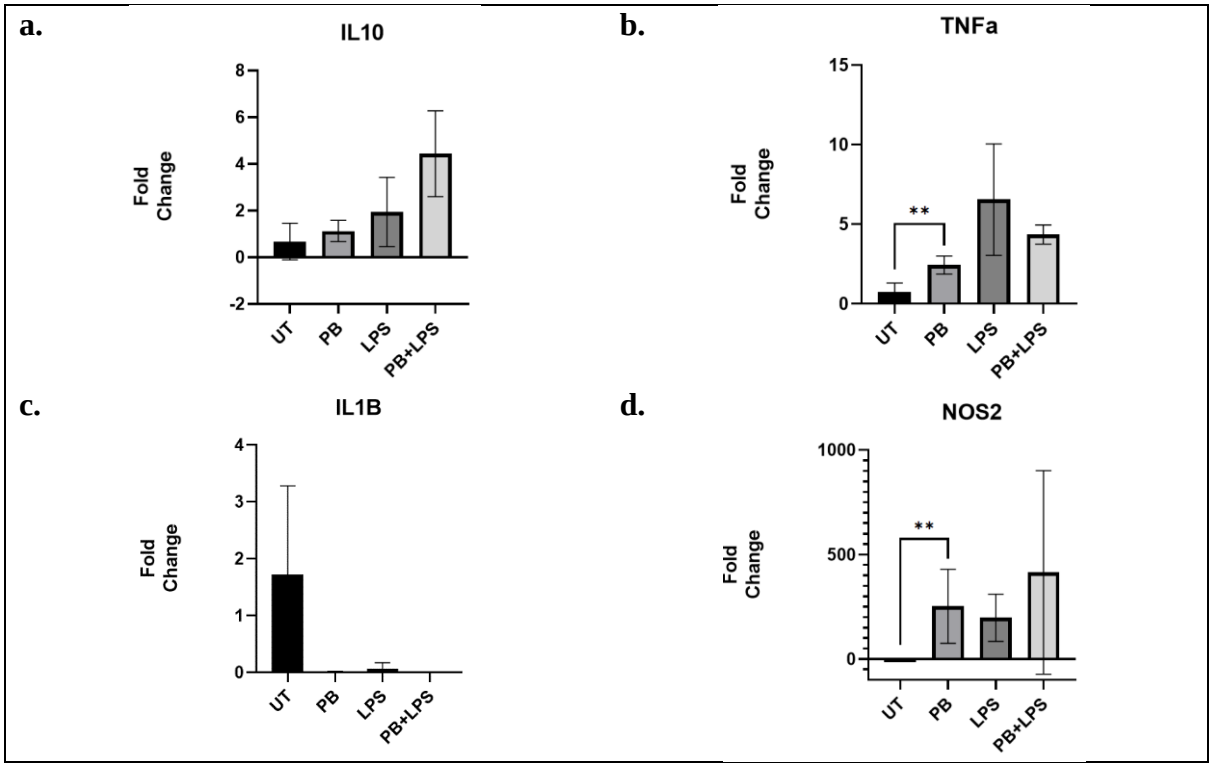


Figure 4.6 Postbiotic 1 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers IL10, TNFa, IL1B and NOS2 RAW-Blues are pre-treated with postbiotic 1 at 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 3 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **.

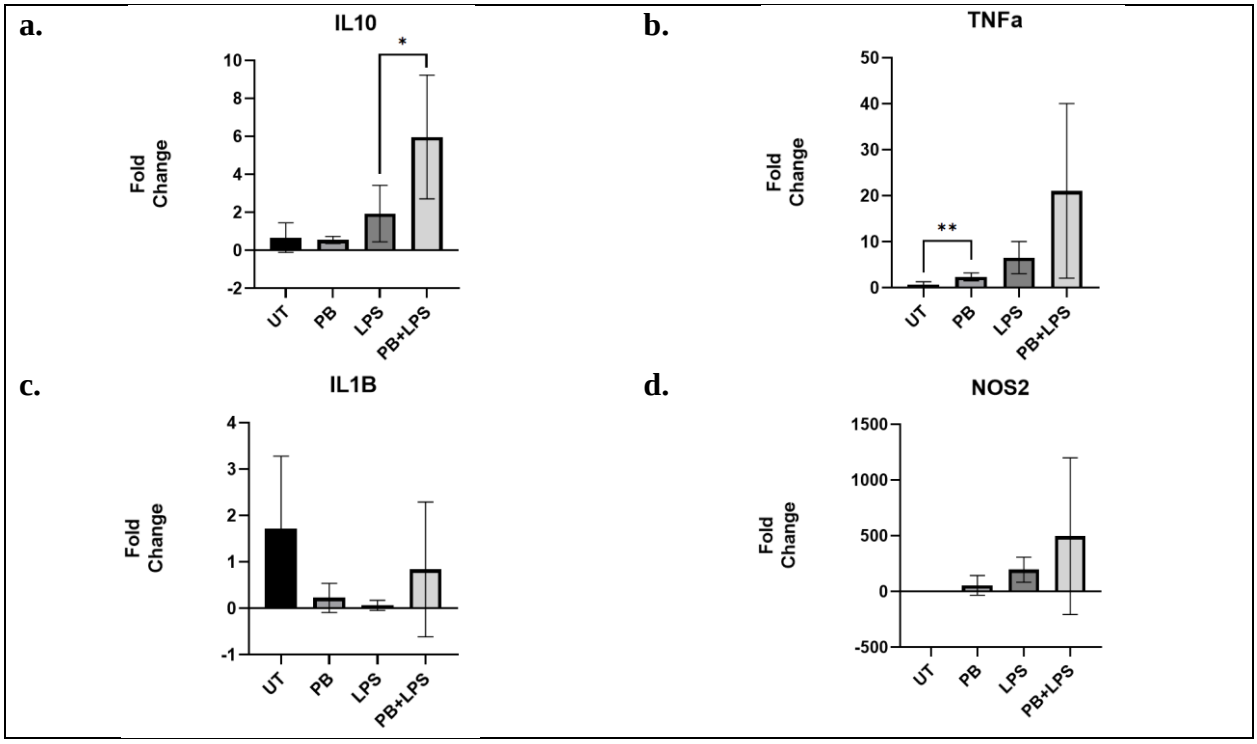


Figure 4.7 Postbiotic 3 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers IL10, TNFa, IL1B and NOS2 RAW-Blues are pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 3 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = * and $p \leq 0.01$ = **.

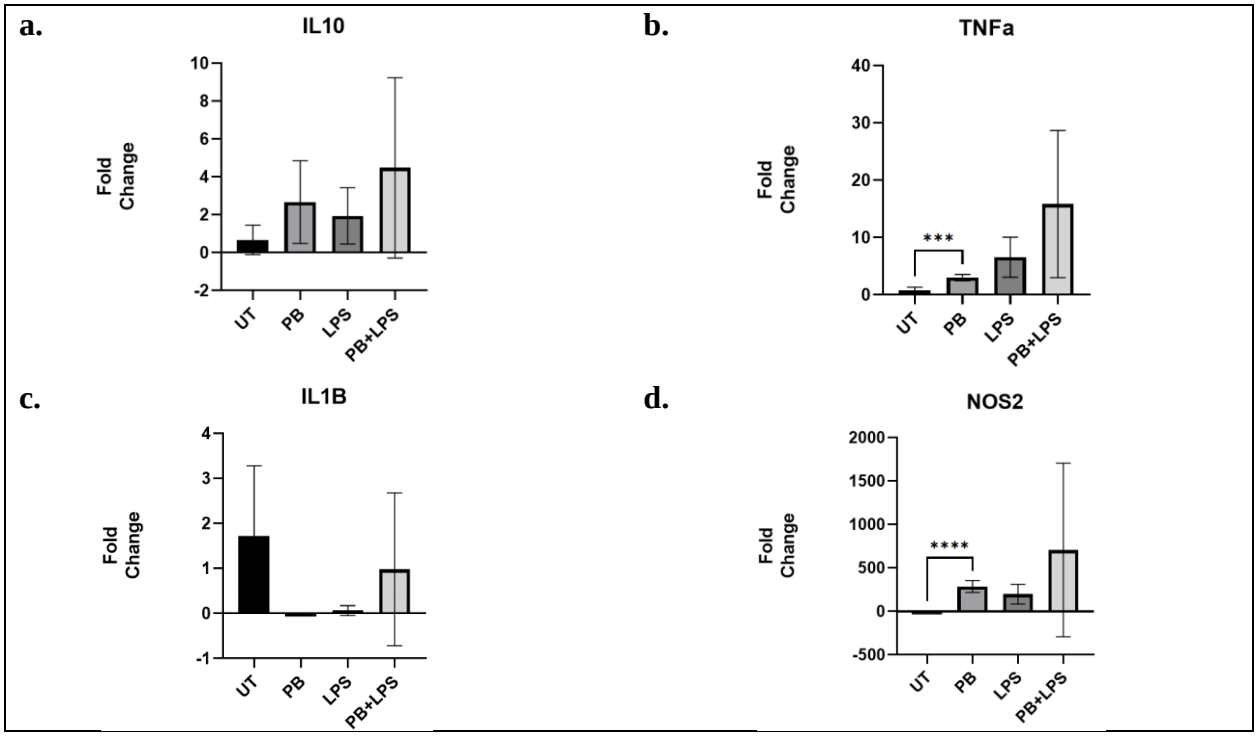


Figure 4.8 Postbiotic 9 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers IL10, TNFa, IL1B and NOS2 RAW-Blues are pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 3 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

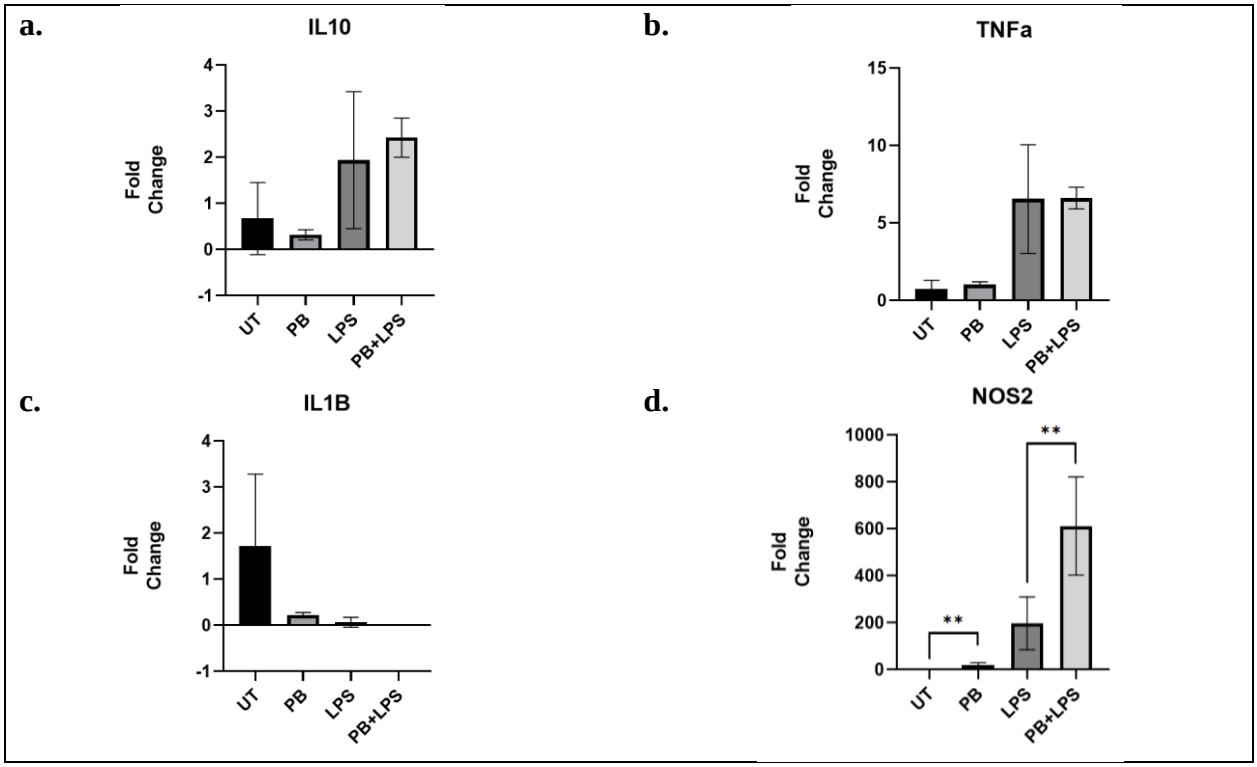


Figure 4.9 Postbiotic 14 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers IL10, TNFa, IL1B and NOS2 RAW-Blues pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 3 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **.

4.2.3.2 Postbiotic treatment +/-LPS treatment modulates cytokine responses

To examine levels of protein excretion in RAW-Blue macrophages pre-treated with postbiotics and challenged with LPS, ELISAs were performed on the cell supernatant from these experiments. Figure 4.10 illustrates the effect of PB 1 on IL10 and IL6 protein expression. PB1 significantly increases both IL10 [Figure 4.10 A], and IL6 [figure 4.10 B]. PB3 increases IL10 in response to LPS stimulus [figure 4.11 A], and significantly increases IL6 both basally and in response to LPS stimulus [figure 4.11 B]. PB9 increases IL10 in response to LPS stimulus [figure 4.12 A], and significantly increases IL6 both basally and in response to LPS stimulus [figure 4.12 B]. PB 14 increases IL10 [figure 4.13 A], and IL6 [figure 4.13 B] in response to LPS challenge, however no change basally.

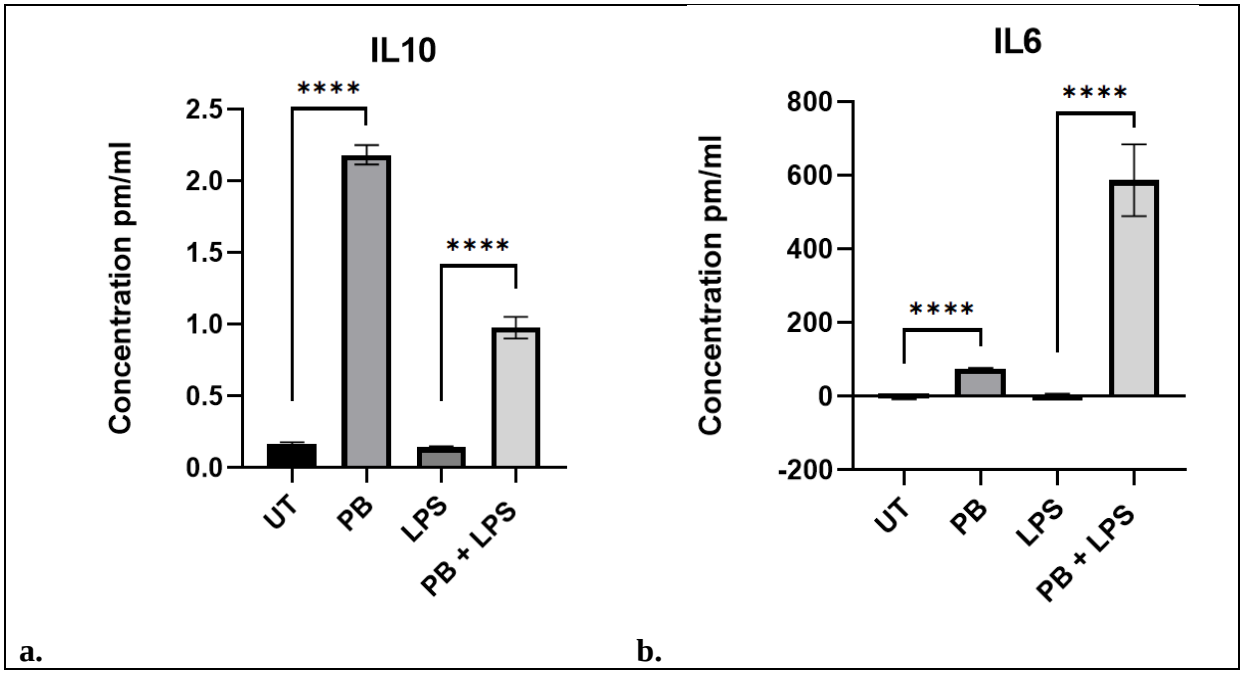


Figure 4.10 Postbiotic 1 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

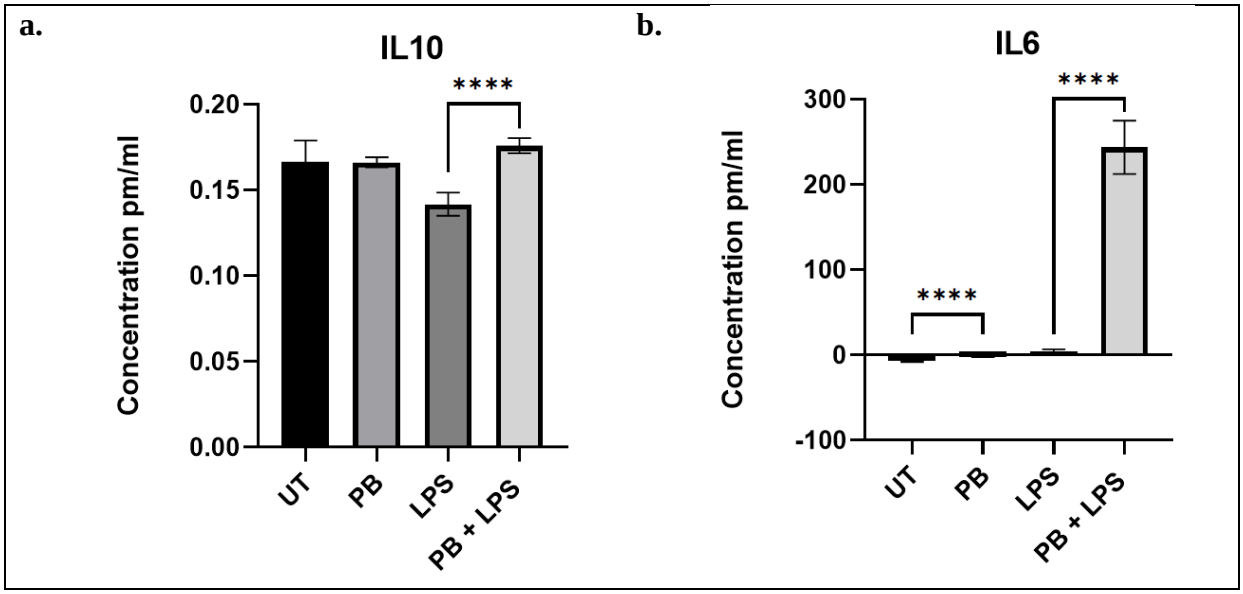
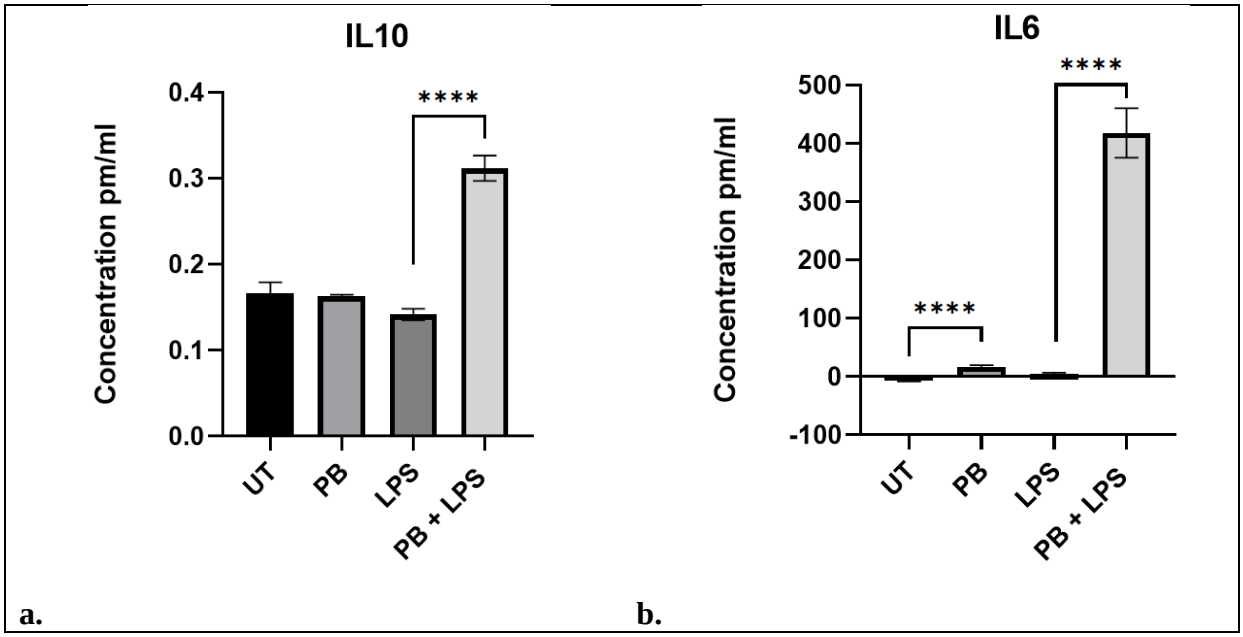


Figure 4.11 Postbiotic 3 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.



a. **b.**

Figure 4.12 Postbiotic 9 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

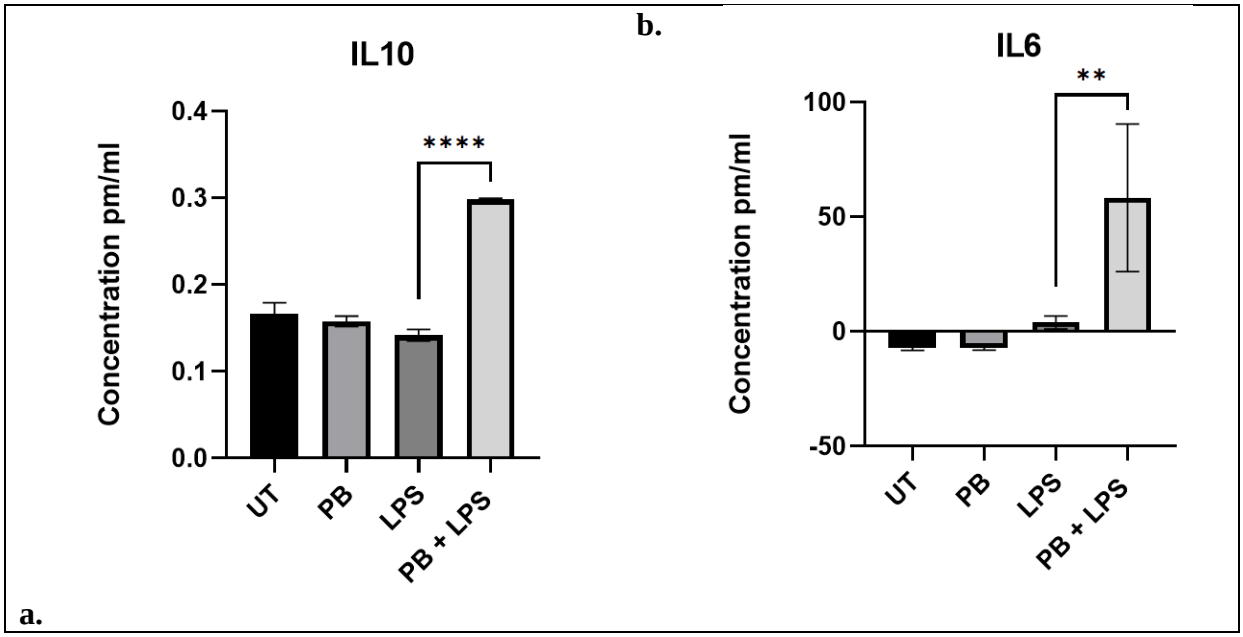


Figure 4.13 Postbiotic 14 +/- LPS stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **, and $p \leq 0.0001$ = ****.

4.2.4 Postbiotic treatment +/- TNF treatment modulates inflammatory response biomarkers

4.2.4.1 Postbiotic treatment +/- TNF treatment modulates mRNA expression of inflammatory response biomarkers

To explore different pathways of inflammation, for this part of the experiment, TNF was used as the infection model stimulus. RAW-Blue macrophages were pre-treated with postbiotic samples at 2.5% concentration and the given a TNF stimulation to induce inflammation. mRNA from the macrophages was collected and analysed using qPCR to quantify levels of different biomarkers related to inflammatory states. Figure 4.14 displays the ability of PB 1 to significantly upregulate levels of IL10 [figure 4.14 A], IL1B [figure 4.14 B], and NOS2 [figure 4.14 C] mRNA expression basally and in response to TNF stimulation. PB 3 [figure 4.15], PB 9 [figure 4.16], and PB 14 [figure 4.17] follow the same trends of significant upregulation, apart from PB 3 having no significant effect on IL10 expression [figure 4.15 A].

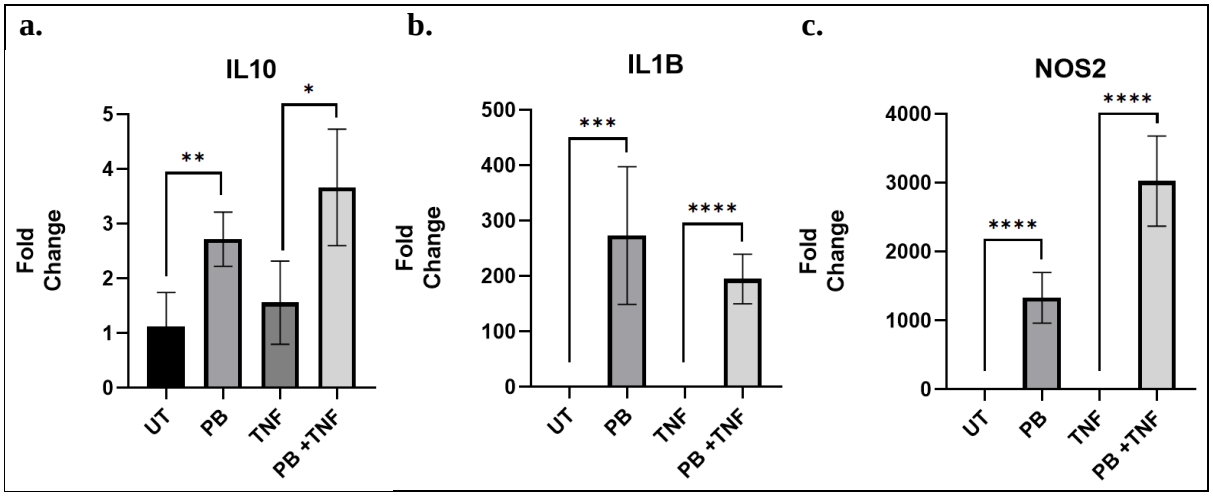


Figure 4.14 Postbiotic 1 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

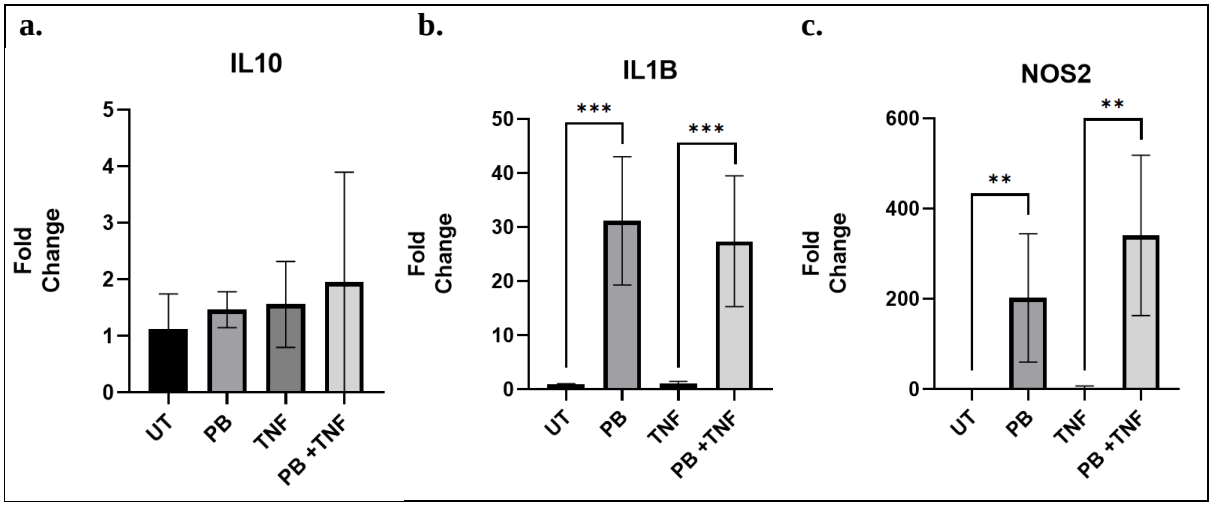


Figure 4.15 Postbiotic 3 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = ** and $p \leq 0.001$ = ***.

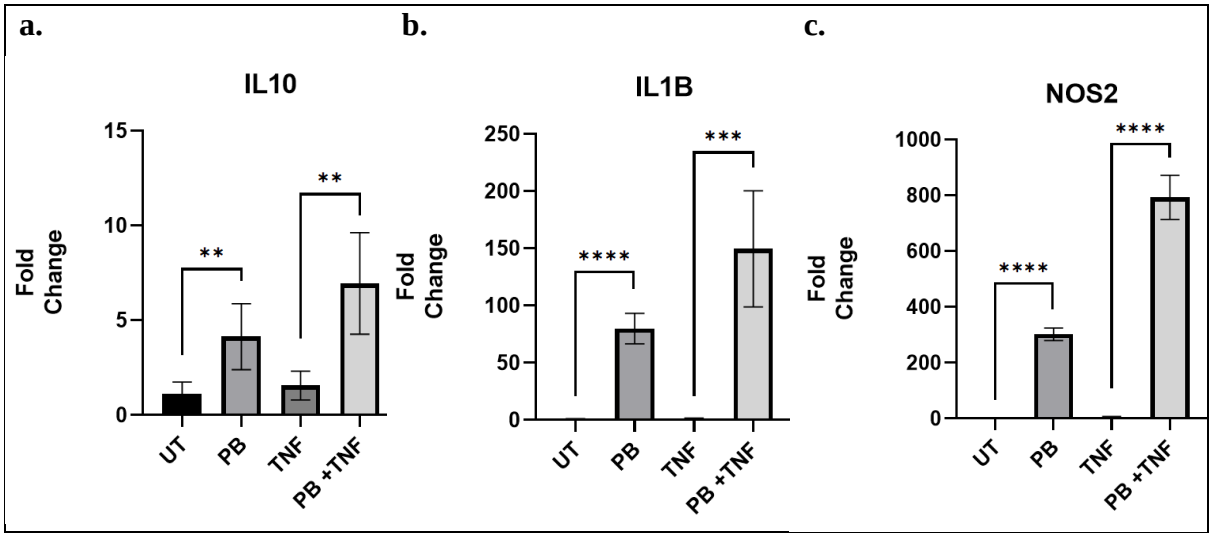


Figure 4.16 Postbiotic 9 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **, $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

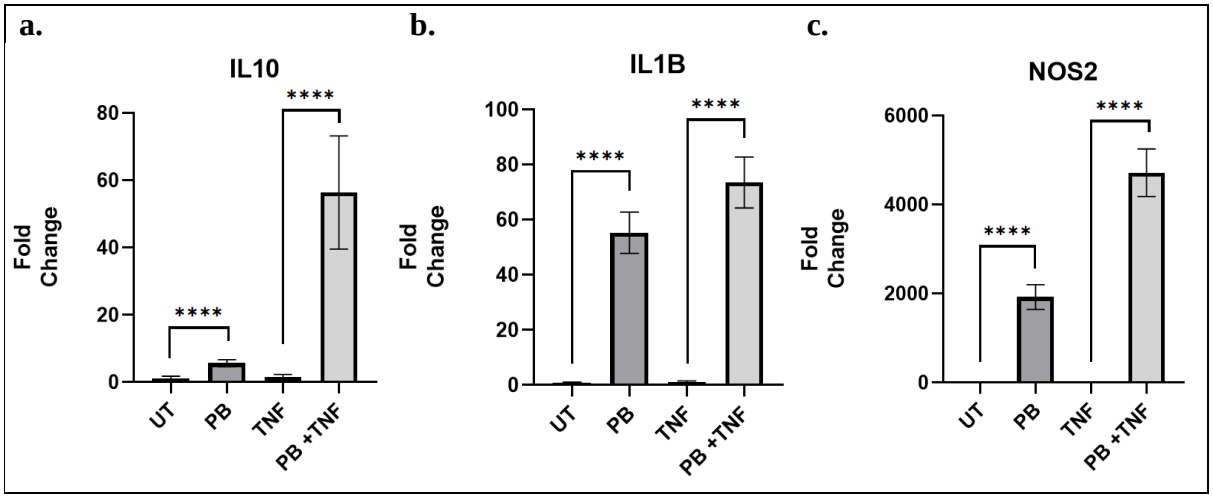


Figure 4.17 Postbiotic 14 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

4.2.4.2 Postbiotic treatment +/- TNF treatment modulates cytokine responses

After studying the mRNA levels in this experiment, excreted protein expression was then measured. This is done by performing ELISAs on the cell supernatant from macrophages pre-treated with postbiotic samples and subsequently challenged with TNF. As shown in figure 4.18, PB 1 significantly increases levels of IL10 [A] and IL6 [B]. This significant trend is also consistent for PB 3 [figure 4.19], PB 9 [figure 4.20], and PB 14 [4.21].

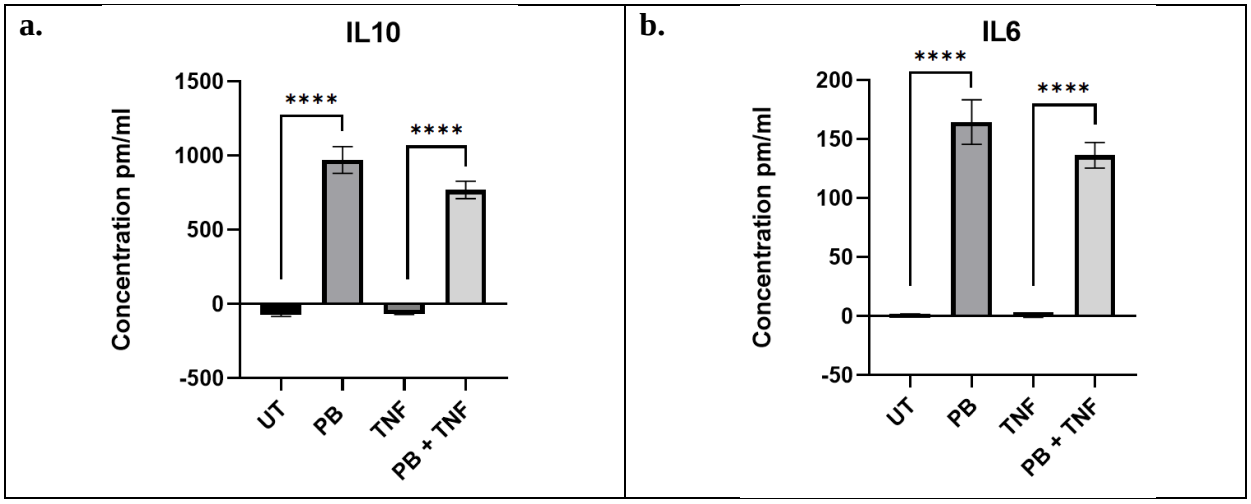


Figure 4.18 Postbiotic 1 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

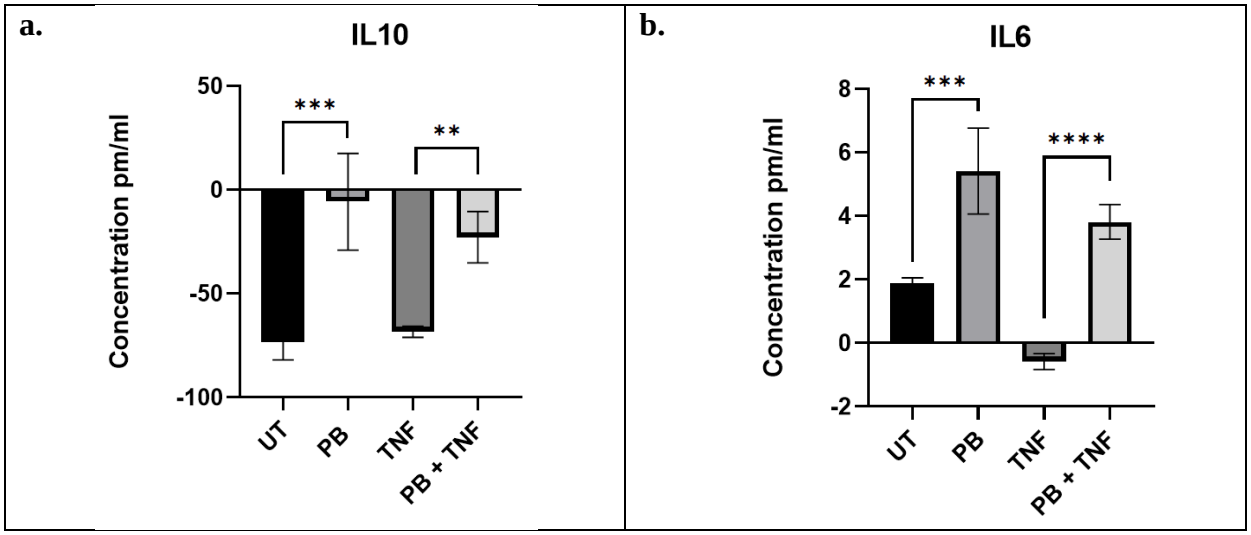


Figure 4.19 Postbiotic 3 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **, $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

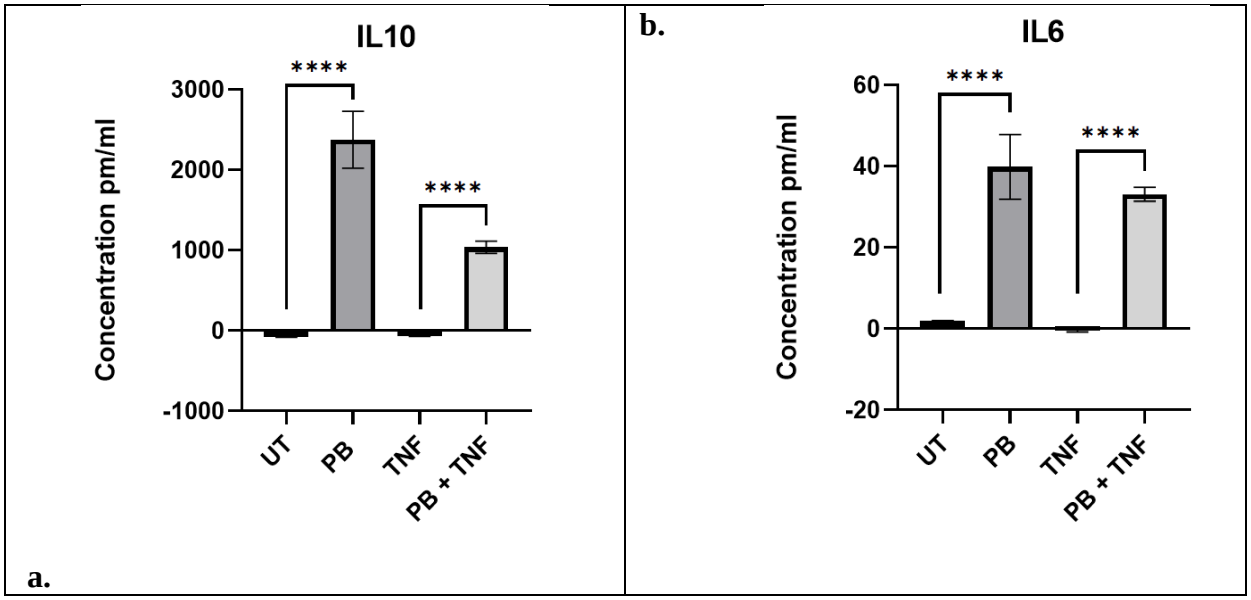


Figure 4.20 Postbiotic 9 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

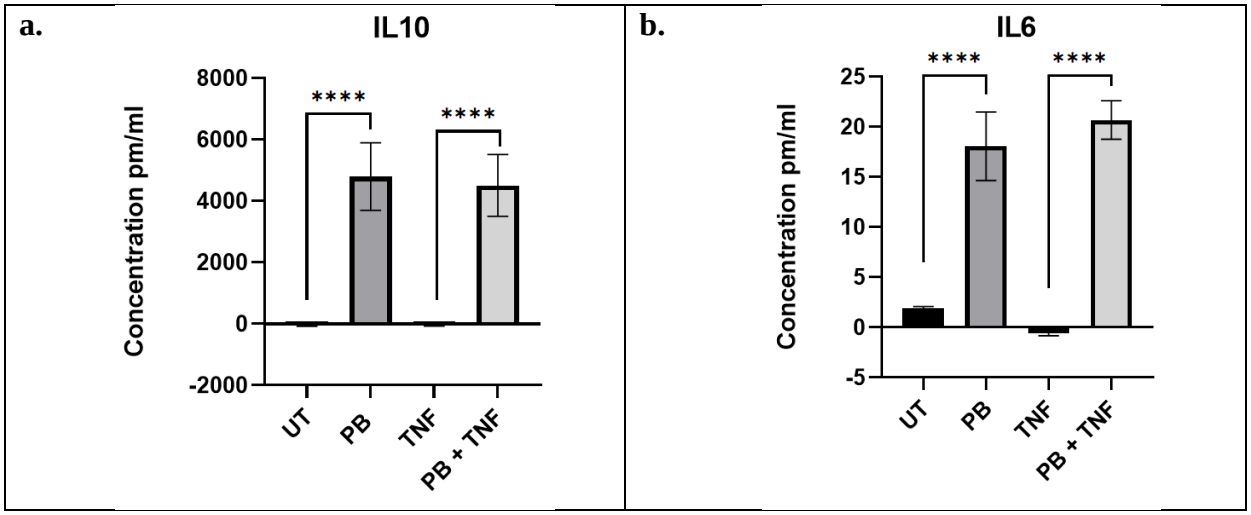


Figure 4.21 Postbiotic 14 +/- TNF stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

4.2.5 Postbiotic treatment +/- PamCSK4 treatment modulates inflammatory response biomarkers

4.2.5.1 Postbiotic treatment +/- PamCSK4 treatment modulates mRNA expression of inflammatory response biomarkers

To continue the investigation, another PAMP was used in the experiments. PAM3CSK4 is used to stimulate infection and inflammation. Macrophages were pre-treated with postbiotic samples and then treated with PAM3CSK4. mRNA from the macrophages was collected and analysed using qPCR, which allows quantification of the different inflammation markers. Figure 4.21 displays the effect of PB 1 pre-treatment; PB 1 significantly increases expression of IL10 [A], TNFa [B], and IL1B [C] both basally and in response to PAM3CSK4 stimulation, and PB 1 significantly increases NOS2 expression basally [D]. As shown in figure 4.22, PB 3 significantly increases expression of IL10 [A], TNFa [B], IL1B [C], and NOS2 [D] both basally and in response to addition of PAM3CSK4. In figure 4.23, PB 9 is shown to significantly increases expression of IL10 [A], IL1B [C], and NOS2 [D] both basally and in response to addition of PAM3CSK4; and TNFa [B] expression is increases only basally. This pattern is held consistent with PB 14 pre-treatment, as shown in figure 4.24.

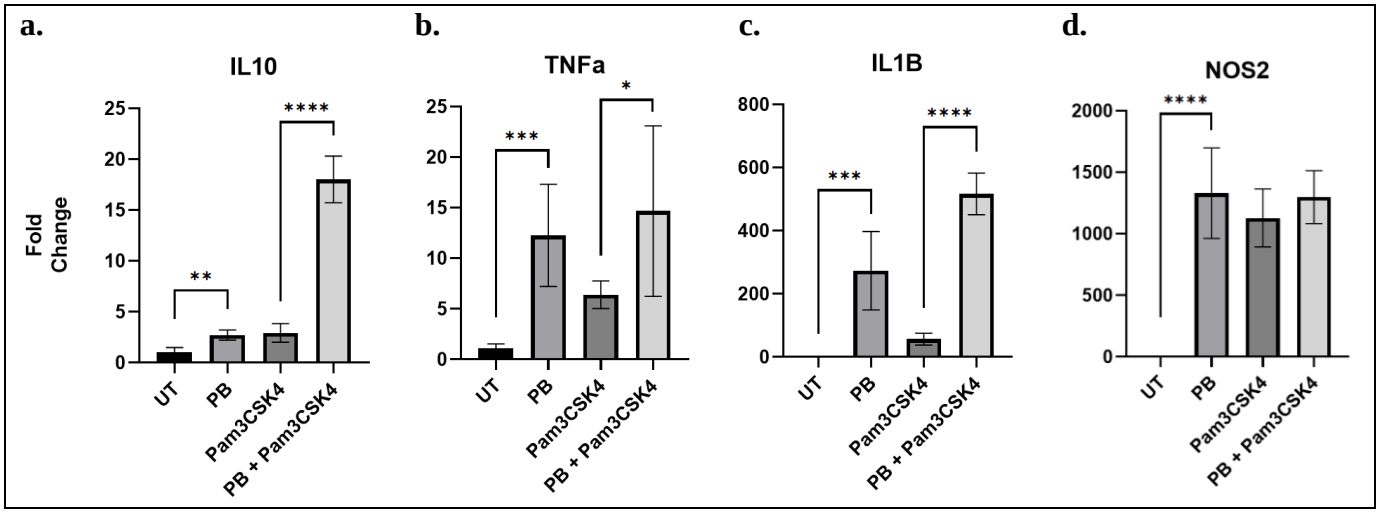


Figure 4.22 Postbiotic 1 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

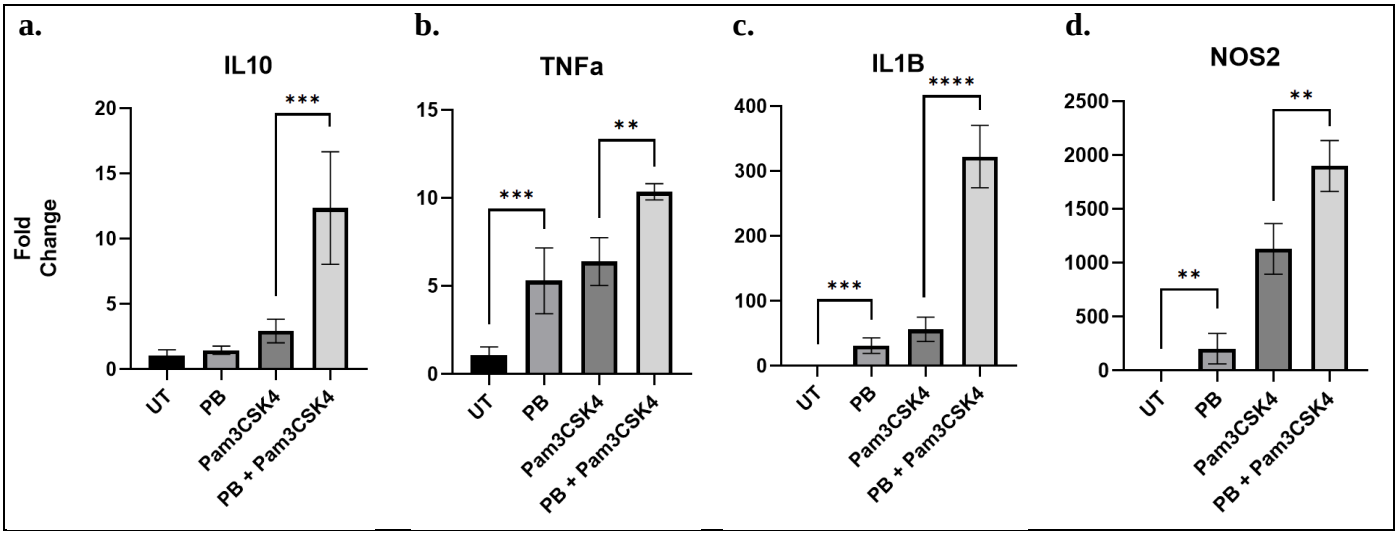


Figure 4.23 Postbiotic 3 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **, $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

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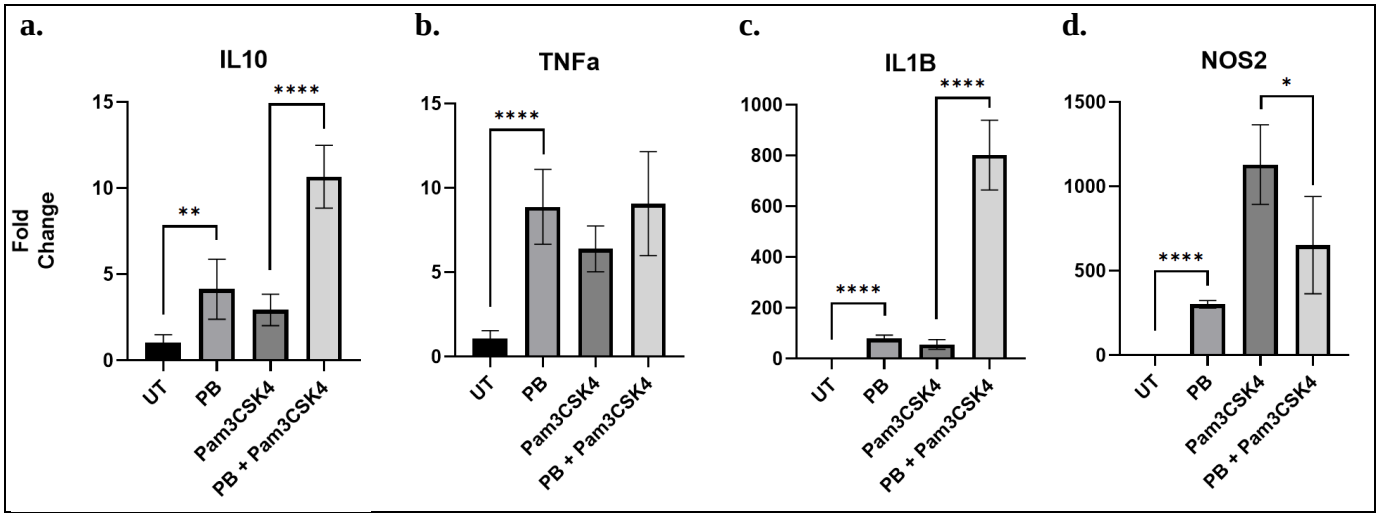

Figure 4.24 Postbiotic 9 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = *, $p \leq 0.01$ = **, and $p \leq 0.0001$ = ****.

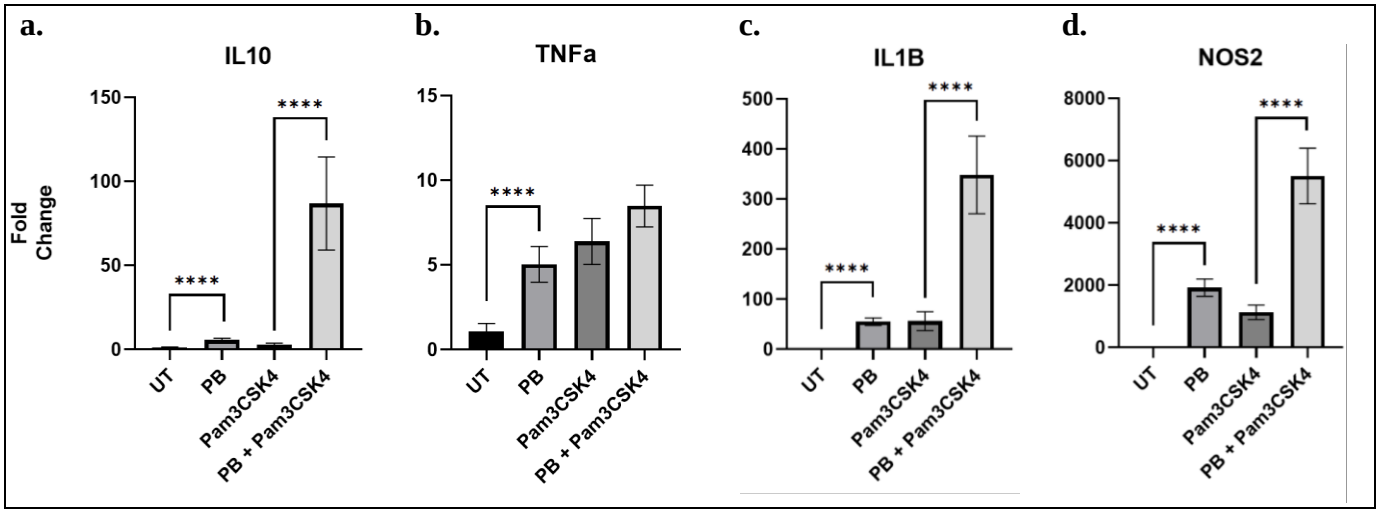

Figure 4.25 Postbiotic 14 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers mRNA expression RAW-Blues are untreated or pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

4.2.5.2 Postbiotic treatment +/- PamCSK4 treatment modulates cytokine responses

After studying the mRNA levels in this experiment, excreted protein expression was then measured. This is done by performing ELISAs on the cell supernatant from macrophages pre-treated with postbiotic samples and subsequently challenged with PAM3CSK4. In response to pre-treatment with PB 1 [figure 4.25], PB 9 [figure 4.27], and PB 14 [figure 4.28], both IL10 [A] and IL6 [B] are significantly upregulated, both basally and in response to PAM3CSK4 challenge. PB 3 [figure 4.26] significantly increases IL10 [A] expression basally, but not in response to PAM3CSK4 treatment, and significantly increases IL6 [B] expression in response to PAM3CSK4 treatment, but not basally.

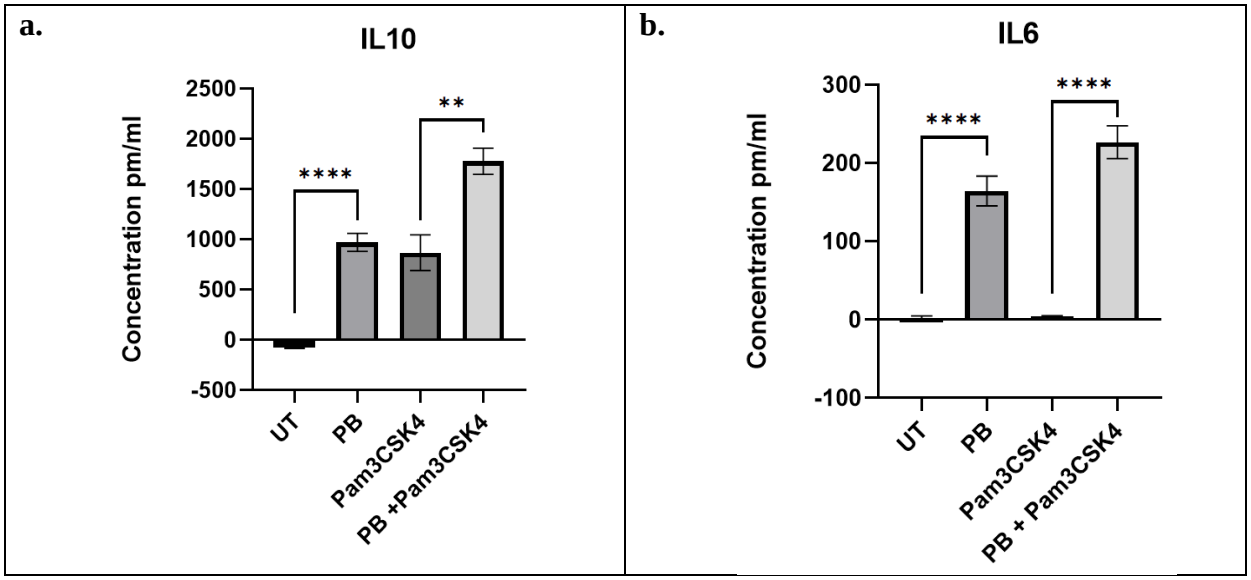

Figure 4.26 Postbiotic 1 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = ** and $p \leq 0.0001$ = ****.

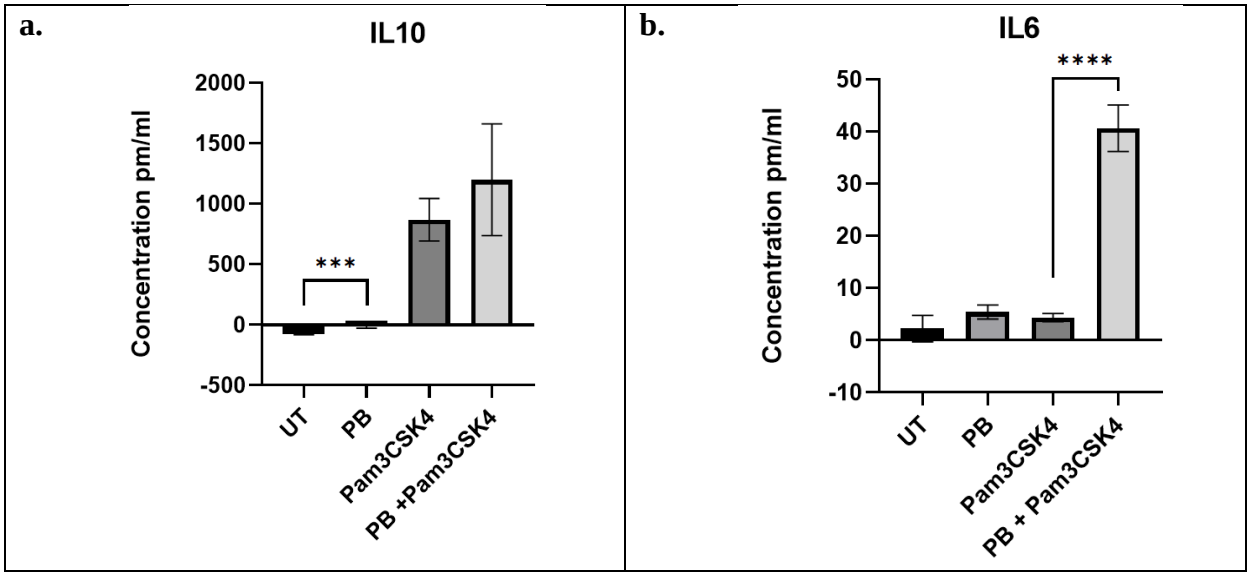

Figure 4.27 Postbiotic 3 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

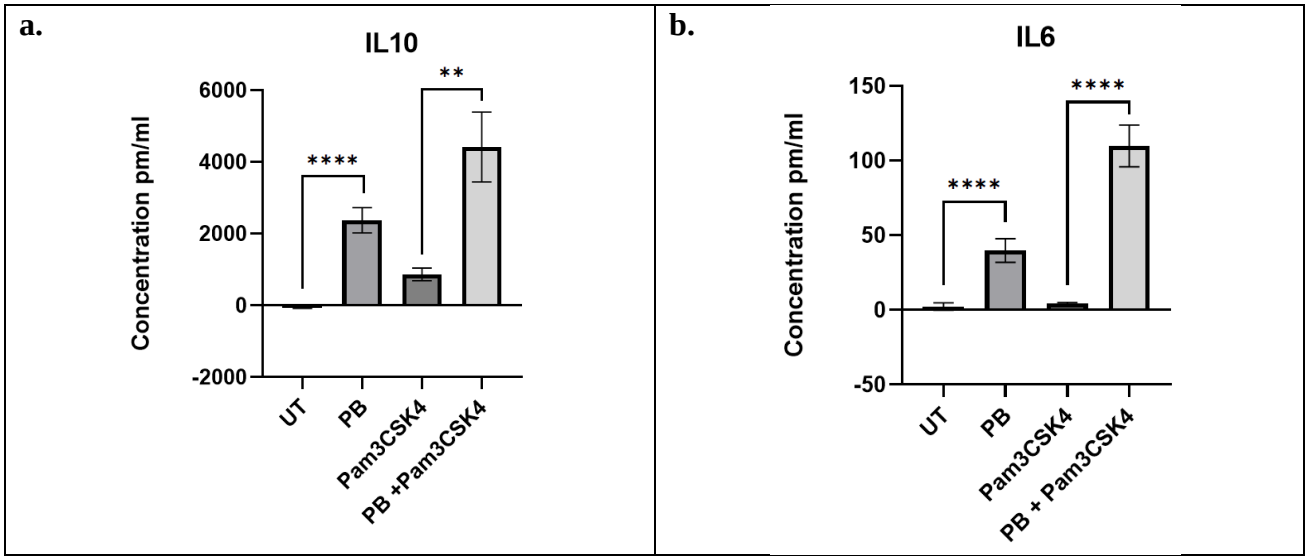

Figure 4.28 Postbiotic 9 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = ** and $p \leq 0.0001$ = ****.

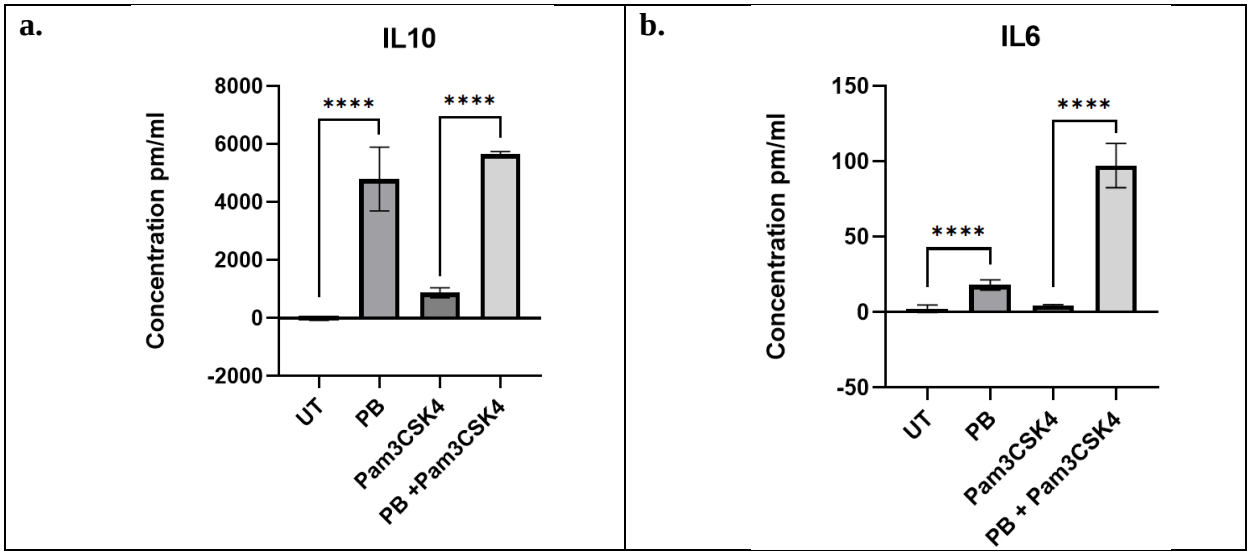

Figure 4.29 Postbiotic 14 +/- Pam3CSK4 stimulation in RAW-Blue macrophages induces changes in key biomarkers protein concentration RAW-Blues are pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 100ug/ml Pam3CSK4 stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

4.2.6 Postbiotic treatment +/- LPS treatment modulates inflammatory response biomarkers in BMDMs

After conducting experiments in RAW-Blue reporter macrophages, the results needed to be verified in a primary macrophage line. Here we used murine bone marrow derived macrophages (BMDMs) to confirm results seen in RAW-Blue macrophages. The same experiments performed in the previous section were performed on BMDMs in this section.

4.2.6.1 Postbiotic treatment +/- LPS treatment modulates mRNA expression of inflammatory response biomarkers in BMDMs

Bone marrow derived macrophages (BMDMs) were pre-treated with postbiotic samples and then challenged with treatment of LPS to simulate an infection. As displayed in figure 4.29, PB 1 significantly increases IL1B [C] and NOS2 [D] basally, however the general trend of increasing levels of IL10 [A], TNFa [B], IL1B [C], and NOS2 [D] both basally and in response to LPS is still shown. As shown in figure 4.30, PB 3 significantly decreases levels of IL10 basally and has no effect on cells treated with LPS [A]. PB 3 seems to slightly decrease TNFa levels both basally and in response to LPS, although not significantly [B]. PB 3 significantly increases levels of IL1B basally and elicits no change in response to LPS [C]. While not significantly, PB 3 slightly increases levels of NOS2 [D]. Figure 4.43 displays the effects of PB 9 pre-treatment. PB 9 increases expression of IL10 [A] and TNFa [B] in response to LPS treatment but not basally. PB 9 increases expression of IL1B [C] basally and elicits no change in response to LPS. Pre-treatment with PB 9 increases expression of NOS2 both basally and in response to LPS treatment [D]. In figure 4.32, it is shown that pre-treatment with PB 14 significantly increases expression of IL10 in response to LPS treatment and elicits no change basally [A]. The same trend is seen in expression of TNFa [B]. PB 14 pre-treatment displays an upward trend in IL1B expression both basally and in response to LPS treatment [C]. PB 14 pre-treatment significantly increases levels of NOS2 [D], both basally and in response to LPS.

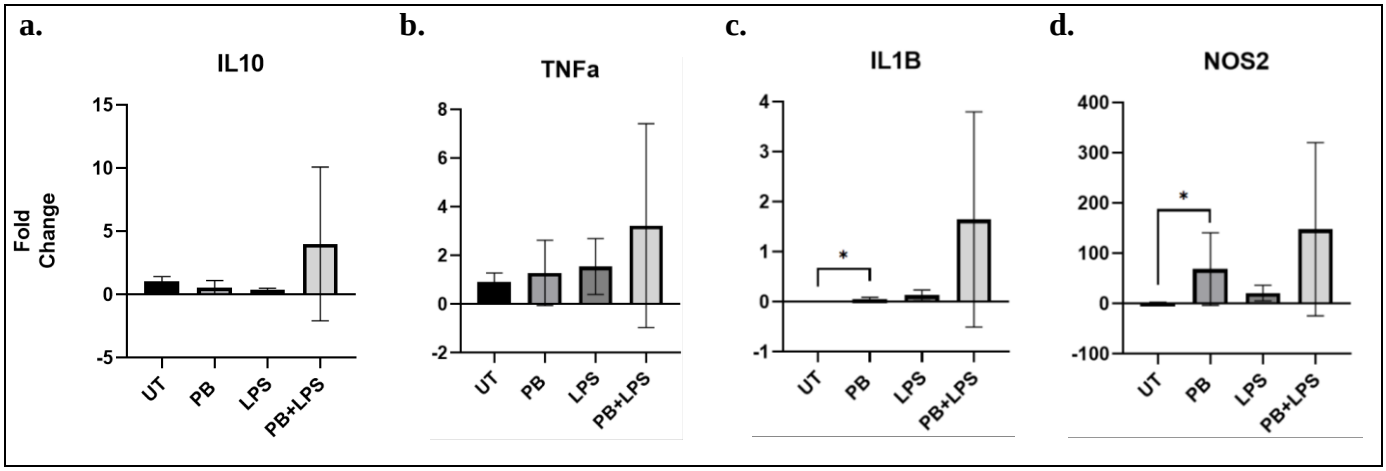

Figure 4.30 Postbiotic 1 +/- LPS stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = *

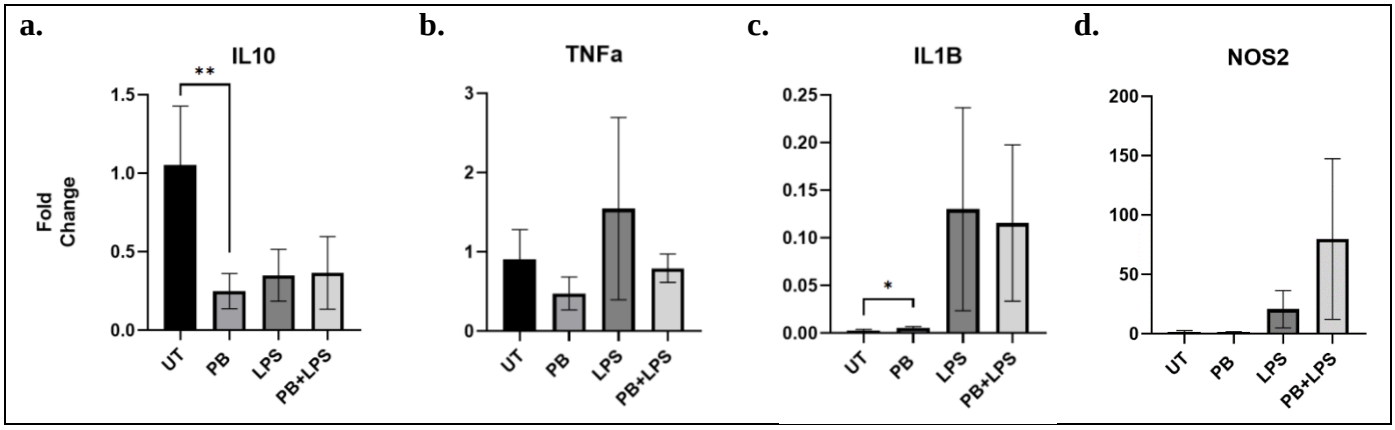

Figure 4.31 Postbiotic 3 +/- LPS stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = * and $p \leq 0.01$ = **.

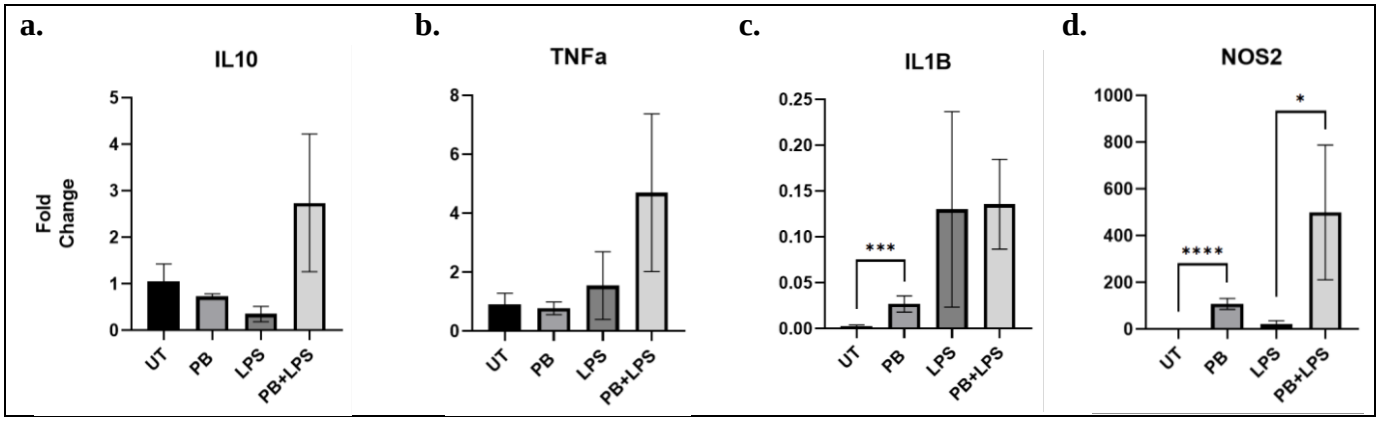

Figure 4.32 Postbiotic 9 +/- LPS stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = *, $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

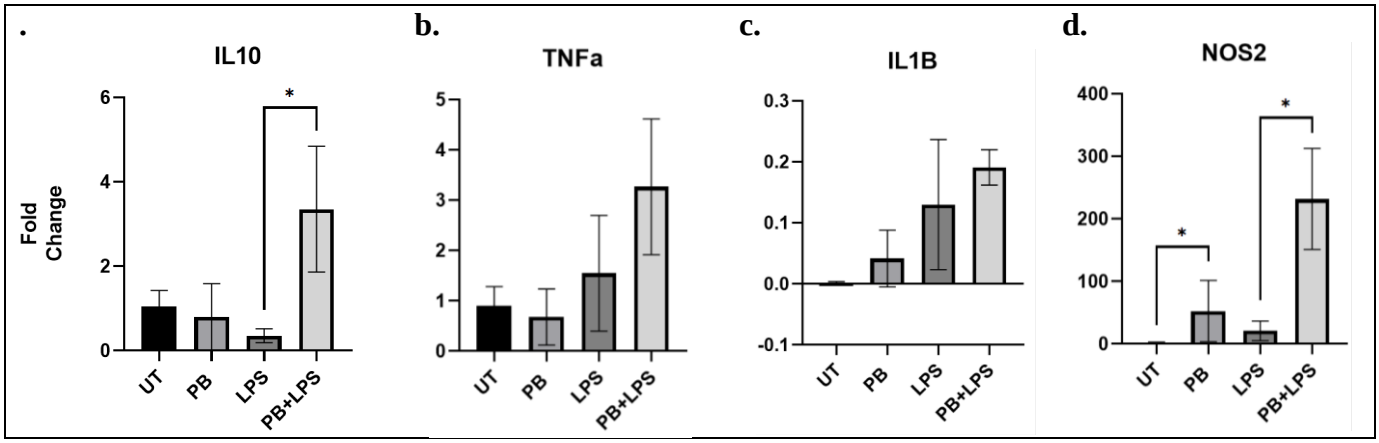

Figure 4.33 Postbiotic 14 +/- LPS stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = *.

4.2.6.2 Postbiotic treatment +/- LPS treatment modulates cytokine response in BMDMs

The next step was analysing the cell supernatants to determine levels of inflammatory biomarkers. Cell supernatant from BMDM cells pre-treated with postbiotic samples and then treated with LPS, was collected for ELISA analysis. As shown in figure 4.33, pre-treatment with PB 1 significantly increases TNFa concentration both basally and in response to LPS [A]. PB 1 pre-treatment significantly increases concentration of IL6 basally and has no effect on cells treated with LPS [B]. Figure 4.34 shows pre-treatment with PB 3 significantly increases TNFa [A], and IL6 [B], both basally and in response to LPS. This same effect is seen with pre-treatment of PB 9 [figure 4.35] and PB 14 [figure 4.36].

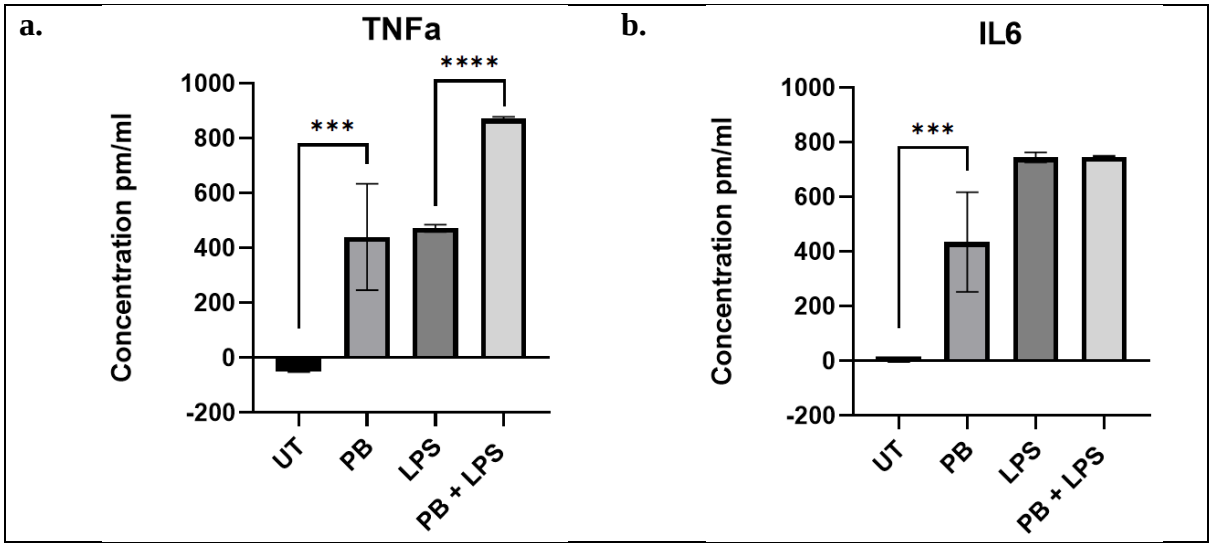

Figure 4.34 Postbiotic 1 +/- LPS stimulation in BMDMs induces changes in key biomarkers protein concentration BMDMs are untreated or pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.001$ = ***, and $p \leq 0.0001$ = ****.

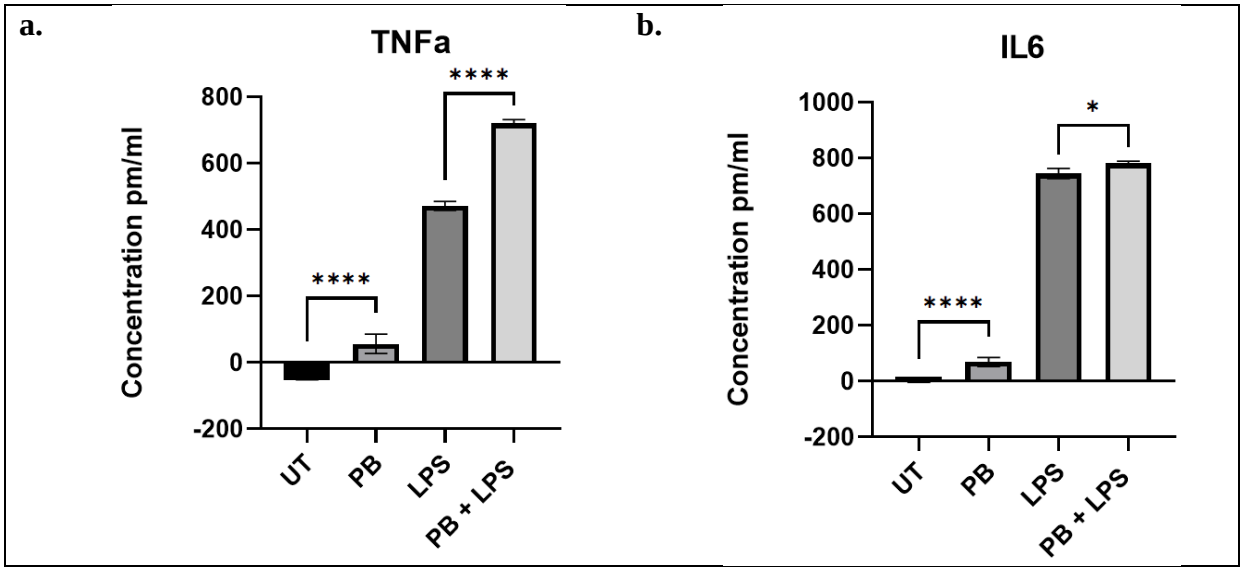

Figure 4.35 Postbiotic 3 +/- LPS stimulation in BMDMs induces changes in key biomarkers protein concentration BMDMs are untreated or pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = * and $p \leq 0.0001$ = ****.

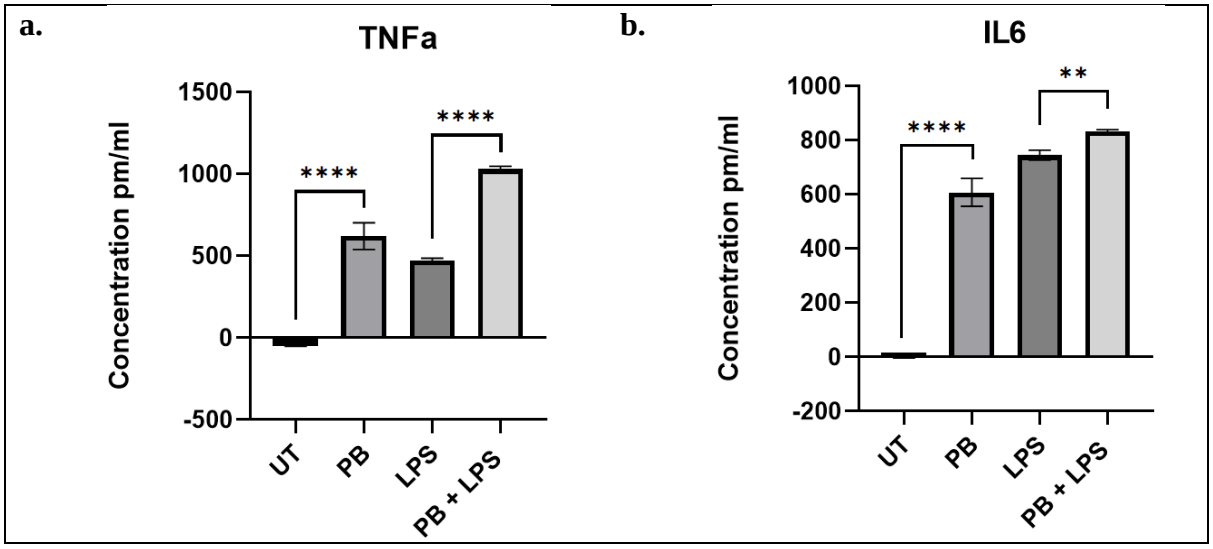

Figure 4.36 Postbiotic 9 +/- LPS stimulation in BMDMs induces changes in key biomarkers protein concentration BMDMs are untreated or pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **, and $p \leq 0.0001$ = ****.

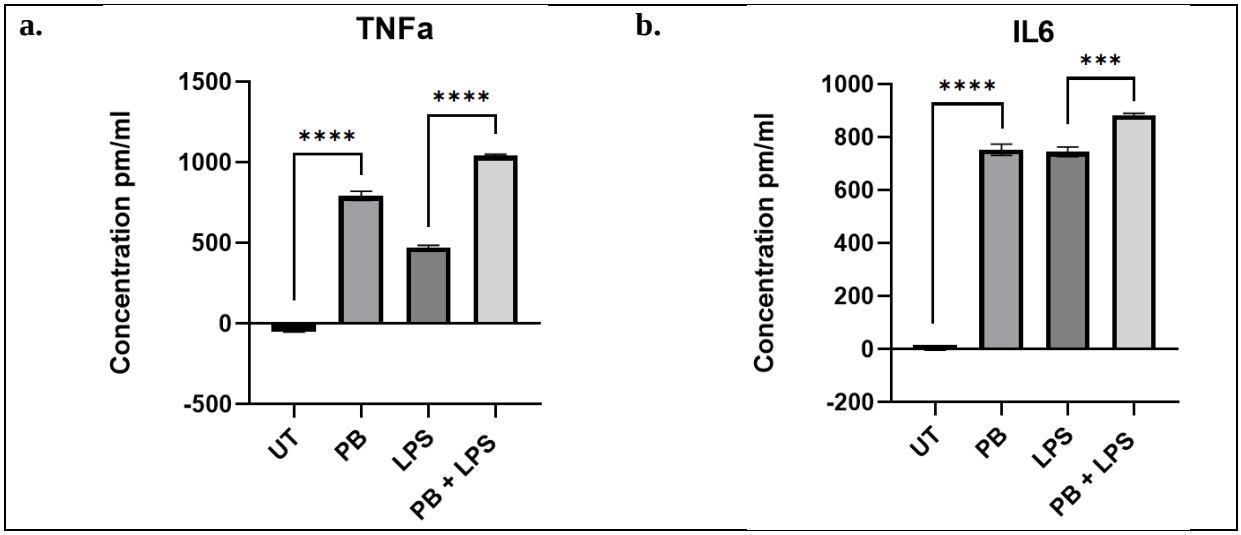

Figure 4.37 Postbiotic 14 +/- LPS stimulation in BMDMs induces changes in key biomarkers protein concentration BMDMs are untreated or pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 10ng/ml LPS stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.001$ = *** and $p \leq 0.0001$ = ****.

4.2.7 Postbiotic treatment +/- TNF treatment modulates inflammatory response biomarkers in BMDMs

After conducting experiments in RAW-Blue reporter macrophages, the results needed to be verified in a primary macrophage line. Here we used murine bone marrow derived macrophages (BMDMs) to confirm results seen in RAW-Blue macrophages. The same experiments performed in the previous section were performed on BMDMs in this section.

4.2.7.1 Postbiotic treatment +/- TNF treatment modulates mRNA expression inflammatory response biomarkers in BMDMs

To determine the mRNA expression of inflammatory related biomarkers, qPCR was used on RNA samples collected from BMDMs pre-treated with postbiotic samples before a TNF treatment. Figure 4.37 displays high error margins, however, PB 1 seems to increase IL10 in response to TNF stimulation [A]. Pre-treatment with PB 1 also shows a trend of increasing expression of IL1B [B] and NOS2 [C] both basally and in response to TNF. In figure 4.38 it is shown that pre-treatment with PB 3 significantly decreases IL10 expression basally [A]. Here the trend of increased expression of IL1B [B] and NOS2 [C] both basally and in response to TNF, is shown as well. In figure 4.39, pre-treatment with PB 9 shows a slight decrease of basal IL10 expression, and slight increase when given TNF treatment [A]. PB 9 pre-treatment significantly increases IL1B basally and slightly increases expression in response to TNF treatment [B]. Figure 4.40 shows the effects of pre-treatment with PB 14. While there is no change basally, PB 14 pre-treatment significantly increases expression of IL10 in response to TNF treatment [A]. PB 14 pre-treatment basally slightly increases expression of IL1B [B] and NOS2 [C], and significantly increases expression in response to TNF treatment.

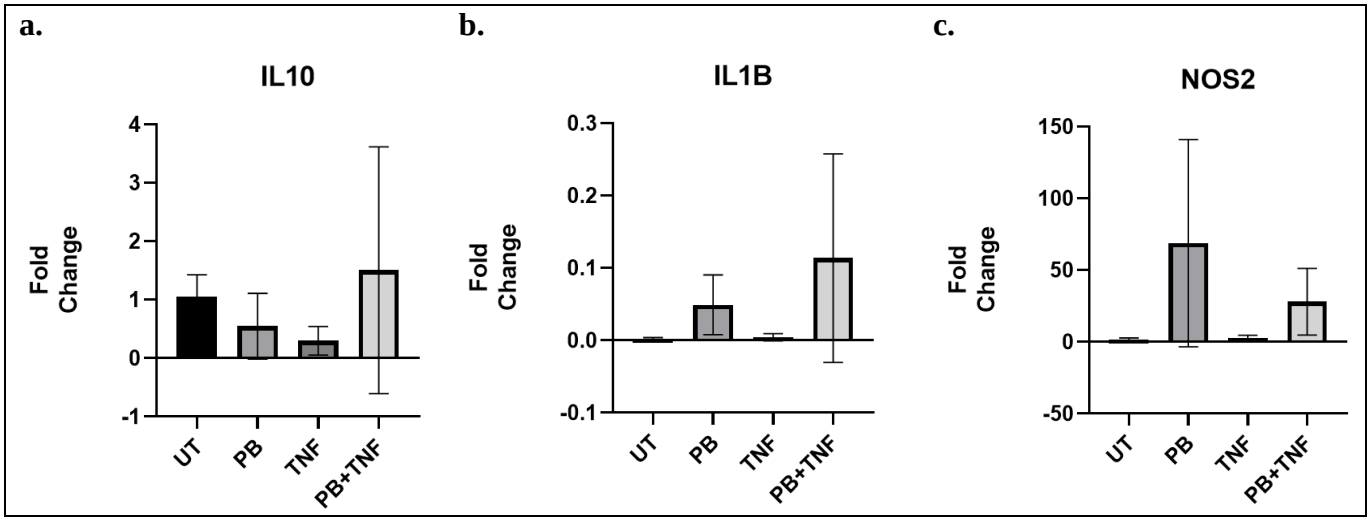

Figure 4.38 Postbiotic 1 +/- TNF stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and no significant change was measured.

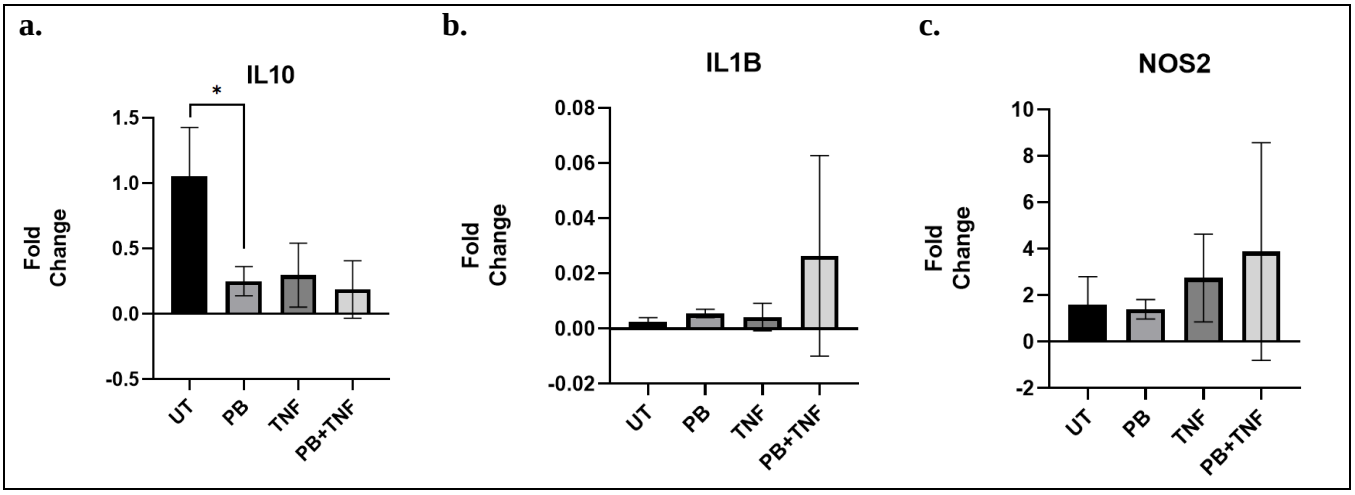

Figure 4.39 Postbiotic 3 +/- TNF stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = *.

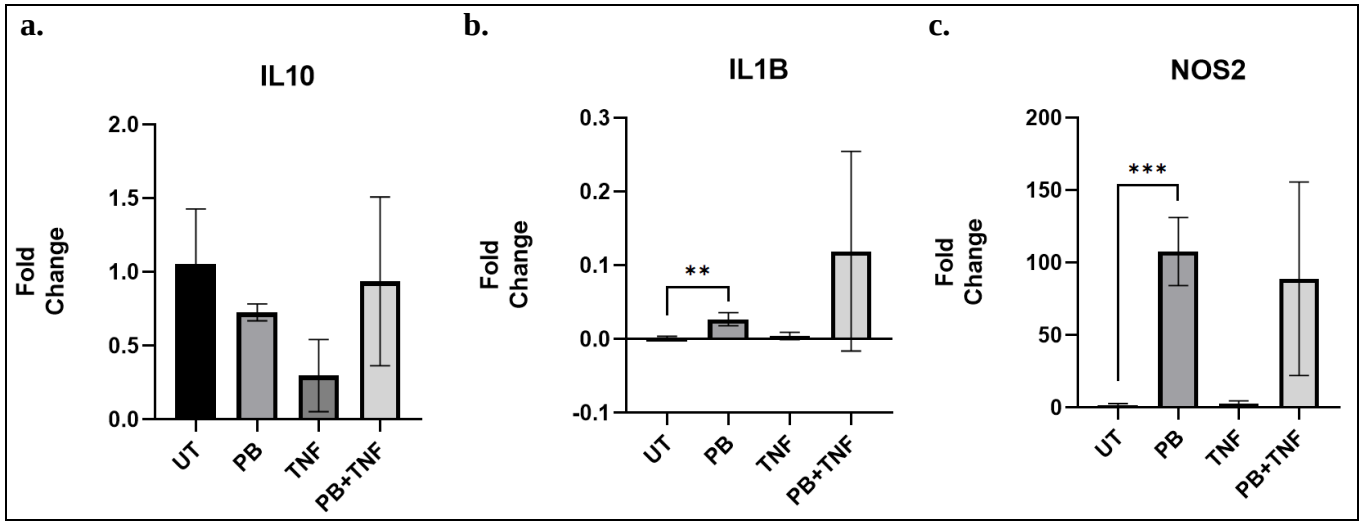

Figure 4.40 Postbiotic 9 +/- TNF stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = ** and $p \leq 0.001$ = ***.

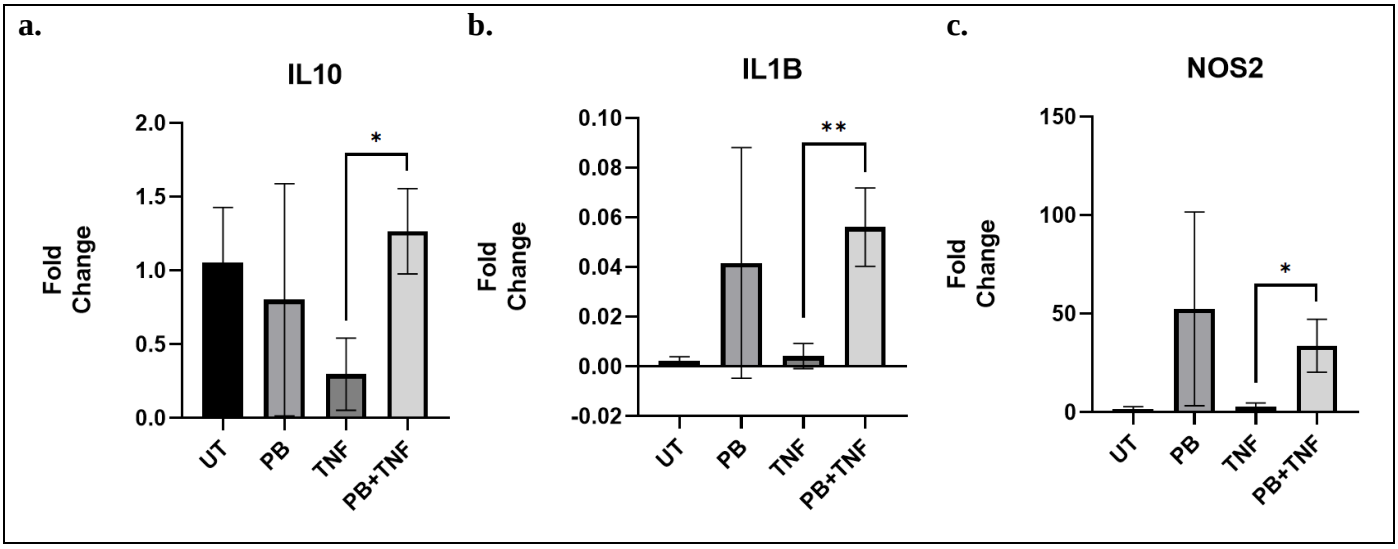

Figure 4.41 Postbiotic 14 +/- TNF stimulation in BMDMs induces changes in key biomarkers mRNA expression BMDMs are untreated or pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p < 0.05$ = * and $p \leq 0.01$ = **.

4.2.7.2 Postbiotic treatment +/- TNF treatment modulates cytokine response in BMDMs

After mRNA expression was examined, next the supernatant was analysed using ELISA. Expression of secreted IL6, an inflammatory biomarker, was measured. Supernatant was collected from BMDMs pre-treated with postbiotic samples and treated with TNF. Figure 4.41 displays that pre-treatment with PB1, significantly increases concentration of IL6 both basally and in response to TNF treatment. This significant effect is seen for the remaining postbiotic samples, PB 3 [figure 4.42], PB 9 [figure 4.43], PB 14 [figure 4.44].

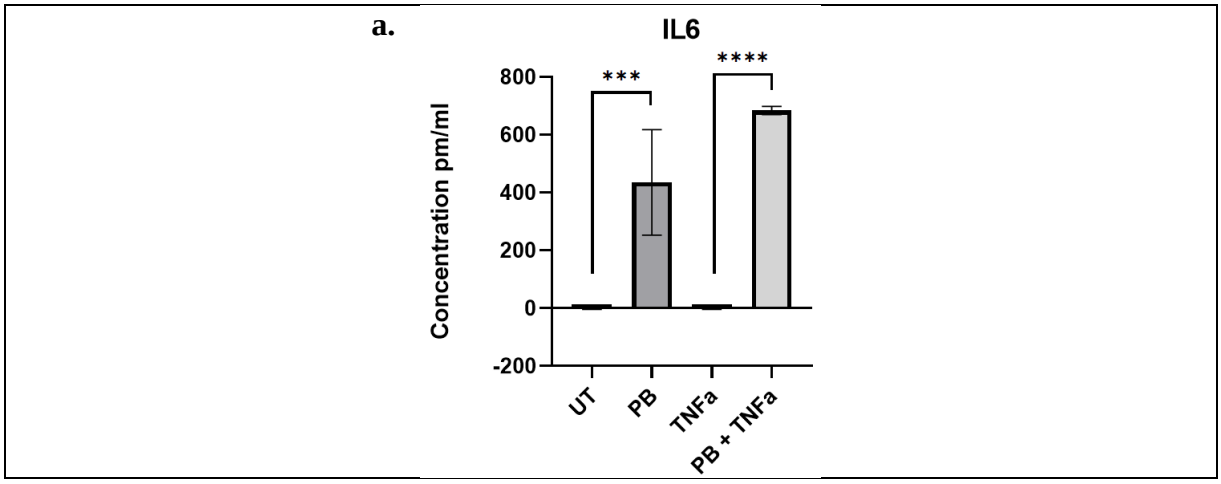

Figure 4.42 Postbiotic 1 +/- TNF stimulation in BMDMs induces changes in key biomarker protein expression BMDMs are untreated or pre-treated with postbiotic 1 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.001$ = *** and and $p \leq 0.0001$ = ****.

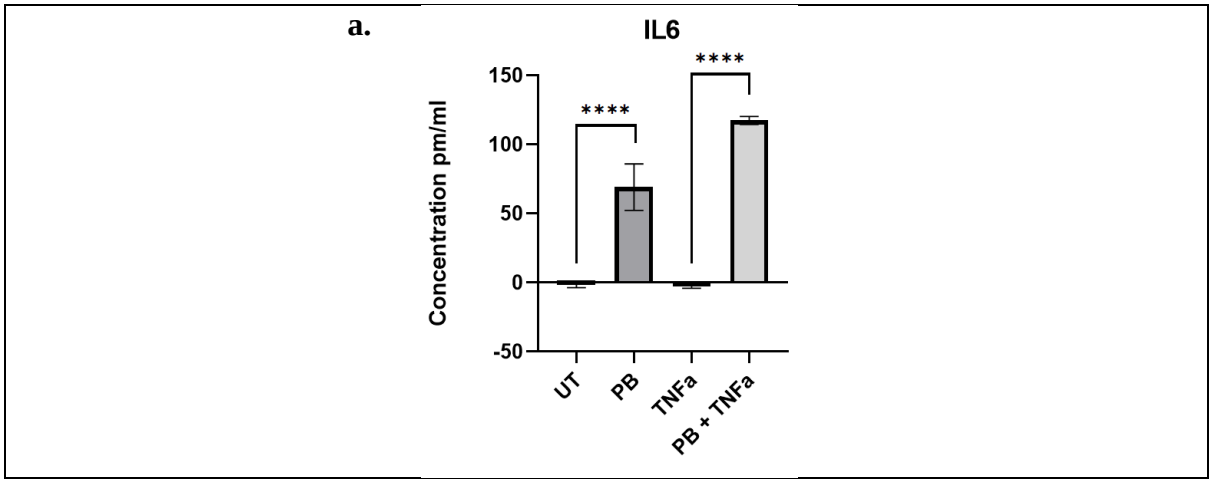

Figure 4.43 Postbiotic 3 +/- TNF stimulation in BMDMs induces changes in key biomarker protein expression BMDMs are untreated or pre-treated with postbiotic 3 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

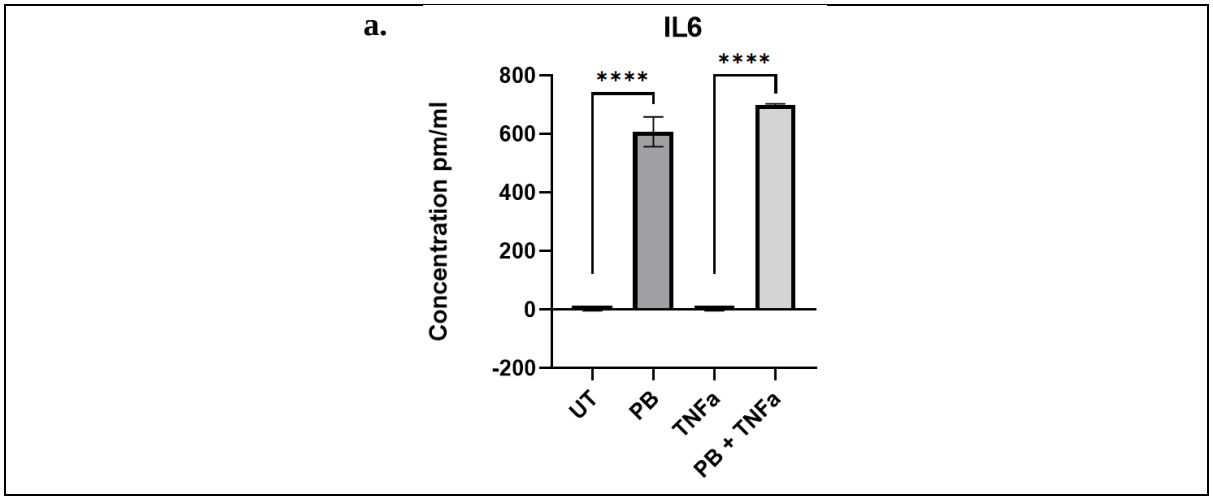

Figure 4.44 Postbiotic 9 +/- TNF stimulation in BMDMs induces changes in key biomarker protein expression BMDMs are untreated or pre-treated with postbiotic 9 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

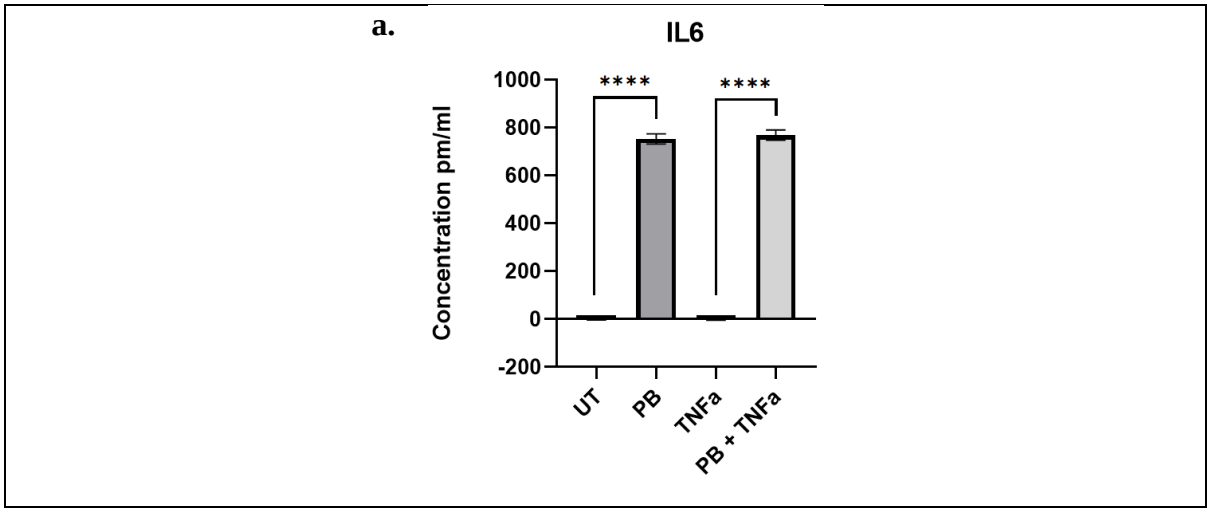

Figure 4.45 Postbiotic 14 +/- TNF stimulation in BMDMs induces changes in key biomarker protein expression BMDMs are untreated or pre-treated with postbiotic 14 at a 2.5% concentration for 24 hours, with a 100ng/ml TNF stimulation after 2 hours. Results are representative of 2 separate experiments with 3 replicates in each. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.0001$ = ****.

4.2.8 Summary of postbiotic treatment influence on key inflammatory biomarkers in macrophages

Next the results from the previous experiments on postbiotic effect on inflammatory biomarkers (both mRNA and protein expression) in macrophages, are summarised in tables. Table 4.1 indicated the effects of postbiotics in RAW-Blue macrophages. Postbiotic 1 significantly upregulated mRNA expression of IL10, TNFa, IL1B and NOS2 basally, and protein expression of IL10 and IL6 basally. In response to LPS stimulation, postbiotic 1 pre-treatment does not illicit any significant change in mRNA expression but does significantly upregulate protein expression of IL10 and IL6. In response to TNFa stimulation, postbiotic 1 pre-treatment significantly upregulates the mRNA expression of IL10, IL1B and NOS2, and protein expression of IL10 and IL6. In response to Pam3CSK4 stimulation, postbiotic 1 pre-treatment significantly upregulated mRNA expression of IL10, TNFa and IL1B, and no significant change in expression of NOS2. However, protein expression of IL10 and IL6 was significantly upregulated.

Postbiotic 3 significantly upregulated mRNA expression of TNFa, IL1B and NOS2 basally, but no significant change in IL10. Protein expression of IL10 and IL6 upregulated by basal treatment of postbiotic 3. In response to LPS stimulation, postbiotic 3 pre-treatment significantly upregulates mRNA expression of IL10 and protein expression of IL10 and IL6. In response to TNFa stimulation, postbiotic 3 pre-treatment significantly upregulates the mRNA expression of IL1B and NOS2, and protein expression of IL10 and IL6. In response to Pam3CSK4 stimulation, postbiotic 3 pre-treatment significantly upregulated mRNA expression of IL10, TNFa, IL1B, and NOS2. Protein expression of IL6 was significantly upregulated.

Postbiotic 9 significantly upregulated mRNA expression of IL10, TNFa, IL1B and NOS2 basally. Protein expression of IL10 and IL6 was also upregulated by basal treatment of

postbiotic 9. In response to LPS stimulation, postbiotic 9 pre-treatment did not significantly change mRNA expression, however, protein expression of IL10 and IL6 were significantly upregulated. In response to TNFa stimulation, postbiotic 9 pre-treatment significantly upregulates the mRNA expression of IL10, IL1B and NOS2, and protein expression of IL10 and IL6. In response to Pam3CSK4 stimulation, postbiotic 9 pre-treatment significantly upregulated mRNA expression of IL10, IL1B, and NOS2, and protein expression of IL10 and IL6.

Postbiotic 14 significantly upregulated mRNA expression of IL10, IL1B and NOS2 basally. Protein expression of IL10 and IL6 was also upregulated by basal treatment of postbiotic 14. In response to LPS stimulation, postbiotic 14 pre-treatment significantly upregulated NOS2 mRNA expression and protein expression of IL10 and IL6 were significantly upregulated. In response to TNFa stimulation, postbiotic 14 pre-treatment significantly upregulates the mRNA expression of IL10, IL1B and NOS2, and protein expression of IL10 and IL6. In response to Pam3CSK4 stimulation, postbiotic 14 pre-treatment significantly upregulated mRNA expression of IL10, IL1B, and NOS2, and protein expression of IL10 and IL6.

Table 4.2 summarises the effects of postbiotic treatment on BMDMs. Postbiotic 1 significantly upregulated mRNA expression of IL1B and NOS2 basally. Protein expression of TNFa and IL6 was also upregulated by basal treatment of postbiotic 1. In response to LPS stimulation, postbiotic 1 pre-treatment did not significantly change mRNA expression, however, protein expression of TNFa was significantly upregulated. In response to TNFa stimulation, postbiotic 1 pre-treatment did not significantly change mRNA expression, but protein expression of IL6 was significantly upregulated.

Postbiotic 3 significantly upregulated mRNA expression of IL19 and IL1B basally. Protein expression of TNFa and IL6 was also upregulated by basal treatment of postbiotic 3. In

response to LPS stimulation, postbiotic 3 pre-treatment did not significantly change mRNA expression, however, protein expression of TNFa and IL6 were significantly upregulated. In response to TNFa stimulation, postbiotic 3 pre-treatment did not significantly change mRNA expression, but protein expression of IL6 was significantly upregulated.

Postbiotic 9 significantly upregulated mRNA expression of IL1B and NOS2 basally. Protein expression of TNFa and IL6 was also upregulated by basal treatment of postbiotic 9. In response to LPS stimulation, postbiotic 9 pre-treatment significantly upregulated mRNA expression of NOS2, and protein expression of TNFa and IL6 were significantly upregulated. In response to TNFa stimulation, postbiotic 9 pre-treatment did not significantly change mRNA expression, but protein expression of IL6 was significantly upregulated.

Postbiotic 14 significantly upregulated mRNA expression of NOS2 basally. Protein expression of TNFa and IL6 was also upregulated by basal treatment of postbiotic 14. In response to LPS stimulation, postbiotic 14 pre-treatment significantly upregulated mRNA expression of IL10 and NOS2, and protein expression of TNFa and IL6 were significantly upregulated. In response to TNFa stimulation, postbiotic 14 pre-treatment significantly upregulated mRNA expression of IL10, IL1B and NOS2, and protein expression of IL6 was significantly upregulated.

a.

RAW-Blue cells		mRNA expression				Protein expression	
Treatment		IL10	TNFa	IL1B	NOS2	IL10	IL6
PB 1	basal	**	***	***	****	****	****
	plus LPS	ns	ns	ns	ns	****	****
	plus TNF	*	N/A	****	****	****	****
	plus Pam3CSK4	****	*	****	**ns**	**	****
PB 3	basal	ns	***	***	**	***	***
	plus LPS	*	ns	ns	ns	****	****
	plus TNF	ns	N/A	***	**	**	****
	plus Pam3CSK4	***	**	****	**	ns	****
PB 9	basal	**	****	****	****	****	****
	plus LPS	ns	ns	ns	ns	****	****
	plus TNF	**	N/A	***	****	****	****
	plus Pam3CSK4	****	ns	****	*	**	****
PB 14	basal	****	ns	****	****	****	****
	plus LPS	ns	ns	ns	**	****	**
	plus TNF	****	N/A	****	****	****	****
	plus Pam3CSK4	****	ns	****	****	****	****

b.

ns	not significant	**Significance of upregulation (students t-test)**	*	$p < 0.05$	***	$p \leq 0.001$
N/A	not applicable		**	$p \leq 0.01$	****	$p \leq 0.0001$

Table 4.1 Summary of postbiotic influence on inflammatory biomarkers in RAW-Blue Macrophages a. This table represents a summary of the effect of postbiotic treatment, basally and in response to LPS, TNF and Pam3CSK4 stimulation, on prevalent inflammatory biomarkers in RAW-Blue macrophages. Both mRNA expression and protein expression are shown on the graph. **b.** This section is the key to the symbols on the table.

a.

BMDMs		mRNA expression				Protein expression	
Treatment		IL10	TNFa	IL1B	NOS2	TNFa	IL6
PB 1	basal	ns	ns	*	*	***	***
	plus LPS	ns	ns	ns	ns	****	ns
	plus TNF	ns	N/A	ns	ns	N/A	****
PB 3	basal	**	ns	*	ns	****	****
	plus LPS	ns	ns	ns	ns	****	*
	plus TNF	ns	N/A	ns	ns	N/A	****
PB 9	basal	ns	ns	***	****	****	****
	plus LPS	ns	ns	ns	*	****	**
	plus TNF	ns	N/A	ns	ns	N/A	****
PB 14	basal	ns	ns	ns	*	****	****
	plus LPS	*	ns	ns	*	****	***
	plus TNF	*	N/A	**	*	N/A	****

b.

ns	not significant	**Significance of upregulation (students t-test)**	*	$p < 0.05$	***	$p \leq 0.001$
N/A	not applicable		**	$p \leq 0.01$	****	$p \leq 0.0001$

Table 4.2 Summary of postbiotic influence on inflammatory biomarkers in BMDMs
a. This table represents a summary of the effect of postbiotic treatment, basally and in response to LPS, TNF and Pam3CSK4 stimulation, on prevalent inflammatory biomarkers in RAW-Blue macrophages. Both mRNA expression and protein expression are shown on the graph. **b.** This section is the key to the symbols on the table.

4.2.9 Postbiotic treatment influences phagocytosis function in macrophages

Next, we wanted to assess the ability of the postbiotics to alter phagocytic ability of macrophages. This is done using a standard phagocytosis assay, where macrophages are treated with postbiotic samples and subsequently infected with *E. coli*. Macrophages are then burst open at different timepoints, and the lysed cells plated on L agar to investigate the cell's phagocytic ability. Firstly, this experiment was performed using RAW-Blue macrophages. Figure 4.45 displays the log CFU values for macrophages treated with postbiotics. PB 1[A] and PB 9 [C] significantly increases the amount phagocytic uptake by the macrophage cells at each timepoint. PB 3 has little to no effect on the phagocytic uptake [C]. Figure 4.46 displays that PB 1 [A] and PB [C] inhibit efficient intracellular killing at the later timepoint (6 hours), and PB 3 has little to no effect on this rate of killing [B]. Next, these results needed to be confirmed in the primary macrophage cell line, BMDMs. As PB 3 had no effect on phagocytic activity, it was left out of the remainder of these experiments. Figure 4.47 displays that PB 1 [A] and PB 9 [B] both significantly increase phagocytic uptake. In figure 4.48, it is shown that in BMDMs, there is insignificant effect on the rate of intracellular killing, however PB 9 [B] slightly increases intracellular killing. To further asses the effect of postbiotics on phagocytosis, RNA was collected from BMDMs during a phagocytosis assay at 3 hours post infection. In figure 4.49 shows that PB 1 decreases the levels of different phagocytic markers NOS2 [A], cdc42 [B] and rhoB [C]. The same effect is seen with treatment of PB 9 in figure 4.50.

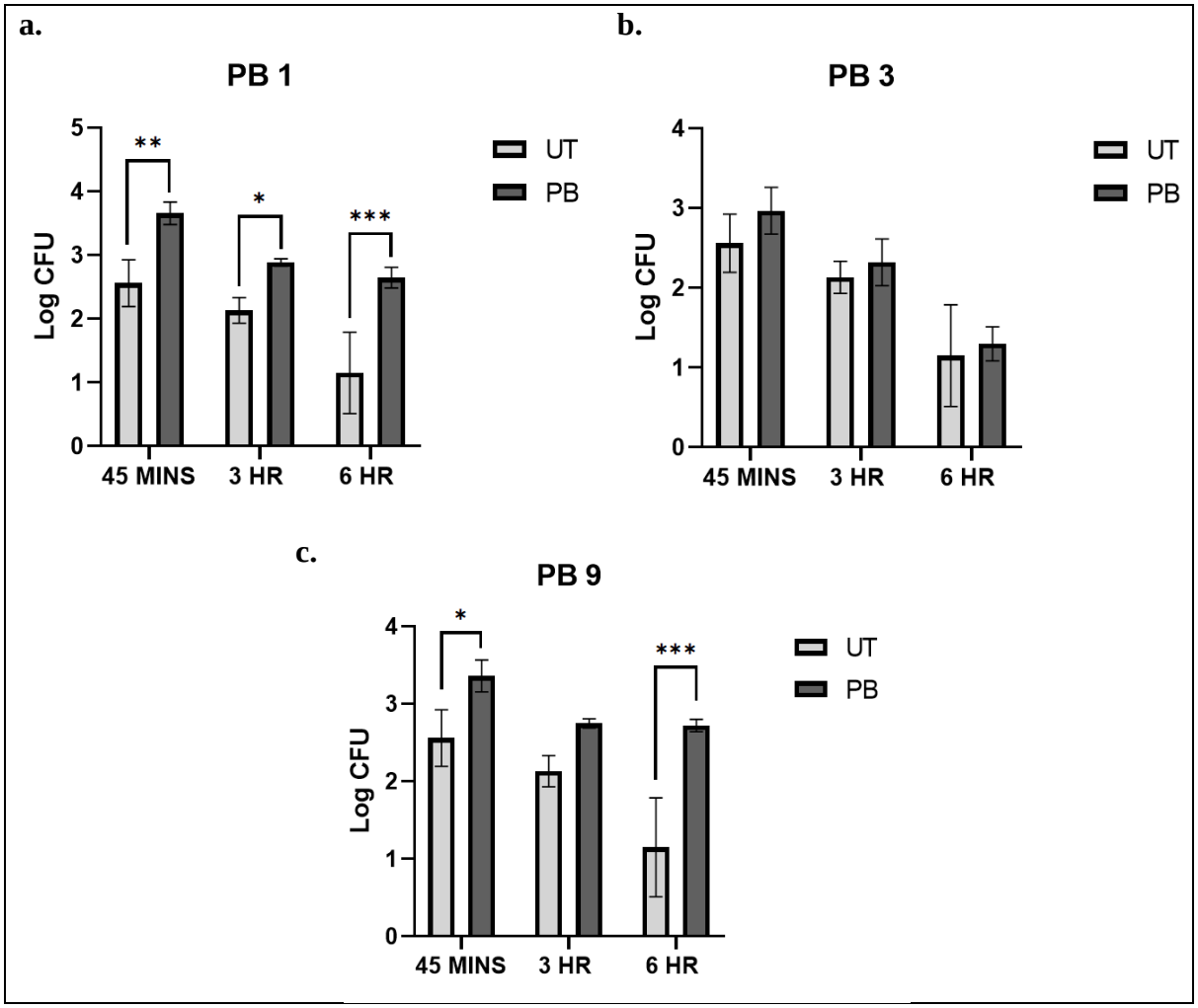

Figure 4.46 Treatment with postbiotics increases phagocytosis in RAW-Blue macrophages RAW-Blue macrophages were pre-treated with postbiotics at 2.5% concentration and then exposed to *E. coli* NCTC 12900 for 30 minutes at an MOI 20:1. Cells were then lysed and plated for CFU counting. Data is representative of three separate experiments with three replicates each. A two-way ANOVA was performed, and significance is indicated as follows: $p < 0.05$ = *, $p \leq 0.01$ = ** and $p \leq 0.001$ = ***.

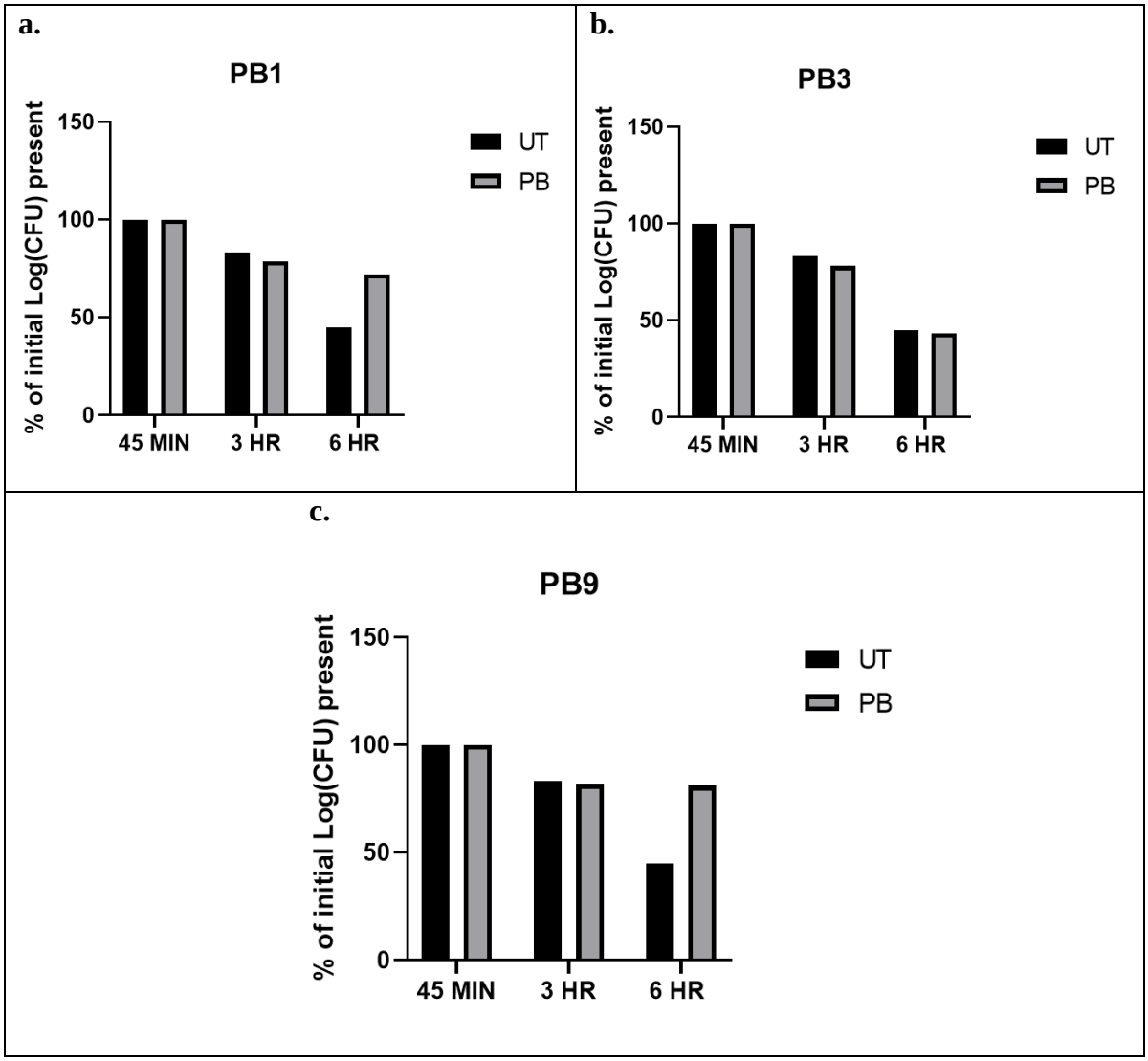

Figure 4.47 Intracellular killing ability of RAW-Blue macrophages treated with postbiotics is decreased This is a reanalysis of figure 4.46, to specifically visualise the intracellular killing ability of each postbiotic treatment. Initial CFU from the 45-minute timepoint was set equal to 100% and other values calculated to reflect the % CFU still present at each subsequent timepoint.

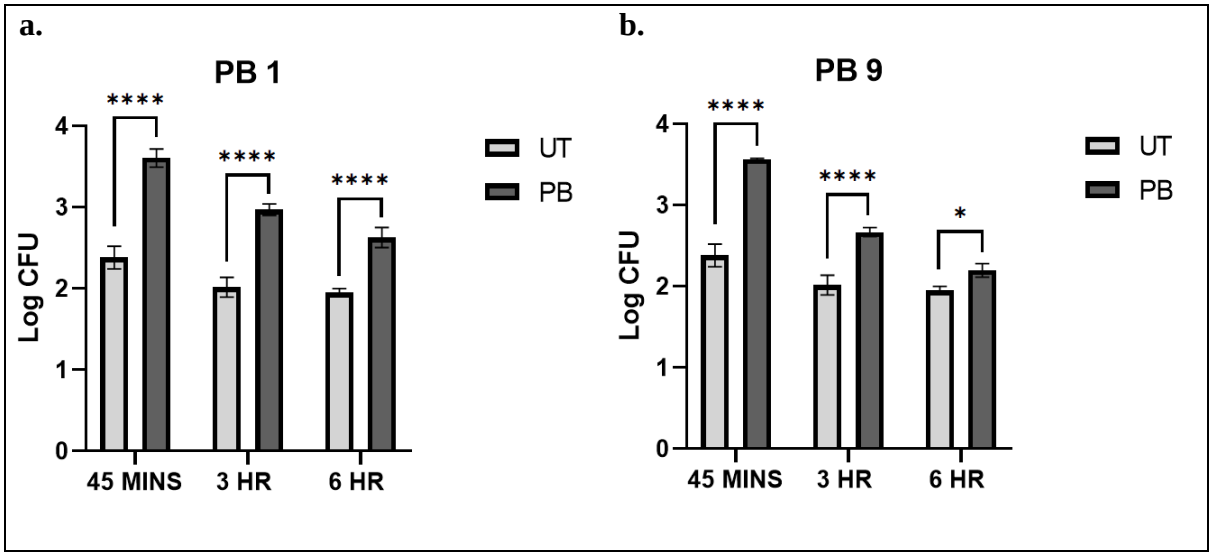

Figure 4.48 Treatment with postbiotics increases phagocytosis in BMDMs BMDMs were pre-treated with postbiotics at 2.5% concentration and then exposed to *E. coli* NCTC 12900 for 30 minutes at an MOI 20:1. Cells were then lysed and plated for CFU counting. Data is representative of two separate experiments with three replicates each. A two-way ANOVA was performed, and significance is indicated as follows: $p < 0.05$ = * and $p \leq 0.0001$ = ****.

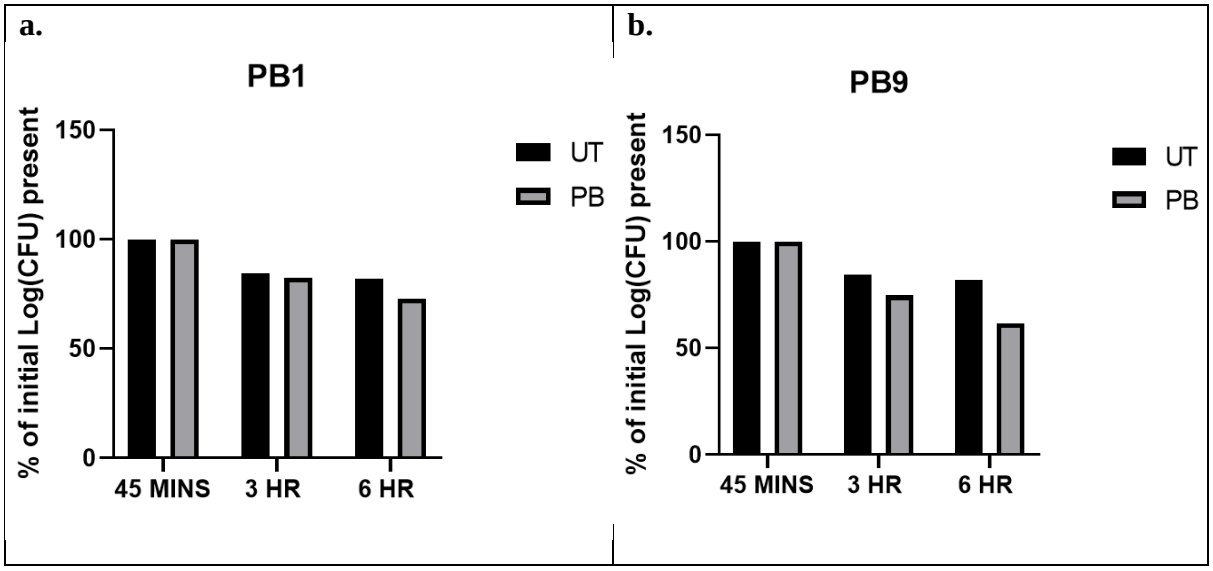

Figure 4.49 Intracellular killing ability of BMDMs treated with postbiotics is increased
This is a reanalysis of figure 4.48, to specifically visualise the intracellular killing ability of each postbiotic treatment. Initial CFU from the 45-minute timepoint was set equal to 100% and other values calculated to reflect the % CFU still present at each subsequent timepoint.

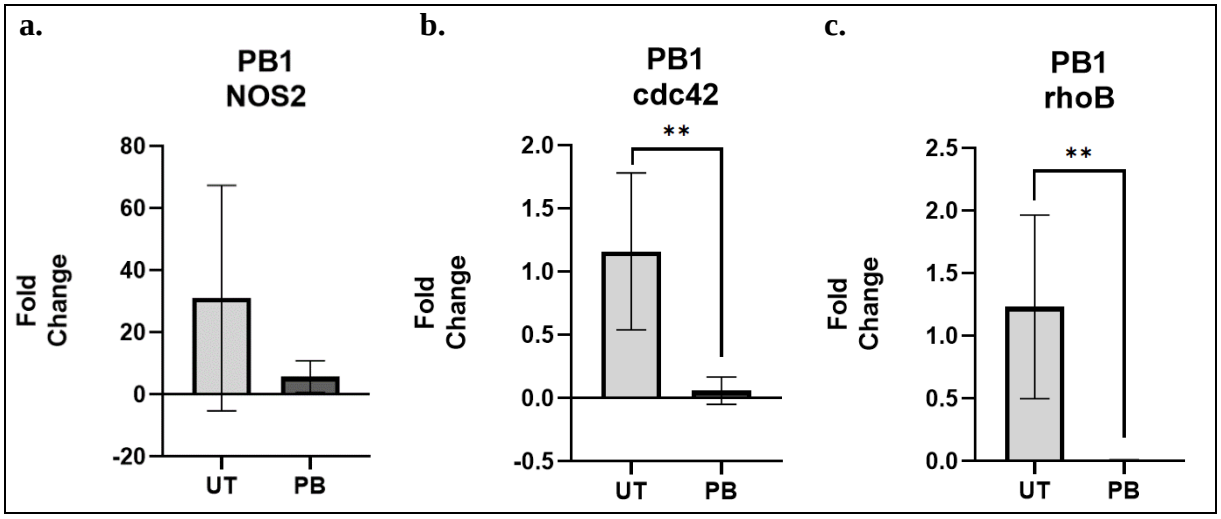

Figure 4.50 Postbiotic 1 decreases expression of phagocytosis markers BMDM cells were treated with postbiotic sample, PB1, for 24 hours and subsequently infected with *E. coli* NCTC 12900, RNA was extracted 3 hours post infection. This data is representative of one experiment containing three biological replicates. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **.

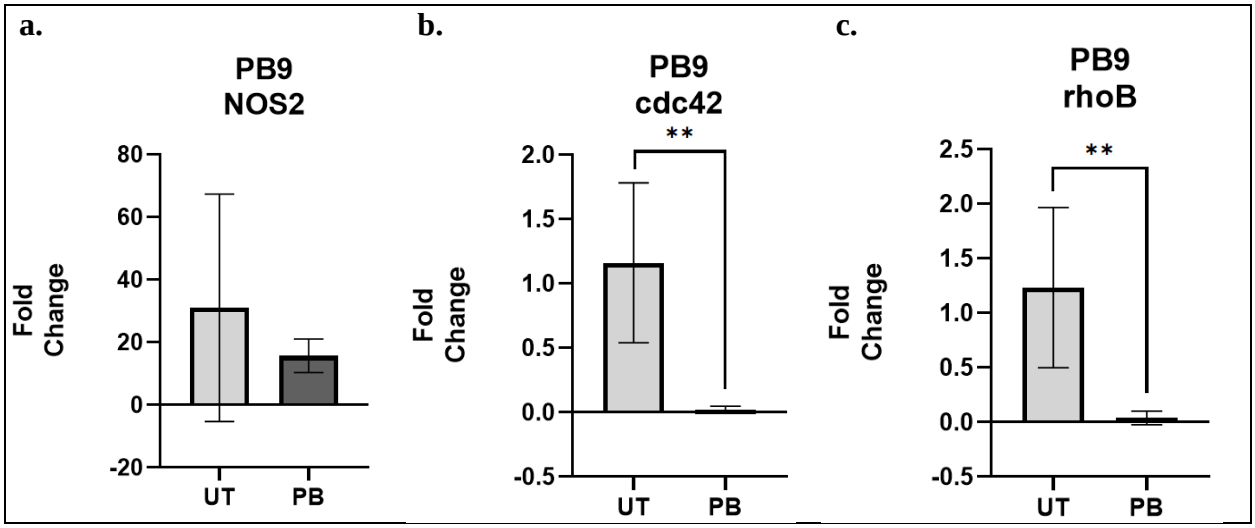

Figure 4.51 Postbiotic 9 decreases expression of phagocytosis markers BMDM cells were treated with postbiotic sample, PB9, for 24 hours and subsequently infected with *E. coli* NCTC, RNA was extracted 3 hours post infection. This data is representative of one experiment containing three biological replicates. A student's t-test was performed, and significance is indicated as follows: $p \leq 0.01$ = **.

4.2.10 Postbiotic 9 shows no significant influence on inflammatory biomarkers in LPS-induced model of sepsis

In an attempt to determine results translated from *in vitro* work to *in vivo*. A mouse trial was conducted using an LPS induced model of sepsis. Mice were treated with PB 9 before LPS induced sepsis model, peritoneal epithelial cells were then collected. ELISA was performed on supernatants to assess concentrations of inflammatory biomarkers. Then RN A was extracted from the epithelial cells themselves to analyse using qPCR for expression of mRNA of inflammatory biomarkers. Lastly, blood samples were taken, and ELISA performed on the serum. Figure 4.51 displays that PB 9 treatment has no significant effect on concentrations of excreted proteins IL1B [A], IL6 [B], and TNFa [C]. Figure 4.52 displays that treatment with PB 9 has no significant effect on the PECs mRNA expression of IL10 [A], TNFa [B], IL6 [C], IL1B [D], and NOS2 [E] after LPS treatment. Figure 4.53 displays that treatment with PB 9 has no effect on the levels of TNFa in the serum of mice undergoing an LPS induced sepsis model.

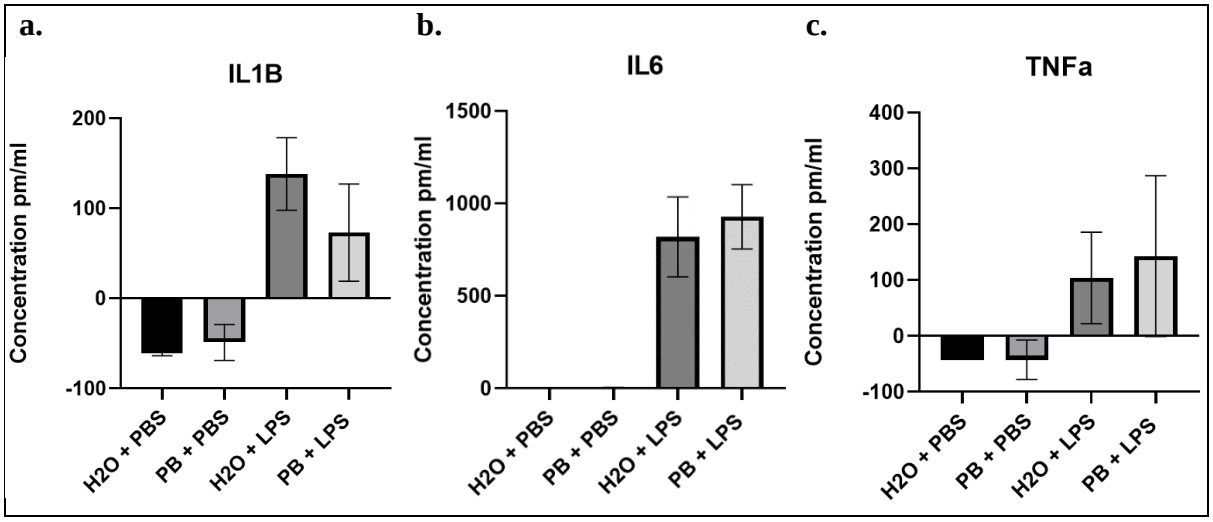

Figure 4.52 PB 9 has no effect on concentrations on IL1B, IL6 or TNFa excreted by PECs of mice having undergone LPS-induced sepsis This data is representative of one experiment containing three biological replicates. A student's t-test was performed, and no significant change was measured.

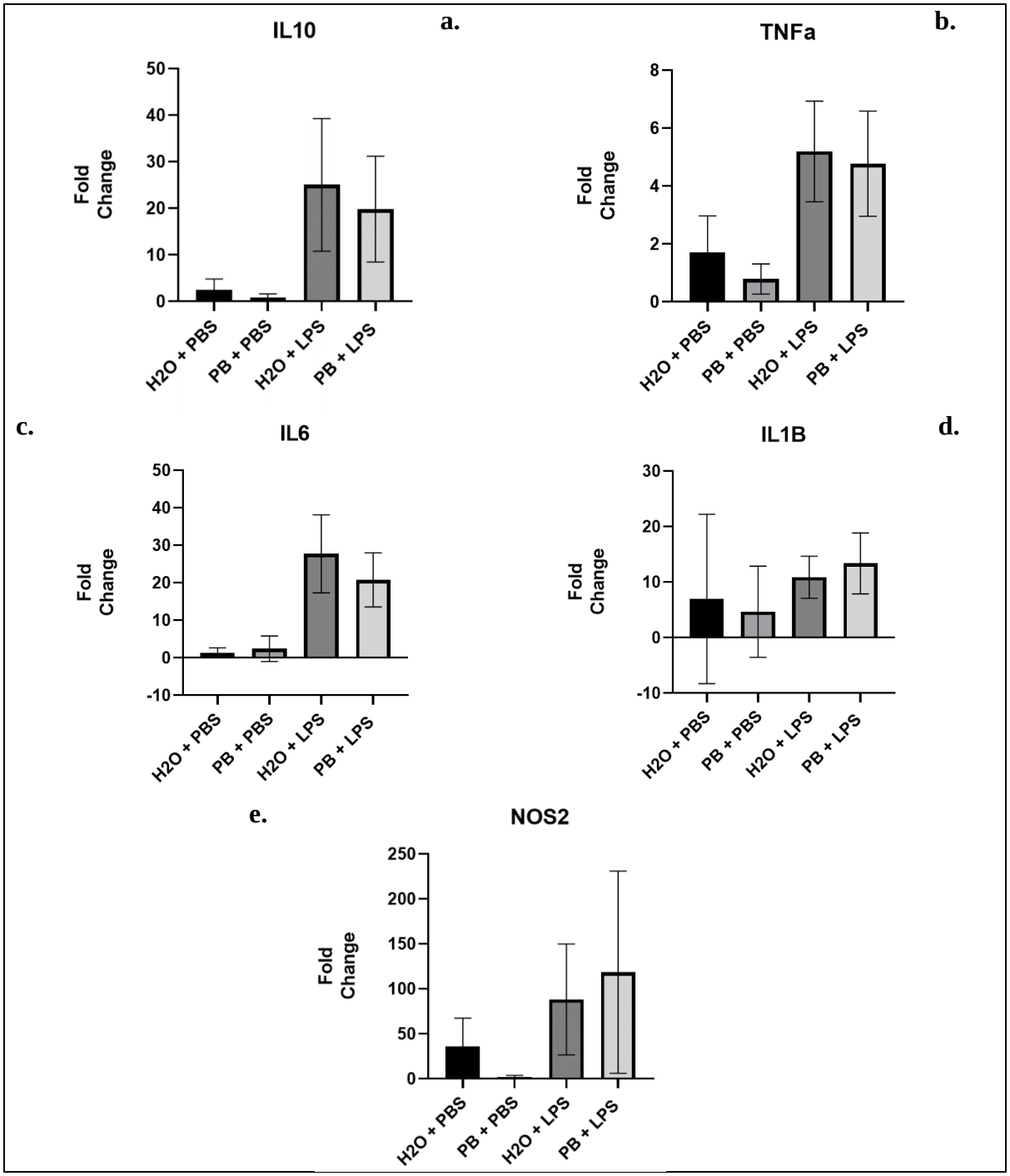

Figure 4.53 PB 9 has no effect on expression of IL1B, IL6 or TNFa in PECs of mice having undergone LPS-induced sepsis This data is representative of one experiment containing three biological replicates. A student's t-test was performed, and no significant change was measured.

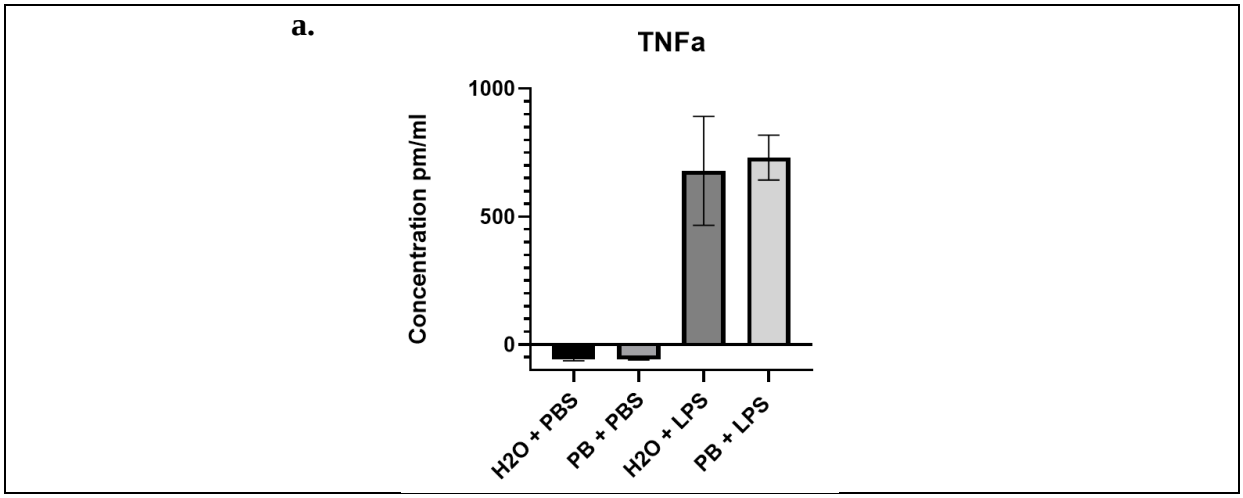

Figure 4.54 PB 9 has no effect on concentrations of TNFa in blood serum of mice having undergone LPS-induced sepsis This data is representative of one experiment containing three biological replicates. A student's t-test was performed, and no significant change was measured.

4.3 Discussion

The intestinal lumen is full of bacteria, both commensal and possibly pathogenic, antigens and other inflammatory stimuli. In this highly complex environment, the host immune system must react accordingly. Dysregulation of the relationship between host immune system and gut microbiome can lead to IBD. Macrophage cells, which are crucial to the initial innate immune response are most populous in the intestinal lamina propria[115]. Macrophage interaction with stimuli in the gut can set the precedent for the entire immune response. As anti-inflammatory therapeutic options for the gut are limited and not consistent, postbiotic supplements are being explored for their anti-inflammatory and immunomodulatory abilities. For this novel postbiotic to be properly identified as a postbiotic, it needs to incur a benefit on the host[83]. In this chapter the anti-inflammatory and immunomodulatory effects of novel postbiotic samples sourced from whisky distillation waste products will be examined and held to the standard of current postbiotic research.

Firstly, we established that postbiotic treatment does not have a significant effect on RAW-Blue macrophage cell viability. Interestingly, postbiotic treatment slightly increased BMDM cell viability. While these results were not significant, we observed visibly more BMDM cells in wells treated with postbiotics and significantly higher RNA yields from wells treated with postbiotics during experiments. An increase in macrophage proliferation could suggest an increase in activation/immune priming[116], which is supported by other results in this chapter.

Health interventions targeting the transcription factor NF-κB, are common anti-inflammatories. NF-κB activation is responsible for the inflammatory response and is shown to be overactive in inflammatory diseases[117], however it does have a paradoxical role as it is also crucial to the innate and adaptive immune system. Inhibition of NF-κB activity has

proven to be a valid therapeutic option for inflammatory diseases[118]. Postbiotic samples previously researched have shown many anti-inflammatory effects, while the mechanism of these effects is not well known[38,72,73]. In this work we assess the possible anti-inflammatory effects of the novel postbiotic samples. We also attempt to understand if NF-κB is involved with the mechanism to help fill this knowledge gap. It is worth noting here that in figure 4.4 TNF stimulation did not induce SEAP, as it would have been expected to. The reason for this discrepancy is not known. While there are no significant changes in NF-κB activity, treatment with postbiotic samples (PB1, PB3, PB9, and PB14) slightly upregulate the activity of NF-κB. This suggests the postbiotics illicit slightly pro-inflammatory effects. NF-κB controls the transcription of pro-inflammatory cytokines, such as TNFa, that are known to increase IBD pathogensis[117] and chronic over-activation of NF-κB is seen in IBD[119]. This implies that the postbiotic samples may not be effective as anti-inflammatory agents but may illicit immune training effects in the gut, as NF-κB activity is central to development of innate immunity[120]. As macrophages are key players in the pathogenicity of IBD, immune priming and tolerance building of macrophages is beneficial.

Next, we observed that postbiotics alter RAW-Blue macrophage responses to LPS, TNF and PAM3CSK4 stimuli. Mostly, each postbiotic sample upregulates mRNA expression of pro-inflammatory cytokines (TNFa, IL1B, IL6) along with anti-inflammatory cytokines (IL10). However, there are some inconsistencies with the upregulation of mRNA expression of IL10 basally (not in response to LPS, TNF, or PAM3CSK4 stimulation), as work in BMDMs showed a downregulation of IL10 and initial experiments in RAW-Blues showed no change in IL10 production. This strictly contradicts other work in RAW-Blues, where IL10, both mRNA and protein levels were significantly upregulated. In general work in BMDMs did not replicate the results seen in RAW-Blues for mRNA expression of different inflammatory markers (IL10, TNFa, IL1B and NOS2). This may be because RAW-Blue is an altered

reporter cell line. More work in primary macrophage cell lines would be required to solidify these results. While there are discrepancies, the main takeaway remains that postbiotic treatment upregulates both pro-inflammatory and anti-inflammatory cytokines.

These results may seem contradictory with most postbiotic samples upregulating both pro-inflammatory cytokines (TNFa, IL1B, IL6) along with anti-inflammatory cytokines (IL10) simultaneously. However, it has been hypothesized that in some cases these seemingly opposite effects work together to prime the immune system to react to a stressor (pro-inflammatory) and then restore homeostasis (anti-inflammatory)[121]. This research begins to fill the knowledge gap on the mechanisms behind postbiotics manipulation of the inflammatory response.

There has been evidence of postbiotics (referred to previously as cell-free bacterial supernatants) augmenting macrophage phagocytosis ability for decades[122,123].To address if our novel postbiotic samples maintain this ability, we observed treatment with PB1 and PB9 increase phagocytic activity in both RAW-Blue macrophages and BMDMs. However, these results were contradictory to the mRNA work which displayed a significant downregulation of the phagocytic markers NOS2, cdc42 and rhoB. The mRNA work was based upon one experiment and would need to be repeated to confirm. In RAW-Blue macrophages, treatment with PB1 and PB9 not only increased phagocytosis but they inhibited intracellular killing ability. This suggests a shift towards the M2 polarization state, or the anti-inflammatory state of macrophages[100]. However, this inhibition of intracellular killing effect was not seen in BMDMs, as postbiotic treatment slightly increased intracellular killing ability. It is also interesting to note here that the postbiotics with effect on phagocytic activity are the cell containing postbiotics (PB1 and PB9), cell free postbiotics such as PB3 and PB14 had no effect. This is likely because whole inactivated microbial cells are known to be better at immune priming[124,125], which is in keeping with current literature on the subject.

No changes were observed in the *in vivo* experiments. Mice subject to an LPS-induced model of sepsis, treated with postbiotics showed no change among common inflammatory cytokines compared to the control group. This experiment involved the administration of the postbiotics through gavage, which may be why the potency of the inflammatory effects were dampened. Perhaps a higher concentration of the postbiotics would illicit a stronger response. Our samples here were also taken from the peritoneal cavity, which could also be the reason for these different results. This experiment was only conduced once and would need to be repeated for validated results. *In vivo* work with postbiotics is limited and needs to be further explored.

While treatment with the postbiotic samples *in vitro* overall increases expression of pro-/anti-inflammatory cytokines, and phagocytic activity. Further research is needed to confirm this, especially as intestinal macrophages have unique adaptations to the intestinal environment. Resident macrophages in most parts of the body when experiencing higher levels of bactericidal and phagocytic activity initiate fully inflammatory immune responses to help fight off infection. However, an unchecked inflammatory immune response to commensal bacteria in the gut would result in inflammatory bowel diseases[126]. So intestinal macrophages have adopted the ability to resist inflammatory responses while experiencing higher levels of phagocytic activity. This can be achieved by the lack of innate immune response receptors, such as the receptors for LPS. As seen in Smythies et al. macrophages isolated from the intestinal lamina propria, when exposed to pro-inflammatory stimuli including phagocytosis, did not produce inflammatory cytokines such as TNFa [110]. More research, perhaps on macrophages isolated from the lamina propria, is needed to observe the effects the postbiotic samples would have on intestinal macrophages that are more suited to building innate immune tolerance.

This chapter on the immunomodulatory effects of postbiotic samples on macrophages concludes that postbiotic treatment induces strong immune responses. Based on the NF-κB activity and cytokines looked at in this study the immune response is pro-inflammatory, with some contradictory IL10 anti-inflammatory activity. However more targets would be needed to truly understand the scope of this immune response. Treatment with postbiotic samples also directly increases phagocytosis ability in macrophages demonstrating immune priming potential. More work is needed to uncover the underlying mechanisms of these immune modulations.

Chapter 5

Discussion

5.1 Final discussion and future perspectives

The overall aims of this thesis were to deepen the understanding of antimicrobial potential and immunomodulatory potential of novel postbiotic samples sourced from whisky distillation waste products. We assessed the antimicrobial and immunomodulatory potential of four postbiotic samples; they are cell containing yeast postbiotic (PB 1), cell free yeast postbiotic (PB 3), cell containing *L. casei* postbiotic (PB 9), and cell free *L. casei* postbiotic (PB 14). Postbiotics have been known to illicit antimicrobial and immunomodulatory effects in other studies, here we investigate the potential of these postbiotics to have these effects in the context of IBD.

Current research on other postbiotics or cell-free supernatant displays their antimicrobial activity against pathogenic bacterial strains[89,90]. In this research we demonstrated that this novel source of postbiotics display antimicrobial potential as do current postbiotics on the market. Our yeast postbiotic samples PB 1 (yeast cell containing postbiotic), and PB 3 (yeast cell free postbiotic) had significant inhibitory effects against different priority pathogens at concentrations of 12.5% and 25% respectively. More research is needed to confirm the specific MICs of these compounds. More strains to investigate would also be beneficial. As novel antimicrobial compounds are in high demand, further research on these postbiotics would be advantageous.

Postbiotics also have been known to have anti-inflammatory/immunomodulatory effects on macrophage cells as well as other cell types[72]. As macrophages are immune cells that, in response to stimuli, initiate inflammatory responses, they are central to the pathogenesis of inflammatory diseases like IBD. In De Macro et al., anti-inflammatory effects in macrophages were observed in response to LPS when treated with postbiotic samples[72]. Our novel postbiotic samples illicit some anti-inflammatory effects such as the increase in anti-inflammatory cytokine IL-10, however, also induce pro-inflammatory responses. The

transcription factor, NF-κB, which is a main control for the inflammatory response in macrophages was investigates using the RAW-Blue macrophage reporter cell line. We saw that postbiotics slightly increase NF-κB activity, which indicates an increase in inflammation. However, as NF-κB is also central to the innate immune response learning in macrophages[120], this may suggest immune priming is taking place. Inflammatory biomarkers were investigated at the mRNA level and protein level after postbiotic treatment followed by LPS, TNF, and PAM3CSK4 treatment, and the results were conflicting. Postbiotics upregulated both pro-inflammatory and anti-inflammatory cytokines simultaneously. This poses the potential for postbiotics to be immune training agents for macrophages, readying both side of the immune response at the same time[121]. Research displays that the novel postbiotic samples do not have fully anti-inflammatory effects, rather a more immune-modulatory effect. This is consistent with current postbiotic research as the debate for these supplements being anti- or pro-inflammatory is divided. Just as the postbiotic samples may contain anti-inflammatory bioagents, there will also be cell fragments such as LPS that are known to be inflammatory[127]. Research on the composition of the novel postbiotic samples would aid in the explanation of these seemingly contradicting results; as well as more research on the mechanisms behind them.

The modulation of phagocytosis ability by postbiotics is an area of research with very mixed results[128,129]. However, having been around longer, several studies have demonstrated the ability of probiotics to increase phagocytosis activity[130]. In this research we saw that postbiotics significantly increase phagocytic ability in both RAW-Blue macrophages and BMDMs. Postbiotics also appear to hinder intracellular killing ability in RAW-Blue macrophages, suggesting a shift towards the anti-inflammatory macrophage polarization, M2[131]. However, this effect on intracellular killing was not seen in BMDM cells. The modulation of basic macrophage function supports the hypothesis that probiotics may have an

immune training effect on macrophages. This is very important for inflammatory diseases such as IBD, as macrophage responses set the precedent for the full immune response that follows. These results help fill a knowledge gap concerning postbiotics and their influence on phagocytosis and macrophage polarization, as well as establishes this novel source of postbiotics as a strong contender in the world of postbiotics.

It is important to emphasize that antibiotics are one of the current treatment options for IBD management. This is contributing to the AMR global health crisis. Another serious implication of IBD is increased host susceptibility to pathogenic bacterial infection[3,132]. Treatment options for intestinal infections are limited, especially as the number of MDR strains grows every year[88]. Antibiotic treatment is Widley used for intestinal infections and has many risks, including the greater global risk of AMR. So novel compounds of any kind with potential to modulate the inflammatory response in the gut, is important research.

Just as fighting AMR with novel solutions is imperative to the future of human health and safety, so is prioritizing sustainable solutions. With the growing threat of climate change looming, promoting a sustainable circular economy is imperative. Our research helps to focus sustainable solutions to today's health crises. Whisky distillation waste known as pot-ale, contributes to pollution of the environment. This is both fiscally and environmentally costly. Our research investigates putting this waste product to use. Using the help of microbes to help break down the biomass of the pot-ale, lessens the carbon footprint as the alternative is an expensive drying process. Taking the treated pot-ale and using it as a postbiotic supplement would help to add some circularity to the whisky distillation process and avoid unnecessary waste.

One major consideration for this research is that the intestinal macrophage is a different cell to the ordinary macrophage. Intestinal macrophages have higher thresholds for phagocytic

activity before inducing an inflammatory state, as well as higher thresholds for other inflammatory stimuli[31,98,133]. Furthermore, research on intestinal macrophages would be necessary to assess the true effect of postbiotics on the immune response in the intestinal environment. This can be done with further *in vitro* work on intestinal macrophage cell lines, and/or to get a full picture of the gut environment, *in vivo* work.

Another factor to consider is the stability of the postbiotics as they are carried through the digestive system. While the question of the microbes reaching the gut alive is no longer relevant when dealing with postbiotics as opposed to probiotics, there are still concerns for the stability of the postbiotics potency. This problem can be solved with different encapsulation methods; however, testing is needed to choose the proper method[134].

Another gap in the research is the characterization of the components of the postbiotic. While it is known that pot ale is made up of yeast, yeast by-products, barley residue, soluble protein, soluble carbohydrates[74]. The amount of yeast metabolites and by products has not been characterized for PB 1 and PB 3. The same is true for PB 9 and PB 14, the amount and composition of *L. casei* by products has not been characterized. It is also possible that the heat-inactivation process to kill live yeast/bacteria, introduce different metabolites and bioactivity[135]. NMR and/or other spectroscopy options may be a good next step. Although, the postbiotic samples will produce a lot of noise as they are highly variable samples. Pot ale, with which the postbiotics are made, consists of a very wide range of organic compounds including residual sugars, organic acids, esters, alcohols and phenolic or aromatic compounds[74]. These components may crowd the spectrum and make interpreting the results more difficult. Postbiotics 1 and 9 are cell containing samples, which further complicates the NMR, although not impossible, more advanced NMR techniques would be required. Metabolomics would be a useful solution here, as it involves separating of the sample using a chromatography technique, followed by spectroscopy[136].

The main difference in activity that correlates with a difference in sample properties is the comparison of the cell-containing postbiotics (postbiotics 1, 9) and their corresponding cell-free postbiotics (postbiotics 3, 14). This difference in composition is the most striking and the results from the phagocytosis assays show this. Cell-containing postbiotics 1 and 9 significantly manipulate macrophage phagocytosis ability and intracellular killing rates, however, the cell-free counterparts do not maintain this ability. The cell containing samples also showed greater anti-microbial ability as seen in chapter 3 results. The other main difference in the sample's composition is the presence of *L. casei*/it's by products in postbiotic samples 9 and 14, compared with yeast in postbiotic samples 1 and 3. The yeast postbiotic samples showed greater anti-microbial activity than the postbiotics created with *L. casei*. However, this fundamental difference did not show any clear difference in immunomodulatory effects. Further research to break down the finer composition of the postbiotic samples will aid in deciphering the active ingredients.

This thesis investigated novel postbiotic samples sourced from the whisky distillation process for antimicrobial and immunomodulatory potential. Postbiotic samples displayed antimicrobial activity in this study, with both inhibitory and bactericidal activity. Postbiotic samples were also seen to be immunomodulatory, increasing activity of inflammatory and anti-inflammatory cytokines. Postbiotics also significantly increase macrophages phagocytosis ability. When the results from this study are considered together, there is possible evidence for immune training activity by the postbiotic samples. Given the importance of mining new antimicrobials in the wake of AMR crisis, and immunomodulatory agents as IBD and other inflammatory diseases rise across the globe, further research on these novel postbiotics would be prudent. Our research provides a deepens our understanding of novel postbiotics, their place among other postbiotics on the market and their potential for human health benefits.

Chapter 6
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