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Journal of Functional Foods, (IF=3.144, Q1)
Research

Immunomodulatory effects of *Streptococcus thermophilus* on U937 monocyte cell cultures

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ABSTRACT

Probiotics are beneficial to the host through its contribution to the development and maintenance of a healthy immune system. Some probiotics are used in the food industry as secondary starter cultures to ferment dairy products including *Streptococcus thermophilus* (ST). ST bacteria were used to determine their modulatory effects on a promonocytic cell line which exhibited differential cytokine induction, in particular, IL-4 and IL-10 which are important in injury, infection and play a central role in anti-inflammatory responses. CXCL8 and GM-CSF are also activated - important for chemotaxis and recruitment of cells at sites of inflammation, and, increased CD11c, CD86, C206, CD209, MHC-1 expression. As ST are used in the dairy industry, are well tolerated when consumed and remain viable during cold storage, their consumption might be a practical approach in modulating immune responses in the host, and be beneficial to an array of diseases, including, autoimmunity and inflammatory bowel diseases.

Keywords:

Probiotics; *Streptococcus thermophilus*; Monocytes; Cytokines; Inflammation

1. Introduction

The regular consumption of probiotics has been shown to contribute to the maintenance of a healthy microbiome in the intestinal tract and associated health benefits (Ahtesh, Stojanovska, & Apostolopoulos, 2018; Hardy, Harris, Lyon, Beal, & Foey, 2013). It has been documented that there are over 1,000 existing species within the microbiome - with 400 well known, which are all essential for the establishment and maintenance of a healthy and functional immune system (Jensen, Drømtorp, Axelsson, & Grimmer, 2015; A. J. Stagg, Hart, Knight, & Kamm, 2004; J. Stagg et al., 2011). Commensal strains of the human intestinal microbiota have been used as probiotic supplements, either in food or as capsules, for a variety of medical issues including diarrhoea, constipation and various infections (Di Caro et al., 2005; Isolauri, Sütas, Kankaanpää, Arvilommi, & Salminen, 2001; Ouwehand, Salminen, & Isolauri, 2002; Vliagoftis, Kouranos, Betsi, & Falagas, 2008). This is based on the role that the microbiome plays in establishing a balanced immune response during early life and maintaining it throughout adulthood (Kelly, King, & Aminov, 2007; Langhendries, 2005, 2006; Mead et al., 1999). These beneficial bacteria were termed “probiotic” by Fuller in 1989 (AFRC, 1989), which were then defined by the Food and Agriculture Organization and the World Health Organization as ‘‘live microorganisms which upon administration in adequate amounts confer a health benefit to the host’’ (Guarner & Schaafsma, 1998; Lebeer, Vanderleyden, & De Keersmaecker, 2008; Vasiljevic & Shah, 2008). Likewise, “ghost probiotics”, i.e. non-viable microbial cells, intact or broken or crude cell extracts also confer benefits to the host (Deshpande, Athalye-Jape, & Patole, 2018).

Most probiotics today belong to the group of lactic acid bacteria (LAB) which represent gram-positive lactic acid producing microorganisms, and include several genera of lactobacilli, bifidobacteria and enterococci; LAB are abundantly present in the intestine, especially in the lower small intestinal lumen and the colon (Fink et al., 2007; Maassen et al., 2000; Michałkiewicz et al., 2003). LABs are commonly supplemented in foods as live probiotic strains and have been shown to confer health benefits to humans (Asarat, Apostolopoulos, Vasiljevic, & Donkor, 2015, 2016; Asarat, Vasiljevic, Apostolopoulos, & Donkor, 2015; Fink et al., 2007; Guarner & Schaafsma, 1998; Salazar et al., 2009). In addition, *Streptococcus* species (a member of the LAB), including exopolysaccharide-producing strains of *Streptococcus thermophilus* (ST) such as *S. thermophilus* ST1342, *S. thermophilus* ST1275 and *S. thermophilus* ST285 (Purwandari & Vasiljevic, 2009; Salazar et al., 2009) are widely used due to their functional properties such as, immunosuppressive effects in the treatment of

acute ulcerative colitis, improving lactose digestion (Iyer, Tomar, Uma Maheswari, & Singh, 2010; Rabot, Rafter, Rijkers, Watzl, & Antoine, 2010; Savaiano, 2014), improving the intestinal barrier function restricting adhesion and invasion of pathogens (Brigidi, Swennen, Vitali, Rossi, & Matteuzzi, 2003; Elli et al., 2006; Kebouchi et al., 2016) as well as their production of bacteriocins and vitamins (Iyer et al., 2010; Ng et al., 2010; Uriot et al., 2017). Furthermore, ST present characteristics that enable them to be used in fermented milk products (i.e. yogurt), flavoring of dairy, and is recognized as the next most important species after *Lactococcus lactis* (Hols et al., 2005). Since 2002, ST has been accepted to be safe and approved by the American Food and Drug Administration (FDA, 2018) and the Qualified Presumption of Safety grade/rank/status from the European Food Safety Authority (Kebouchi et al., 2016). However, in contrast with other LAB, using the term probiotic for ST is still a matter of debate (Mohammadi, Sohrabvandi, & Mohammad Mortazavian, 2012; Uriot et al., 2017; Vasiljevic & Shah, 2008).

In studies of human primary macrophages, ST bacteria induce the anti-inflammatory interleukin (IL)-10 cytokine, although pro-inflammatory IL-12 cytokine is also produced (Latvala, Miettinen, Kekkonen, Korpela R., & I., 2011). Furthermore, ST1275 and *Bifidobacterium longum* BL536 were shown to stimulate high levels of transforming growth factor (TGF)-beta, important for the differentiation of regulatory T cells (Treg) and T-helper (Th)-17 cells from bulk cultures of peripheral blood mononuclear cells (Donkor et al., 2012a). *S. salivarius*, *S. equinus* and *S. parasanguinus* have been shown to induce IL-8, tumor necrosis factor (TNF)-alpha and IL-12 in human dendritic cells (DC). *Streptococcus* and *Veillonella* often co-occur in bio-environments and can potentially have metabolic collaboration; in fact their combination collectively show immunomodulatory effects. Whilst *Veillonella parvula* was only able to stimulate IL-6 production; combinations of *Streptococcus* and *Veillonella* were able to down regulate IL-12 whilst up regulating IL-6, IL-8, IL-10 and TNF-alpha (van den Bogert, Meijerink, Zoetendal, Wells, & Kleerebezem, 2014). In mice, administration of ST either orally or intraperitoneally, was shown to enhance immune responses by activating phagocytic activity of macrophages and increased antibody production by B cells (Perdigon, Nader de Macias, Alvarez, Oliver, & Pesce de Ruiz Holgado, 1987). Mice with dextran sodium sulphate induced colitis showed reduced clinical signs of disease and decreased cellular infiltration (associated with inflammation) in the colon following ST oral administration (Bailey, Vince, Williams, & Cogan, 2017). Conversely, in a human clinical study, 20 participants with positive skin prick tests and atopic history consumed yogurt that contained live ST and *Lactobacillus bulgaricus* did not show any improvement in immune cell

parameters; phagocytic function, antibody responses, cytokine secretion by T cells (IFN-gamma, IL-2, IL-4), number and function of natural killer (NK) cells and neutrophils (Wheeler et al., 1997). Thus, although probiotics are able to modulate host immune responses, much is still unknown regarding their direct effect on immune cells such as monocyte/macrophages (Lebeer et al., 2008). Thus, we chose to investigate three strains of *S. thermophilus* (ST1275, ST285, ST1342), to determine their direct effects on the human pro-monocytic cell line, U937 cells that were differentiated into monocyte/macrophage cells using vitamin D_3. (Mogensen, 2009; Suresh & Mosser, 2013). Pattern recognition receptors present on monocytes and macrophages have been shown to be responsible for the recognition of bacteria, therefore these cells were used in the current study to determine the direct effect (cell surface markers and cytokine expression) of *S. thermophilus* bacteria on these cells.

2. Material and Methods

2.1. Bacterial strains

Pure bacterial cultures of *S. thermophilus* 1342 (ST1342), *S. thermophilus* 1275 (ST1275) and *S. thermophilus* 285 (ST285) were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). Stock cultures were stored in 40 % glycerol at −80° C. Prior to each experiment the cultures were propagated in M17 broth (Oxoid, Melbourne Australia) and were incubated at 42° C. Bacteria were also cultured in M17 agar (1.5 % w/v agar) for characteristics and assessment of their purity, morphology and gram status by gram staining.

2.2. Preparation of live bacterial cell-suspensions

All media were prepared and sterilized by autoclaving at 121 °C for 15 min. Prior to actual experiments, the cultures were grown 3 times in M17 broth, at 37 °C for 18 hours with a 1 % inoculum transfer rate. *S. thermophilus* start to synthesize autolysins at the end of the exponential growth phase (Husson-Kao et al., 2000), or during or after the transition from exponential to stationary growth phase (Sandholm & Sarimo, 1981). Our cultures were obtained from Victoria University culture collection, which are cultured at 37-42° C for 24 hours (Purwandari & Vasiljevic, 2009). We kept our culture growth time consistent 18 hours (at the end of the exponential growth phase) and before stationary growth phase to prevent cell

lysis. Growth rate varies for various subspecies as well as their temperature (30-50° C) (Tarrah et al., 2018). On the day of experiment, bacteria were harvested during stationary growth phase, by centrifugation (6000×g for 15 min at 4 °C, Beckman J2/HS centrifuge, JA-14 rotor, Palo Alto, CA, USA), washed twice with phosphate-buffered saline (PBS) (Gibco, Australia) and resuspended in RPMI 1640 culture media. These samples constituted the live-cell suspensions.

2.3. Enumeration of bacterial cells

Bacterial strains were scraped from M17 agar and transferred into Dulbecco's PBS (Invitrogen, Pty Ltd. Australia) adjusted to a final concentration of 10^8 cfu/ml by measuring the optical density at 600 nm, and washed twice with PBS before co-culturing with monocyte cell cultures.

2.4. Culture, differentiation and stimulation of U937 cells

U937 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine at 37 °C, 5 % CO_2. For differentiation of U937 cells into monocytes, U937 cells were adjusted to 3×10^5 cells/ml and 100 nM vitamin D_3 was added followed by incubation for 72 h. The resulting cells have characteristics of monocytes with CD14, CD11b, CD86 and MHC class II surface expression (Table 1).

Differentiated U937 cells (5×10^5 cells/ml) were stimulated with 1.5×10^8 live probiotic bacteria (ST1342, ST1275 or ST285) or lipopolysaccharide (LPS, 1 μg/ml; internal positive control) or non-stimulated as reference background control. The ratio of cells to bacteria is usually 1:10, however this ratio is usually for PBMC in which there is only 10-13 % monocytes present. Although there are only a few studies that use pure monocyte cultures, 1:300 ratio of cells to bacteria has been reported (Jensen et al., 2015); hence in our experiments, 1:300 ratio cells to ST bacteria was used. All cell cultures were incubated at 37 °C, 5 % CO_2 for either 24 hours or 48 hours. Supernatants were centrifuged and filtered to remove bacteria and were used for cytokine analysis and cells were used for cell surface marker expression by flow cytometry. Similar protocols have been used for other probiotic bacteria and on epithelial cells or PBMC (Asarat, Apostolopoulos, et al., 2015; Asarat, Vasiljevic, et al., 2015; Donkor et al., 2012b).

2.5. Cytokine analysis

Cytokine concentrations of supernatants were measured by commercially available capture and detection antibodies in a Bio-Plex assay using a 9-plex kit (BioRad, Melbourne Australia) to measure IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFNγ, and TNFα. Supernatants were collected and the assay procedures were performed according to the manufacturer's instructions. Data was collected and expressed as the mean cytokine response minus background (pg/ml) of each treatment from 4 replicate wells, plus or minus the standard error of the mean.

2.6. Flow cytometry assay for cell surface markers

Following stimulation of differentiated U937 cells with probiotics, cells were centrifuged and 5×10^5 cells were incubated with Fc block (BD Life Sciences) for 45 minutes on ice. After washing, cells were labelled with cell surface marker antibodies (Biolegend and BD Life Sciences) linked to fluorochrome and incubated on ice for 45 minutes. The antibodies were diluted in PBS/FBS at the following dilutions according to the manufacturers recommendations (CD11b-PE 1:400; CD83-Alexafluor488 1:400; CD14-BV421 1:200; CD40, CD80, CD83, CD86Alexafluor 488 1:400; CD16-PE 1:400; CD206, CD209-PE/Cy7 1:200; MHCI, MHCII-BV510 1:200). Samples were analyzed using a BD fluorescence activated cell sorter (FACS) Canto II. Data was acquired using Cell Quest program (BD Life Sciences), and analysis performed using FACS Diva software (BD Life Sciences) for percentage of expressed markers; isotype antibody controls (Biolegend and BD Life Sciences) were used as background quadrants set up.

2.7. Statistics

Significant differences between all treatment groups were tested by analysis of variance (ANOVA) followed by a comparison between treatments performed by Fisher's least significant difference (LSD) method, with a level of significance of $p < 0.05$.

3. Results and Discussion

3.1. S. thermophilus bacterial strains activate monocytes necessary for the innate immune response

The innate immune system is the first line of defence against invading pathogens which react quickly and non-specifically. Following this non-specific encounter cytokines (such as, IL-1β, IL-6, TNFα and IFNγ) and chemokines are secreted by innate cells (monocytes, macrophages, dendritic cells, NK cells, granulocytes) which play an important role in the innate immune response. This results in inflammation at the site of infection to aid in pathogen clearance (Parihar, Eubank, & Doseff, 2010). IL-1β, IL-6, TNFα and IFNγ are pro-infammatory cytokines which also aid to recruit and activate T and B cells to mount an adaptive immune response {Lacy, 2011 #104}. Secretion of IL-1β by monocytes is involved in regulating immune and inflammatory responses to infections and injury, hence its role in innate immunity (Lopez-Castejon & Brough, 2011). *S. thermophilus* ST1342 stimulated high levels of IL-1β ($p < 0.001$), whereas, ST1275 ($p < 0.05$) and ST285 ($p < 0.07$) did not induce IL-1β cytokine by differentiated U937 cells (Figure 1). IL-6 regulates both innate and adaptive immune responses and is secreted by monocytes to stimulate immune responses during infection (Jones, 2005). TNFα is a pro-inflammatory cytokine and a main trigger of the inflammatory response by causing vasodilation and vascular permeability allowing the influx of immune cells to the site of infection (Matsuki & Duling, 2000). High levels of TNFα was secreted by monocytes in the presence of ST1342, ST1275 and ST285 ($p < 0.001$) (Figure 1). It has been shown that IL-1β, LPS and TNFα induce IL-6 production by monocytes, and IL-6 is required for resistance against bacteria (Tosato & Jones, 1990). A trend towards increased levels of IL-6 was noted, although this was not significant for all probiotic strains ST1342, ST1275 and ST285 (Figure 1). In addition, all three ST1342, ST1275 and ST285 strains activated high levels of IFNγ secretion (Figure 1); a pro-inflammatory cytokine that is crucial in both innate and adaptive immune responses and has both anti-bacterial and anti-viral properties. It is clear that ST1342, ST1275 and ST285 activate cytokine secretion by monocytes, required for activation of the innate immune response and responsible for pathogen elimination. Similarly, it was noted that the probiotic *L. paracasei* DG commonly used in commercial probiotic products, has been shown to have immunostimulatory properties by increasing expression of IL-6, TNFα and CCL20 in the human monocyte cell line, THP-1 (Balzaretti et al., 2017).

3.2. S. thermophilus bacterial strains activates CXCL8 and GM-CSF: role in chemotaxis and recruitment of cells at sites of inflammation

IL-8 (also known as chemokine CXCL8) is an important cytokine of the innate immune system. IL-8 induces chemotaxis of neutrophils and other granulocytes toward the site of infection and it is a key mediator associated with inflammation; it also induces phagocytosis at the site of infection (Baggiolini & Clark-Lewis, 1992). The probiotic *L. paracasei* DG has been shown to increase expression of IL-8 in the human monocyte cell line, THP-1 (Balzaretti et al., 2017). In addition, short chain fatty acids, produced by probiotic bacteria, also stimulate IL-8 secretion and mRNA levels in the human epithelial cell line HT-29 (Asarat, Vasiljevic, et al., 2015). Likewise, ST1342 ($p < 0.005$), ST1275 ($p < 0.07$) and ST285 ($p < 0.001$) activated monocytes to secrete high levels of IL-8 compared to non-stimulated cells (Figure 2). GM-CSF stimulates the production of white blood cells, in particular, it rapidly increases macrophages *in vivo*, important cells necessary for fighting infections. It also enhances the anti-bacterial activity of monocytes and modulates macrophage/dendritic cell phenotypes; as such, molecular targeting of the GM-CSF pathway has recently been developed to treat a number of autoimmune disorders (Ushach & Zlotnik, 2016). Of interest, ST1275 and ST285 induced monocytes to secrete high levels of GM-CSF ($p < 0.001$) while, conversely, ST1342 stimulated lower levels of GM-CSF ($p < 0.001$) (Figure 2).

3.3. S. thermophilus bacterial strains activate anti-inflammatory cytokines

IL-4 is an anti-inflammatory cytokine which differentiates naïve $CD4^+$ Th0 cells to Th2 cells. IL-4 stimulates B cells and T cells and is a key regulator of humoral and adaptive immune responses at sites of injury. IL-4 promotes M2 anti-inflammatory macrophages and inhibits classical M1 pro-inflammatory macrophages. IL-4 together with IL-10 are important at sites of injury or infection by inhibiting bacterial mediated induction of pro-inflammatory cytokines. In addition, IL-4 and IL-10 are important cytokines required for anti-inflammatory responses against inflammatory diseases such as, autoimmunity and allergies (Mitchell et al., 2017). The probiotic *Bifidobacterium (B) breve* but not *Lactobacillus (L) casei* has been shown to induce IL-10 producing intestinal Treg cells as well as intestinal $CD103^+$ IL-10/IL-27 secreting DCs in mice (Jeon et al., 2012). Oral *B. breve* administration ameliorates colitis in mice but not in IL-10 knockout mice, demonstrating preventive effect of *B. breve* on colonic inflammation

(Jeon et al., 2012). Likewise, *L. reuteri* and *L. lactis* strains given in mice orally stimulates anti-inflammatory IL-10 and Treg cells (Levkovich et al., 2013; Souza et al., 2016). Furthermore, co-culturing PBMC with selected bacteria (LAVRI-A1, *L. rhamnosus* GG, *Bifidobacteria* and *L. acidophilus*) induce anti-inflammatory cytokines IL-4, IL-10 and TGF-beta (Donkor et al., 2012b; Donkor, Shah, Apostolopoulos, & Vasiljevic, 2010). These cytokines inhibit the production of IL-12, IFNγ and other pro-inflammatory cytokines which are beneficial for autoimmune and allergic responses. Here we show that, ST1342 stimulated IL-4 production by monocytes ($p < 0.001$) and to a lesser degree ST1275 ($p < 0.07$) and ST285 ($p < 0.005$), (Figure 3). Similarly, IL-10 was secreted by monocytes in the presence of ST1342, ST1275 and ST285 ($p < 0.001$), with ST1275 and ST285 stimulating higher levels (Figure 3). It is clear that ST probiotic bacteria have potential anti-inflammatory properties which could have positive implications in chronic inflammatory diseases (autoimmunity and allergies) and warrant further investigation.

3.4. S. thermophilus bacterial strains upregulate the expression of cell surface markers on differentiated U937 cells; role in initiating innate and adaptive immune responses

Monocytes are major constituent cells of the innate immune system, which also play a role in the adaptive immune response. The expression of cell surface markers on monocytes is crucial in the ensuing immune responses. The specific markers presented on monocytes is dependent on their environment and their exposure to pathogens and/or pathogenic peptides and pathogen derived metabolites; with these factors causing alterations in the profile of monocyte markers, accordingly (Ziegler-Heitbrock, 2015). The human pro-monocytic histiocytic lymphoma cell line, U937 cells, are commonly used to study the behavior and differentiation of monocytes. They exhibit pro-monocytic characteristics by displaying monoblast morphology, produce lysozymes and have esterase activity (dos Santos et al., 2009; Sundstrom & Nilsson, 1976). They are not phagocytic, they express low levels of CD14, CD54, CD86, and major histocompatibility complex (MHC)-class II is not detectable (Azam et al., 2006). However, upon stimulation with viral or bacterial fragments, or, vitamin D_3, they express markers demonstrating monocyte/macrophage morphology, with increased expression of CD14 (dos Santos et al., 2009; Koss, Lucero, & Koziner, 1996; Santegoets, Van Den Eertwegh, Van De Loosdrecht, Scheper, & De Gruijl, 2008).

Our data shows that U937 cells incubated with ST1342, ST1275 or ST285 results in enhanced expression of CD14, CD11c, CD86, CD206, CD209 and MHC1 cell surface markers at varying levels; CD11b, CD16, CD40, CD80 and CD83 were also up regulated, albeit at a much lower level (Table 1). In other studies, the combination of 3 probiotics (*L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. bifidum*) stimulated increased expression of cell surface markers, CD14, MHC class II and CD80 (Gutkowski et al., 2010).

CD14 is expressed on the surface of monocytes and macrophages and primarily binds to bacterial LPS; although other bacterial cell wall constituents also bind to CD14 such as, lipid A, *Staphylococcus aureus, Escherichia coli* and lipoteichoic acid (Bron, Tomita, Mercenier, & Kleerebezem, 2013; Lee, Tomita, Kleerebezem, & Bron, 2013; van Baarlen, Wells, & Kleerebezem, 2013). The interaction between CD14 and its ligands initiates the innate immune response (Bedell et al., 2018), as well as further up regulating its expression (CD14 expression) (Landmann et al., 1996). Indeed, ST1342, ST1275 and ST285 up regulated CD14 expression on U937 cells after 24 and 48 hours incubation, with ST285 being the most significant at 48 hours (Table 1).

CD11c is a type I transmembrane protein expressed by DCs, monocytes, macrophages and neutrophils (Dyer, Garcia-Crespo, Killoran, & Rosenberg, 2011). The presence of CD11c on these cells allows their adherence to endothelial cells, phagocytosis of complement positive cells (important for innate immune defence) and activates cellular immune responses. Selected strains of *Lactobacillus* (*L. reuteri*, *L. plantarum* Lb1 and *L. fermentum*) cultured with murine bone marrow cells and GM-CSF, induce high levels (85-90 %) of $CD11c^{+}$ cells (Christensen, Frøkiær, & Pestka, 2002). Basal expression levels of CD11c on U937 cells was 26-27%, which almost doubled following LPS (48-49%) and ST1342 (48-50%) stimulation; significant up regulation was also noted with ST1275 (37-43%) and ST285 (43-46%) after 24 or 48 hours respectively (Table 1). Interestingly, there were no major differences in CD11c expression, whether cells were stimulated for 24 or 48 hours.

CD86 (B7-2) expression on antigen presenting cells (DCs, macrophages, B cells) is involved in co-stimulatory signalling that is required for the priming and proliferation of T cells (Fleischer et al., 1996). Monocytes express low levels of CD86 which is up regulated following stimulation with IFN-gamma or other ligands. In fact we showed that expression of CD86 increased significantly from 8.6% to 33.4% (ST1342), 28.1% (ST1275) and 38% (ST285) after 24 hour co-culture, which was lower than that after LPS stimulation (46.3%) (Table 1). The up regulation of CD86 was transient and after 48 hours the levels decreased significantly. It is clear that *S. thermophilus* bacteria promote CD86 expression levels, required for T cell

activation and the maintenance of immune responses (Fleischer et al., 1996). Similarly, *L plantarum* WCFS1 and *L. fermentum* GR1485 have been shown to upregulate CD86 cell surface expression on monocytes, however, *L. rhamnosus* and *L. delbruekii* reduce cell surface expression of CD86 (Esmaeili et al., 2018).

CD206 (mannose receptor, MR) (Geurtsen et al., 2009), is primarily present on the surface of macrophages and immature DCs (Kerrigan & Brown, 2009), and functions to arrest antigens and pathogenic components, followed by processing and presentation to T cells (Engering et al., 2004). The MR recognizes mannose, fucose and N-acetylglucosamine residues (Kerrigan & Brown, 2009; Mitchell et al., 2017) commonly expressed on the surface of microorganisms (such as *Pneumocystis*, *Candida*, *Mycobacterium*, *Leishmania*), and capsular polysaccharides of *Streptococcus* and *Klebisella* (Geurtsen et al., 2009; Kerrigan & Brown, 2009; Zamze et al., 2002), which results in the destruction of bacteria (innate immune response) and activation of the adaptive immune response (cellular responses). Poly-mannose (mannan) linked to protein antigens as a model, targets the MR on DCs and macrophages resulting in stimulation of either pro- or anti-inflammatory responses, significant in a number of diseases from cancers to autoimmunity (Apostolopoulos, Barnes, Pietersz, & McKenzie, 2000; Apostolopoulos & McKenzie, 2001; Apostolopoulos, Pietersz, Gordon, Martinez-Pomares, & McKenzie, 2000; Apostolopoulos, Pietersz, Loveland, Sandrin, & McKenzie, 1995; Apostolopoulos, Pietersz, & McKenzie, 1996; Sheng et al., 2006). Here we show that U937 cells co-cultured with ST1342, ST1275 or ST285 up regulated the expression levels of CD206 within 24 hours (ST285 inducing the highest levels) which subsided by 48 hours, but did not reach basal level expression (Table 1, Figure 4). In addition, CD209 (DC-SIGN), a C-type lectin receptor expressed on the surface of macrophages and DCs also binds to mannose residues present on bacteria, viruses and fungi. The interaction between CD209 and mannose moieties activates phagocytosis as well as endocytosis for processing and presentation to T cells (Apostolopoulos et al., 2014; Cambi et al., 2003; Proudfoot, Apostolopoulos, & Pietersz, 2007; Sheng et al., 2008; Sheng, Pietersz, Wright, & Apostolopoulos, 2005). U937 cells cultured in the presence of ST strains also up regulated the expression of CD209 with maximal up regulation noted within 24 hours (Table 1); ST285 stimulation resulted in the highest up regulation at both 24 and 48 hours. Thus, *S. thermophilus* strains induce CD206 and CD209 expression, as a result have a positive role in activating both the innate and adaptive immune responses (Apostolopoulos et al., 2006; Apostolopoulos et al., 2014).

The major histocompatibility complex class I (MHC-I) is expressed by all nucleated cells and presents processed antigenic peptides on its surface to activate CD8+ T cells (Neefjes,

Jongsma, Paul, & Bakke, 2011). U937 cells express low levels of MHC-I which is up regulated within 24 hours in the presence of ST1342, ST1275 or ST285 and remains up regulated after 48 hours of stimulation (Table 1). Hence, *S. thermophilus* strains are beneficial in upregulating MHC-I molecules on monocyte/macrophage cells for enhanced CD8+ T cell stimulation, required for the elimination of tumour cells and viruses.

3.5. Conclusion

Activation of monocyte cells with *Streptococcus thermophilus* such as *S. thermophilus* ST1342, *S. thermophilus* ST1275 and *S. thermophilus* ST285 strains, and secretion of IL-1β, IL-6, TNFα and IFN-γ suggests their role in the subsequent activation of the immune responses aiding in the elimination of pathogens. In addition, *S. thermophilus* strains, up regulated the secretion of IL-8, a chemokine involved in chemotaxis and phagocytosis, as well as up regulating the secretion of GM-CSF, a major cytokine for increasing the number of macrophages at the site of infection. Clearly, *S. thermophilus* strains up regulated cytokine levels by monocytes, required for activation of the innate immune response. Furthermore, the activation of anti-inflammatory cytokines (IL-4 and IL-10) could be beneficial in modulating chronic inflammatory conditions and allergies. Moreover, *S. thermophilus* strains up regulated monocyte cell surface markers, CD14, CD11c, CD86, CD206, CD209 and MHC-I suggestive of their potential benefit to activate innate and adaptive immune responses. These findings support a role for these probiotic strains in the healthy modulation of monocyte activity and their roles in innate and cellular immunity. The results also present a potential role for these strains in modulating the inflammatory response, which warrants further investigation. Overall, these findings are in agreement with the body of research that supports the role that the regular consumption of probiotics (including *S. thermophilus*) has in the establishment and maintenance of a healthy immune system and opens pathways to further determine the mechanisms by which these strains modulate immune responses.

Acknowledgements

VA was supported by the Centre for Chronic Disease and the Institute for Health and Sport, Victoria University and would like to thank the College of Health and Biomedicine start-up funds at Victoria University, Australia.

Conflict of interest

The authors declare no conflicts of interest.

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Table 1
Proportion (%) of cell surface marker expression shown, as analyzed by flow cytometry at 24 and 48 hours of stimulation of U937 cells with *S. thermophilus* strains

	Control		**LPS**		**ST1342**		**ST1275**		**ST285**	
	24	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
CD11b	6.1	4.1	13.9	9.1	12.1	8.6	12	7.5	11.8	21
CD11c	27.3	26.2	48	49	50	48	37	43	43	47
CD14	6.6	4	13.5	19	15.6	21	19.1	16	19.8	35
CD16	3	4	7.1	6.5	7.5	6	8.9	4	9.1	12
CD40	1.6	4	6	6	6.2	5.8	8.5	5	6	13
CD80	4	4	5.5	5.5	7.5	5.7	7.5	5.2	5.5	11
CD83	1.7	4	7	6.5	6.8	4.7	13	5	7.1	10
CD86	8.6	4.5	46.3	16	33.4	13	29.8	12.5	38	16.5
CD206	17	7	40.9	30	38.5	30	36.4	34	47.8	34.5
CD209	4	4.5	37.1	20	38.7	18.8	30	16.8	39	31
MHCI	4.2	10	18.7	23	18.9	24	20.8	22.5	22.7	24

Figure Legends

Fig. 1. ***S. thermophilus*** **bacterial strains activate monocytes necessary for the innate immune response.** U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) - ST1342, ST1275 or ST285 for 24 hours and secretion of IL-1β, IL-6, TNFα and IFNγ were measured. LPS was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$ and *** $p < 0.001$.

Fig. 2. ***S. thermophilus*** **bacterial strains activate CXCL8 and GM-CSF essential for recruitment of cells at sites of inflammation**. U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) - ST1342, ST1275 or ST285 for 24 hours and secretion of IL-8 and GM-CSF were measured. LPS was was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Significant differences between treatments were tested by analysis of variance (ANOVA). Symbols represent *p* value for Tukey Test (One way ANOVA) where # $p < 0.07$, ** $p < 0.005$ and *** $p < 0.001$.

Fig. 3. ***S. thermophilus*** **bacterial strains activate anti-inflammatory cytokines.** U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) - ST1342, ST1275 or ST285 for 24 hours and secretion of IL-8 and GM-CSF were measured. LPS was was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Symbols represent *p* value for Tukey Test (One way ANOVA) where # $p < 0.07$, ** $p < 0.005$ and *** $p < 0.001$.

Fig. 4. ***S. thermophilus*** **(ST) bacterial strains increase cell surface marker expression.** U937 cells were differentiated into monocytes and stimlated with ST1342, ST1275 or ST285 for 24 or 48 hours and cell surface marker expression assessed. Upregulation of CD14, CD11c, CD86, CD206, CD209 and MHC class I are shown at 24 hours for ST285. LPS was was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control).

Figure 1

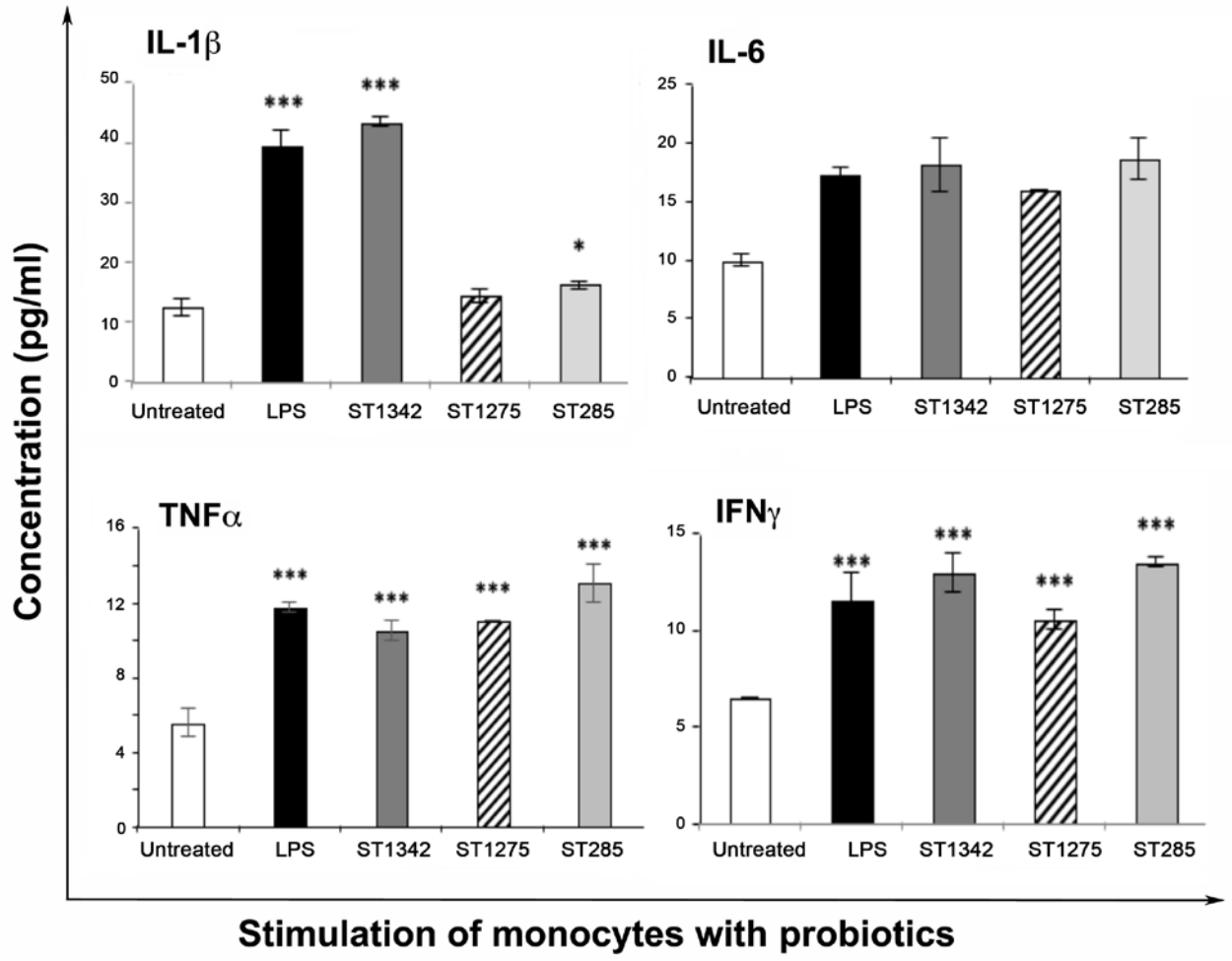

IL-1β
IL-6
TNFα
IFNγ
Concentration (pg/ml)
Untreated
LPS
ST1342
ST1275
ST285
Stimulation of monocytes with probiotics

Figure 2

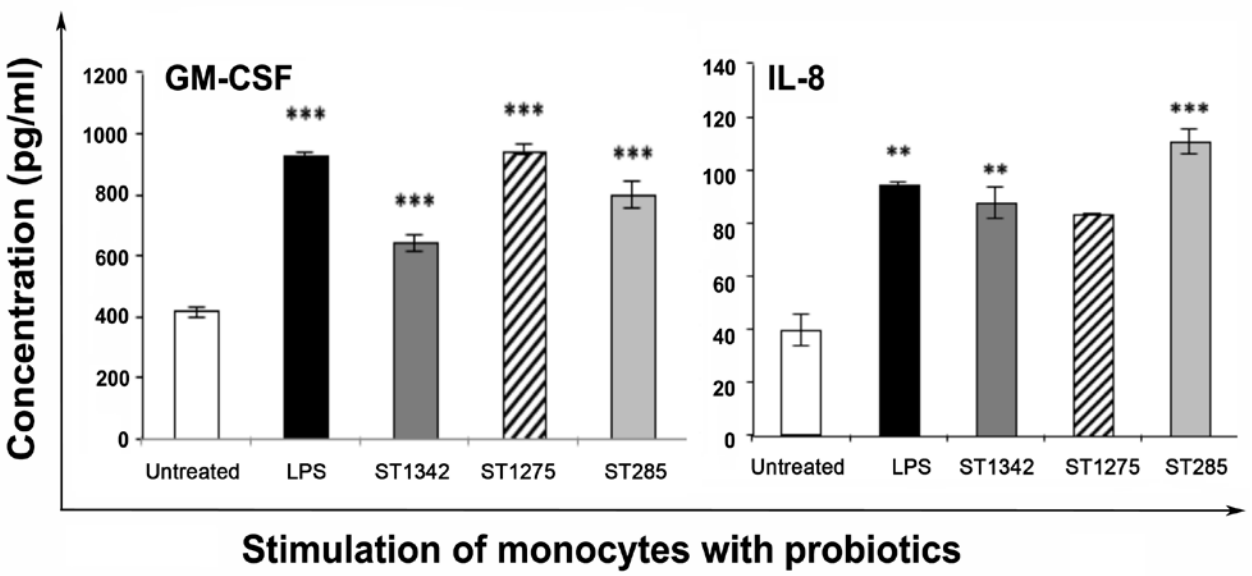


Figure 3

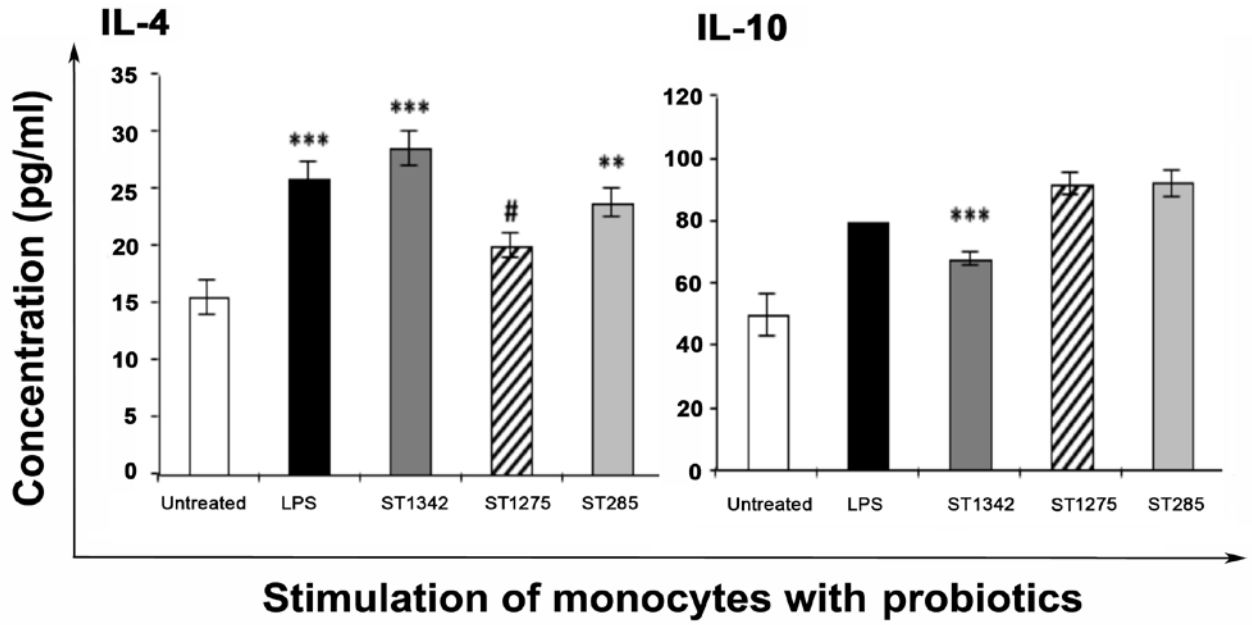
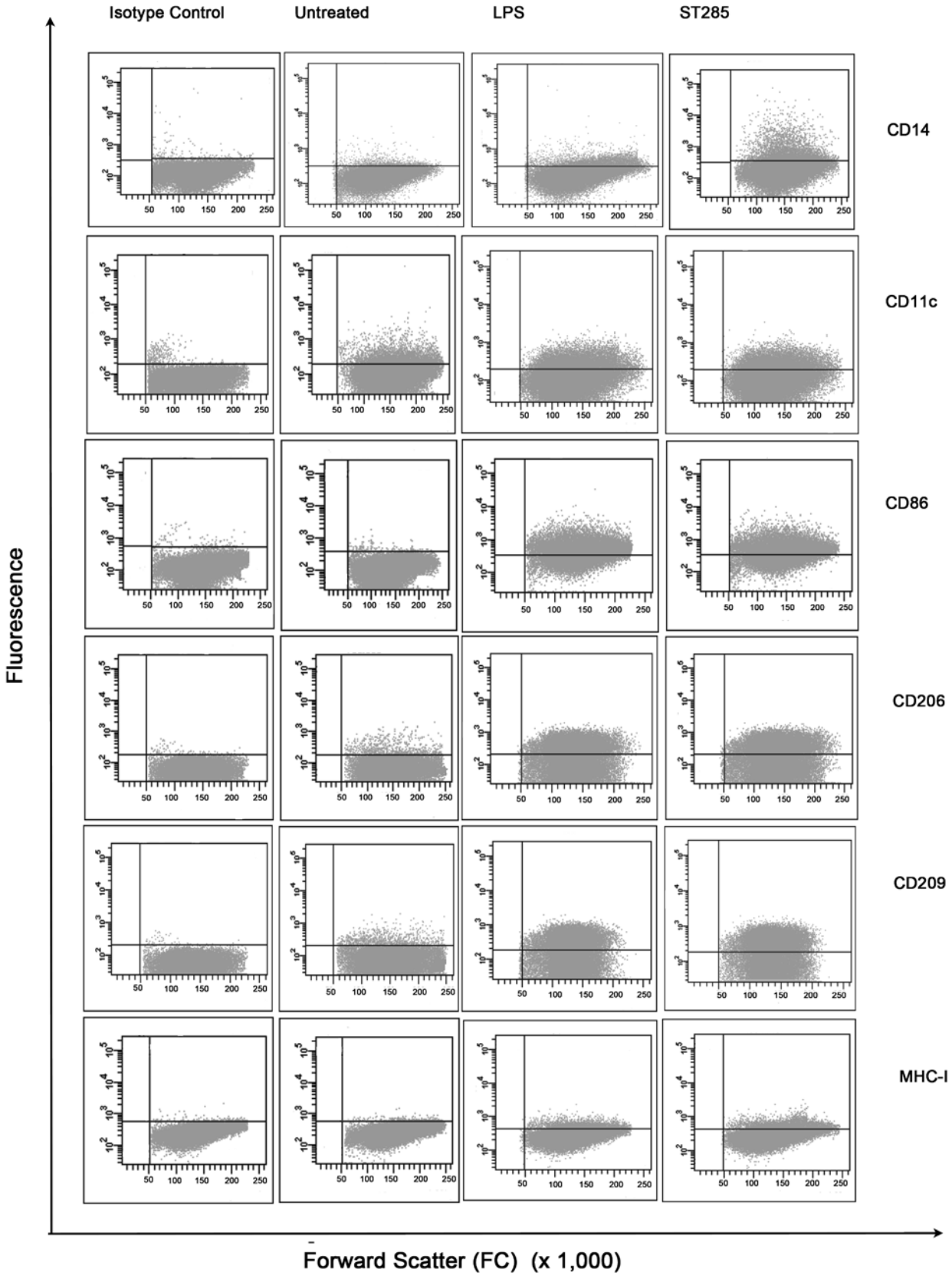


Figure 4


Isotype Control
Untreated
LPS
ST285
CD14
CD11c
CD86
CD206
CD209
MHC-I
Fluorescence
Forward Scatter (FC) (x 1,000)