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Novel Dairy Fermentates Have Differential Effects on Key Immune Responses Associated with Viral Immunity and Inflammation

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Abstract: Fermented foods and ingredients, including fermentates derived from lactic acid bacteria (LAB) in dairy products, can modulate the immune system. Here we describe the use of reconstituted skimmed milk powder to generate novel fermentates from *Lactobacillus helveticus* strains SC232, SC234, SC212, and SC210, and from *Lacticaseibacillus casei* strains SC209 and SC229, and demonstrate using *in vitro* assays that these fermentates can differentially modulate cytokine secretion by bone marrow derived dendritic cells (BMDCs) when activated with either the viral ligand, loxoribine, or an inflammatory stimulus, lipopolysaccharide. Specifically, we demonstrate that SC232 and SC234 increase cytokines IL-6, TNF-α, IL-12p40, IL-23, IL-27 and IL-10, and decreased IL-1β in primary bone marrow derived dendritic cell (BMDCs) stimulated with a viral ligand. In contrast exposure of these cells to SC212 and SC210 resulted in increased IL-10, IL-1β, IL-23, and decreased IL-12p40 following activation of the cells with the inflammatory stimulus LPS. Interestingly SC209 and SC229 had little or no effect on cytokine secretion by BMDCs. Overall, our data demonstrates that these novel fermentates have specific effects and can differentially enhance key immune mechanisms that are critical to viral immune responses, or can suppress responses involved in chronic inflammatory conditions, such as Ulcerative Colitis (UC), and Crohn's disease (CD).

Keywords: fermentates; functional food; immune boosting; immunomodulation; anti-inflammatory; viral immunity; chronic inflammation

1. Introduction

In recent years there has been a clear shift in the interest of consumers in food for health, with people more than ever before being aware of the ancient saying "Let food be thy medicine and medicine be thy food". Food can be a critical contributor to a healthy, disease-free, quality of life. The corollary is also true in that global dietary risk factors are estimated to cause 11 million deaths and 255 million disability-adjusted life years annually [1]. It is a long known fact that what we eat influences our body, and vital nutrients are essential for growth, cellular function, tissue

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development, energy and immune defence [2]. There is growing evidence of the role of specific foods and food components as immunomodulators, improving immune defence function and increasing resistance to infection, while maintaining tolerance [3]. Furthermore, deficiencies in key nutrients can result in the development of disease, putting an individual at greater risk of disease development or susceptibility to viral infections.

The term "fermentates" generally refers to "a powdered preparation, derived from a fermented [food] product and which can contain the fermenting microorganisms, components of these microorganisms, culture supernatants, fermented substrates, and a range of metabolites and bioactive components" [4]. Our previous work outlined the potential of fermentates as immune boosting functional food ingredients for the support of the immune system in the defence against viral invasion in macrophage models (Finnegan et. al.,Submitted). Furthermore, there is growing popularity within the industry and increased demand for functional food components as natural immune fitness boosters [5]. This demonstrates the commercial desire for functional food ingredients, such as fermentates, for boosting of the immune system and support of immune fitness. Indeed with the recent increases of viral outbreaks including SARS-CoV-2 virus, Monkeypox virus, the Langya virus, as well as the yearly outbreaks of seasonal influenza, there is heightened global interest in maintaining and enhancing viral immunity [5](Finnegan, 2024 Submitted). This study is one of the first to explore the effects of novel fermentates from *Lb. helveticus* SC234, SC232, SC212, and SC210, and *Lacticaseibacillus casei* SC229 and SC209 on immune mechanisms that are critical to viral immunity, as well as those critical to anti-inflammatory immune responses.

The discovery of novel anti-inflammatory food ingredients is important given that the modern diet consists of greater meat and animal product consumption, and observational studies have linked such dietary patterns to the risk and development of inflammatory bowel diseases (IBD), such as Crohn's Disease (CD) and Ulcerative Colitis (UC) [6]. These chronic digestive diseases affect over 10 million people worldwide and have no known cause or cure (EFCCA, 2022). IBDs are issues within the body associated with gut-associated symptoms as a result of immunological imbalances within the intestinal mucosa associated with cells of the adaptive immune system [8]. IBDs arise as a result of the immune system responding inappropriately and triggering chronic inflammation.. Dietary approaches to mitigate the effects of IBDs include enteral nutrition in the form of hydrolysed or intact protein formulas; however there is much interest in identifying more palatable and easier to use dietary approaches [6]. Otherwise, nonsurgical strategies involve medications to control symptoms, including aminosalicylates, antibiotics, biologics and corticosteroids, which often have significant side effects [9,10]. The prevalence of IBDs is on the increase [11,12] and thus finding a functional food, that could aid in the control and management of such a prevalent and debilitating disease, in a natural way, would be of huge medical significance.

In this study we examine the effects of a range of fermentates (SC232, SC234, SC212, SC210, SC209 and SC229) on dendritic cell function in the context of viral immunity as well as chronic inflammation, such as that associated with inflammatory bowel diseases.

We used murine dendritic cells challenged with either a viral immune stimulus Loxoribine (LOX), or inflammatory immune stimulus lipopolysaccharide (LPS; *Escherichia coli* 055:B5). LOX is a TLR2 ligand and mimics the immune response triggered by a viral infection, while LPS is a TLR4 ligand and mimics the immune response seen in chronic inflammation, in diseases such as UC, and CD. The effects of these fermentates on cell viability, and cytokine secretion for quantification of interleukin (IL)-1β, IL-6, IL-10, tumour necrosis factor (TNF)-α, IL-12p40, IL-23, and IL-27, were investigated in toll like receptor ligand (TLR) activated dendritic cells, in order to establish the specificity or differential effects of these fermentates on these cells. We demonstrate that fermentates SC232 and SC234 significantly increased the secretion of cytokines IL-6, TNF-α, IL-12p40, and IL-23 as well as causing smaller increases in IL-10; SC212 and SC210 increased IL-10, and decreased IL-1β, and IL-12p40; while SC209, and SC229 had no significant effect on cytokine secretion in bone marrow derived dendritic cells (BMDCs). These data clearly show the differential effects of these novel fermentates on cyokine production by these cells.

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2. Materials and Methods

2.1. Generation of Reconstituted Skim Milk (RSM)-Based Fermentates

Skim Milk Powder (SMP) was used as a substrate for the generation of the fermentates used in this study. SMP was reconstituted at 10% w/v in distilled water, autoclaved, cooled and stored at 4°C for a maximum of 7 days. Working cultures were prepared for each strain from the respective frozen mother culture stocks of strains *Lb. helveticus* SC232, *Lb. helveticus* SC234, *Lacticaseibacillus casei* SC229, *Lb. casei* SC209, *Lb. helveticus* SC210 and *Lb. helveticus* SC212 (all previously prepared in 10% w/v RSM), and incubating for 24hrs at 37°C under aerobic conditions without agitation. From these cultures, a further inoculum was added to 10% w/v RSM and incubated for 24hrs at 37°C under aerobic conditions again without agitation. Fermentates were then heat-treated to inactivate the respective LAB strain. After cooling to room temperature, the pH of the fermentates was neutralized. These fermentates were aliquoted and immediately frozen at -80°C until further analysis. Non-fermented RSM samples subjected to the same heat-treatment mentioned above were used as negative controls for all experiments described herein. The codes SC232, SC229, SC209, SC210, SC212 and SC234 will hereafter be used when referring to the individual fermentates produced.

2.2. Cell Culture

Bone Marrow Derived Dendritic cells (BMDCs), harvested from the bone marrow of a female BALB/c mouse of 6-8 weeks old obtained from Charles River (Margate, UK), were cultured in complete medium RPMI-1640 (Biosciences, Ireland), containing 5ng/ml GM-CSF (Merck, Haverhill, UK), and supplemented with 10% FCS, and 1% Pen-Strep. Cells were cultured for 7 days at 37°C, with 5% CO_2 and 95% humidified air. Cell seeding for experimentation was carried out at concentration of $1x10^6$cells/mL. Cells were left overnight to settle before proceeding with experimentation.

2.3. Cell Viability

Cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay and conducted as per manufacturer's instructions (MyBio, Kilkenny, Ireland). Macrophage were seeded at a concentration of 1 x 10^6 cells/mL in a flat bottom 96-well plate, and incubated for 24 hours at 37°C in a 95% humidified air, and 5% CO_2 atmosphere. Cells were treated with 25mg/mL of the fermentate for 1 hour and incubated under the same conditions, before stimulation with LOX 0.5mM and, LPS 100ng/mL for 24 hours. Dimethylsulfoxide (DMSO) was included as a positive control to induce cell death. After 24 hours, 20µl of the thawed CellTiter96® Aqueous One Solution was added to each well of the 96-well plate, incubated at 37°C for 3 hours in a humidified, 5% CO_2 atmosphere. Absorbance was read at 490 nm using Versamax™ 96-well plate reader. Cell viability was expressed as the percentage viability of the treatment group relative to the control group.

2.4. Enzyme Linked ImmunoSorbent Assay (ELISA)

Determination of the effects of the fermentate samples on cytokine production in activated dendritic cells required harvesting of the cell supernatants, and subsequent analysis using commercial DuoSet ELISA kits (R&D Systems Europe, Abdingdon, Oxon, UK) according to the manufacturer's instructions. This allowed for the quantification of the cytokines IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-23 and IL-27.

2.5. Metabolomics

Metabolomic analysis of the fermentate samples was performed using a nuclear magnetic resonance (^{1}H-NMR) spectroscopy approach (N=54). Following centrifugation at 13500 x g for 15 minutes at 4°C, the samples were filtered using 3kDa ultra centrifugal filters and sample filtrate was frozen at -80°C until further analysis (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). On the day of analysis, the resulting filtrate was combined with 10µL sodium trimethyl silyl [2,2,3,3-2H4]

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propionate (TSP) and 200µL deuterium oxide (D_2O). Spectra were acquired with a 600 MHz Varian Spectrometer (Varian Limited, Oxford, United Kingdom). Spectra were acquired using 16,384 complex points and 256 scans. All ^{1}H-NMR spectra were referenced to TSP at 0.0 parts per million (ppm) and processed manually with the Chenomx NMR Suite (version 7.7) using a line broadening of 0.2 Hz, followed by phase and baseline correction. In total, 45 metabolites were identified based on the Chenomx 600 MHz Library and the Human Metabolome Database (HMBD).

2.6. Statistical Analysis

Statistical analysis was carried out using a one-way ANOVA to compare variance among the means of different sample groups. A Newman-Keuls post-hoc test was used to determine significance among the samples. The level of statistical significance was indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). Statistical testing for significant differences were carried out (n=3) with respect to non-fermented RSM samples, which were used as negative controls for all experiments described herein.

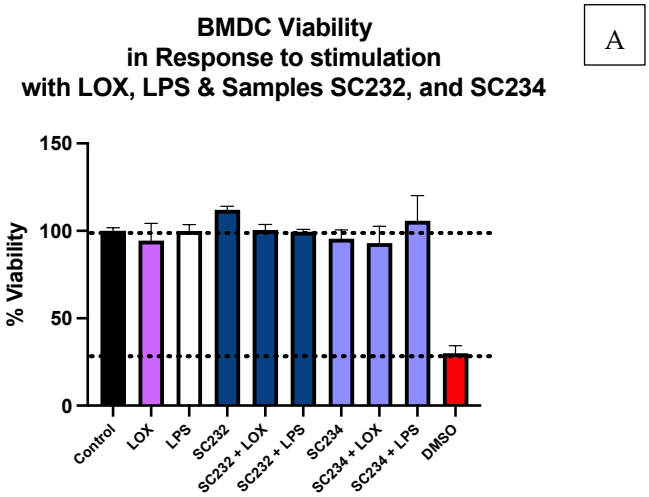
2.7. Ethical Statement

The care, treatment, and experiments involved in this study were approved by the Research Ethics Committee (REC) of Dublin City University (Approval ID: DCUREC/2023/187).

3. Results

3.1. Cell Viability Is Not Affected by the Presence of Fermentate Samples

An MTS assay was performed to determine whether fermentates SC232, SC234, SC212, SC210, SC209, or SC229, in the presence/absence of LOX or LPS, had any significant effect on BMDC cell viability. Figure 1 demonstrates that there was no significant change in cell viability following exposure of BMDCs, to LOX or LPS, in the presence of SC232 or SC234, (Figure 1A), SC212 or SC210, (Figure 1B) or SC209 or SC229 (Figure 1C). The use of the positive control of 10% DMSO provides a clear demonstration of cytotoxic effects on cell viability.



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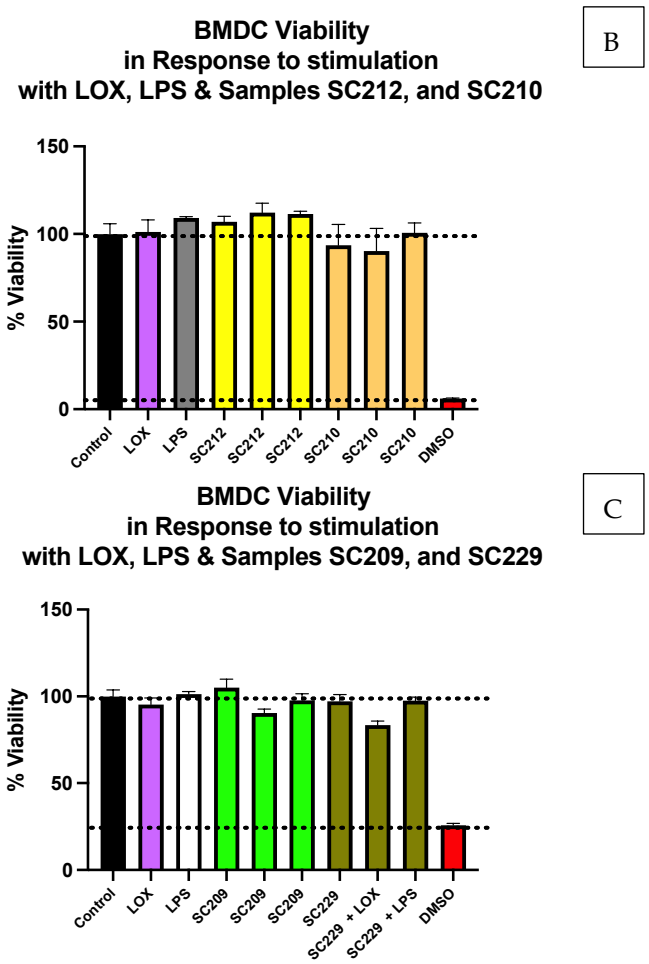

Figure 1. Exposure of LOX, and LPS activated BMDCs to 25mg/ml fermentates does not affect cell viability. BMDC cells were seeded at 1x10^6 cells/mL and incubated overnight at 37°C in 5% CO_2. The following day cells were stimulated with 25mg/ml fermentates as follows: SC212 and SC210; SC232 and SC234; or null SC209 and SC229. These were incubated for 1 hour at 37°C in 5% CO_2 and subsequently exposed to LOX 0.5mM and LPS 100ng/mL before incubating overnight under the same conditions. After 24 hours cells were incubated with MTS cell titre aqueous one solution for 3 hours at 37°C in 5% CO_2. Viability is represented as a percentage, comparing sample to viable untreated control cells. Data is presented as mean ± SEM of three replicates.

3.2. Effects of Fermentates on Cytokine Secretion

An ELISA was then performed in accordance with manufacturer's instructions in order to assess the effects of our novel fermentates in primary dendritic cells in the absence/presence of LOX or LPS.

Figure 2 shows that our novel fermentates SC232 and SC234 significantly affect the secretion of cytokines in response to immune challenges with LOX and LPS when compared to the respective control/TLRs in BMDCs. IL-1β ($p<0.001$), IL-6 ($p<0.001$), IL-12p40 ($p<0.002$), and TNF-α ($p<0.033$) were significantly increased in the presence of LOX, with only low levels of IL-10, IL-23, and IL-27, and no IL-12p70 detectable. IL-6, IL-12p40, and IL-27 were significantly increased in the presence of LPS ($p<0.001$), as well as TNF-α ($p<0.002$), with only low levels of IL-1β, IL-10, and IL-23, and similarly no IL-12p70. Concentrations of IL-12p70 secreted were so low it was not considered statistically significant.

In the presence of LOX, SC232 increased IL-6 ($p<0.001$), TNF-α ($p<0.001$), IL-12p40 ($p<0.001$), IL-27 ($p<0.001$), IL-10 ($p<0.002$), and IL-23 ($p<0.002$). In contrast, SC234 increased IL-10 ($p<0.001$), TNF-α ($p<0.001$), and IL-6 ($p<0.002$), but decreased IL-1β ($p<0.001$) in the presence of LOX. In the presence of LPS, SC232 increased TNF-α ($p<0.001$), but decreased IL-12p40 ($p<0.002$). SC234 increased IL-10 ($p<0.001$), TNF-α ($p<0.001$), and IL-6 ($p<0.002$), but decreased IL-12p40 ($p<0.002$), and IL-27 ($p<0.002$)

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in the presence of LPS. Interestingly in the absence of TLR stimulation SC234 increased IL-10 ($p<0.033$), TNF-α ($p<0.033$), and IL-6 ($p<0.002$).

Figure 3 demonstrates that fermentates SC212, and SC210 significantly affect the secretion of cytokines in response to immune challenges with LOX and LPS, when compared to the respective controls/TLRs in BMDCs.

In the presence of LOX, SC212 increased IL-10 ($p<0.001$) and IL-6 ($p<0.002$), but decreased IL-1β ($p<0.001$) and IL-12p40 ($p<0.001$), with no significant effects on the other cytokines measured when compared to the control cells. SC210 increased IL-10 ($p<0.001$) and IL-6 ($p<0.033$), but decreased IL-1β ($p<0.001$) and IL-12p40 ($p<0.001$) in the presence of LOX with no significant effects on the other cytokines. In the presence of LPS, SC212 increased IL-10 ($p<0.001$) and IL-23 ($p<0.001$), but decreased IL-12p40 ($p<0.033$). SC210 increased IL-1β ($p<0.002$), IL-10 ($p<0.001$), and IL-23 ($p<0.001$) in the presence of LPS. Interestingly, in the absence of TLR stimulation, SC212 alone increased IL-1β ($p<0.002$), IL-6 ($p<0.001$), IL-10 ($p<0.001$), TNF-α ($p<0.001$), IL-23 ($p<0.002$), and IL-27 ($p<0.001$). Similarly, SC210 in the absence of TLR stimulation increased IL-1β ($p<0.002$), IL-6 ($p<0.001$), IL-10 ($p<0.001$), TNF-α ($p<0.001$), IL-23 ($p<0.001$), and IL-27 ($p<0.001$), but in addition also increased IL-12p40 ($p<0.033$).

Figure 4 demonstrates that fermentates SC209, and SC229 significantly affect the secretion of cytokines in response to immune challenges LOX and LPS when compared to the respective controls/TLRs in BMDCs.

In the presence of LOX, SC209 increased IL-12p40 ($p<0.001$), IL-6 ($p<0.002$), and IL-27 ($p<0.002$), but decreased IL-1β ($p<0.001$). SC229 increased IL-12p40 ($p<0.001$), and IL-6 ($p<0.002$) in the presence of LOX. In the presence of LPS, SC209 increased IL-1β ($p<0.002$). In contrast, SC229 increased IL-12p40 ($p<0.001$) in the presence of LPS but has no significant effect on other cytokines. Interestingly in the absence of TLR stimulation SC229 increased IL-1β ($p<0.001$), TNF-α ($p<0.002$), and IL-12p40 ($p<0.033$). In contrast, SC209 has no significant effect on any cytokines secreted in the absence of TLR stimulation.

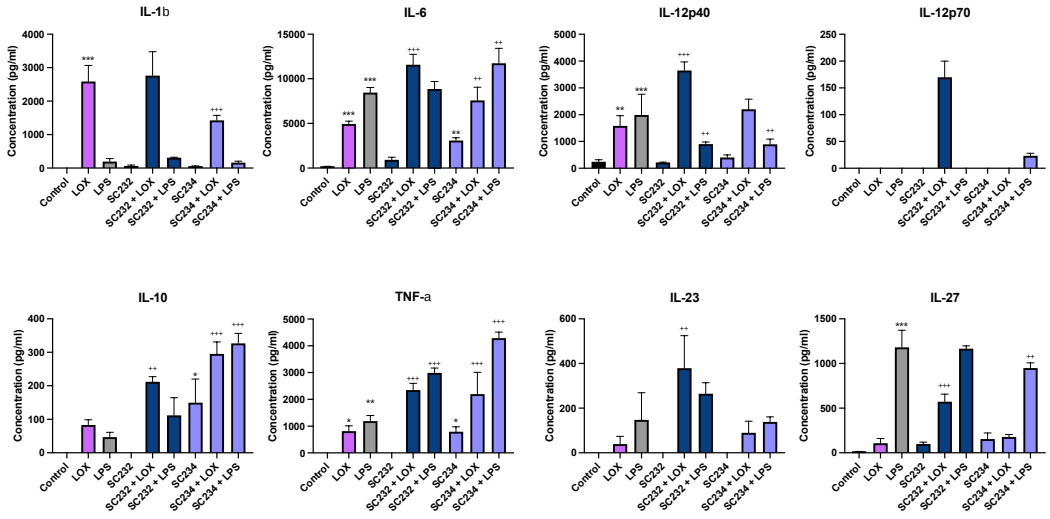

Figure 2. Exposure of LOX and LPS activated BMDCs to 25mg/ml fermentates results in the secretion of for IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-23, and IL-27. BMDC cells were seeded at $1x10^6$ cells/mL and incubated overnight at 37°C in 5% CO_2. The following day cells were stimulated with 25mg/ml fermentate, incubated for 1 hour at 37°C in 5% CO_2 and subsequently exposed to LOX 0.5mM and LPS 100ng/mL before incubating overnight under the same conditions. Supernatants were removed after 24 hours and ELISA was performed for IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-23, and IL-27. Data is presented as mean ± SEM of three replicates. Significance determined using one- way ANOVA with a Newman-Keuls post-test. Output P value style APA: .12 (ns), .033 (*), .002(**) and <.001 (***); 1) comparing control cells, to LOX and LPS, and unstimulated samples"*", 2) comparing TLR, to sample + TLR "+".

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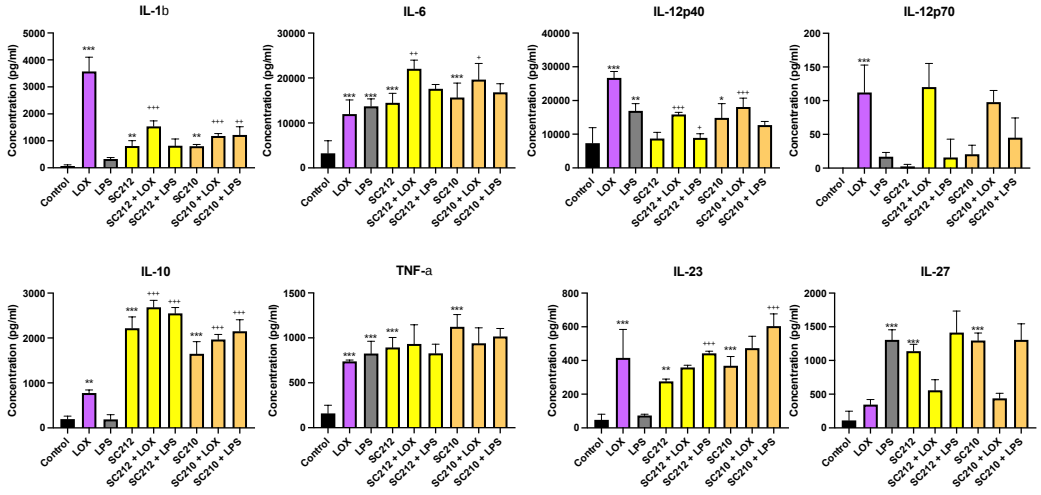

Figure 3. Exposure of LOX and LPS activated BMDCs to 25mg/ml fermentates results in the secretion of IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-23, and IL-27. BMDC cells were seeded at 1x10^6 cells/mL and incubated overnight at 37°C in 5% CO_2. The following day cells were stimulated with 25mg/ml fermentate, incubated for 1 hour at 37°C in 5% CO_2, and subsequently exposed to LOX 0.5mM and LPS 100ng/mL before incubating overnight under the same conditions. Supernatants were removed after 24 hours and ELISA was performed for IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-23, and IL-27. Data is presented as mean ± SEM of three replicates. Significance determined using one- way ANOVA with a Newman-Keuls post-test. Output P value style APA: .12 (ns), .033 (*), .002(**) and <.001 (***); 1) comparing control cells, to LOX and LPS, and unstimulated samples"*", 2) comparing TLR, to sample + TLR "+".

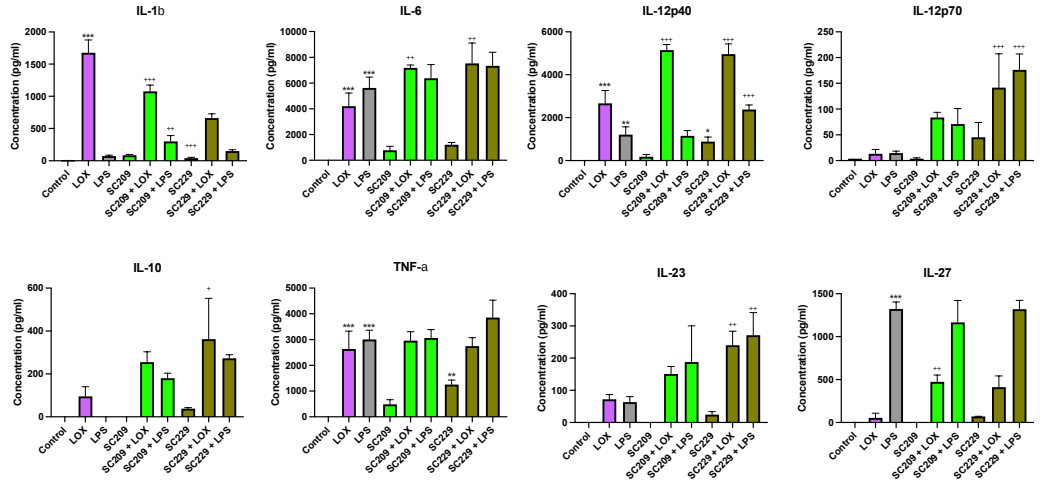

Figure 4. Exposure of LOX and LPS activated BMDCs to 25mg/ml fermentates results in the secretion of IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-23, and IL-27. BMDC cells were seeded at 1x10^6 cells/mL and incubated overnight at 37°C in 5% CO_2. The following day cells were stimulated with 25mg/ml fermentate, incubated for 1 hour at 37°C in 5% CO_2, and subsequently exposed to LOX 0.5mM and LPS 100ng/mL before incubating overnight under the same conditions. Supernatants were removed after 24 hours and ELISA was performed for IL-1β, IL-6, IL-10, TNF-α, IL-12p40, IL-12p70, IL-23, and IL-27. Data is presented as mean ± SEM of three replicates. Significance determined using one- way ANOVA with a Newman-Keuls post-test. Output P value style APA: .12 (ns), .033 (*), .002(**) and <.001 (***); 1) comparing control cells, to LOX and LPS, and unstimulated samples"*", 2) comparing TLR, to sample + TLR "+".

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3.5. Metabolite Levels in Fermentates

Given our data which demonstrates differential effects of the fermentates on cytokine production we carried out metabolomic analysis to determine whether there were differences between the metabolite levels of the fermentates also. Metabolite levels varied in the skim milk powder fermentate with higher levels of metabolites such as desaminotyrosine, succinate, glutamate, and lactate in SC212. Interestingly glutamate was also elevated in SC218 while succinate was also elevated in SC232.

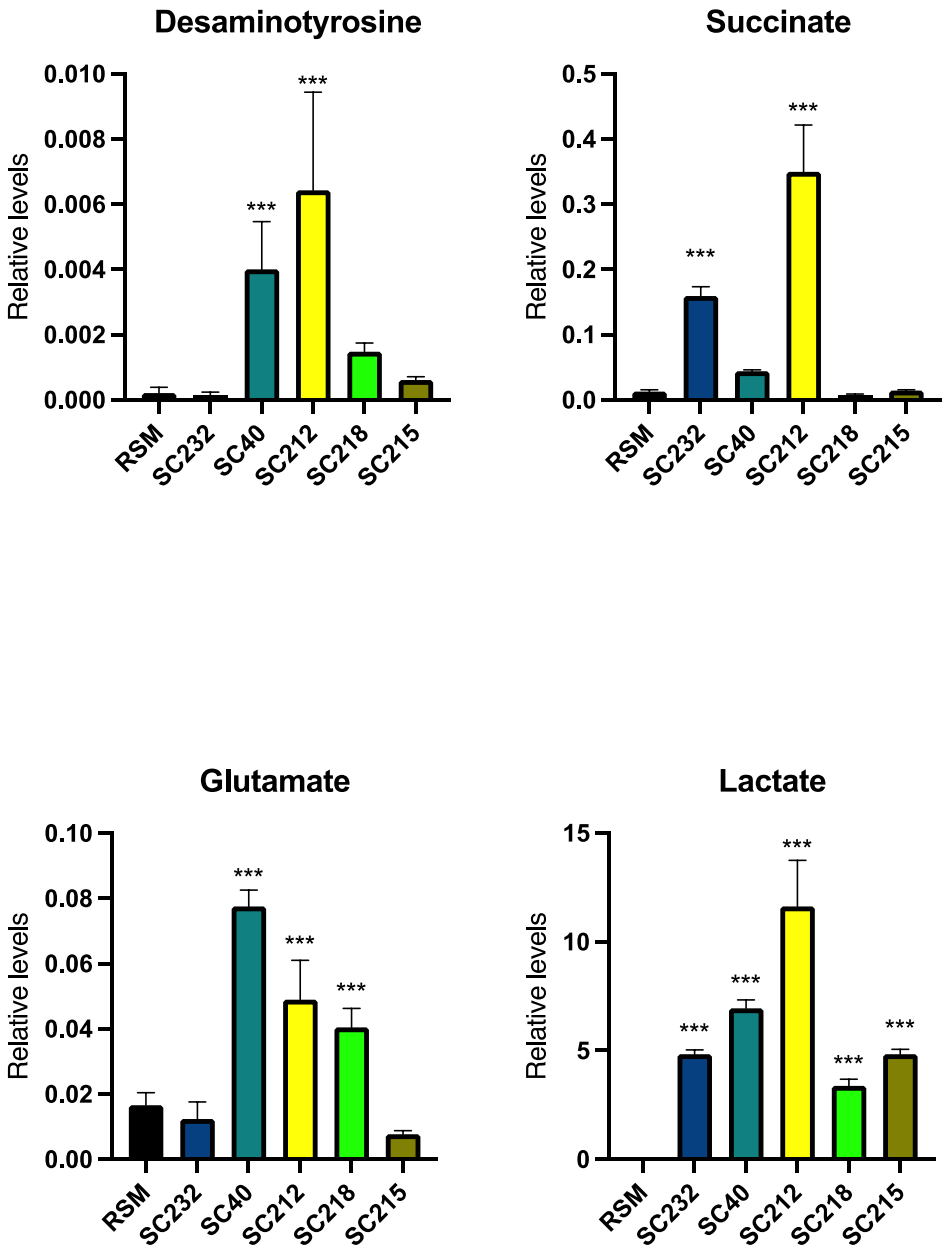

Figure 4. Metabolite levels in fermentates. Data is illustrated represented using GraphPad Prism V10. Data is presented as mean ± SEM of six replicates. Significance determined using one- way ANOVA with a Newman-Keuls post-test. Output P value style APA: .12 (ns), .033 (*), .002(**) and <.001 (***); comparing fermentate samples to the RSM.

4. Discussion

We have previously demonstrated a role for novel fermentates SC232 and SC234 as potential immune boosting supplements through their ability to enhance key macrophage functions, including cytokine secretion, chemokine secretion, nitric oxide production, phagocytosis, and cell surface

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marker expression (Finnegan, 2024 submitted). In this study we aimed to expand this work to assess their effects in another key immune cell, dendritic cells and to compare these effects to a range of other novel fermentates. Dendritic cells are considered the master regulators of the immune response, and play critical roles in antigen presentation to capture, process, and present antigens to lymphocytes to initiate and regulate the adaptive immune response [13]. They play a critical role in viral immunity and chronic inflammation [14,15] therefore, any effect on these cells by fermentates could indicate their ability to have significant influence on shaping immune responses. The TLR7 ligand, LOX, was used in order to mimic an immune response to a viral antigen, while the TLR4 ligand, LPS, was used in order to mimic an inflammatory immune response, similar to that seen in chronic inflammatory disorders such as UC and CD. Assessing fermentate effects in LOX and LPS activated dendritic cells enabled us to determine any specificity the fermentates may have.

We demonstrate that cytokine secretion in BMDCs is differentially influenced by the presence of the fermentates and that these effects differ depending on the mode of activation of the cell. This study demonstrates the specificity of SC232 and SC234 as immune boosting fermentates in the context of a viral infection, and SC212 and SC210 as anti-inflammatory fermentates. Furthermore, SC209 and SC229 had little or no effects on dendritic cell cytokine secretion and thus emphasis the unique specificity of the fermentates.

BMDCs when activated with the viral ligand LOX in the presence of SC232, and SC234 show enhanced levels of secretion of key viral cytokines IL-6, TNF-α, IL-12p40, IL-23, and IL-27, as well as IL-10, which supports viral clearance, thus they have potential to support viral immunity. These effects were not the same in the presence of LPS, with showed decreased IL-12p40 and IL-27, which further supports their possible specificity in enhancing viral immunity. Given the importance of IL-6, TNF-α, IL-12p40, IL-23, and IL-27 in aiding the immune system during viral infection and supporting viral immunity, a fermentate that can enhance these cytokines could indeed be beneficial.

It is well established that viral infections such as COVID-19 are characterised by increases in pro-inflammatory cytokines such as IL-6, TNF-α, IL-12p40, and IL-23 [16,17], while influenza and HIV-1 infections are characterised by increased production of IL-1β, IL-6, and TNF-α [18,19]. These cytokines are released in an attempt by the immune system to prevent or resolve the infection caused by the invading virus. IL-6, TNF-α, IL-12p40, and IL-23 are proinflammatory cytokines with key immunomodulatory and anti-viral roles, including supporting CD4+ and CD8+ cell differentiation, B cell activation, promoting additional cytokine secretion, inhibition of viral replication, leukocyte trafficking, immune complex clearance, and defence of intracellular organisms against invading viruses and pathogens [20–27]. When increased to a small degree, IL-10 plays a supportive role in viral clearance, through regulation of the adaptive immune response and IL-10 secreting T cells [28,29].

Previous research has shown fermentation products as well as food derived compounds can influence cytokines associated with a viral immune response. Specifically, Kawashima et al demonstrated that in a randomized, double-blind, placebo-controlled clinical trial, *Pediococcus acidilactici* K15, was shown to increase levels of both IL-6 and IL-10, ultimately increasing sIgA concentrations at mucosal sites in humans, aiding in host defence against pathogens and maintaining symbiosis with microorganisms present in the small intestine [29]. Furthermore, other studies by Takeda et al. showed that LAB, in particular strain *Lactiplantibacillus plantarum* 06CC2, from cows cheese, increased the production of IL-12, and IL-12p40 *in vitro* and *in vivo* [30]. *Lb. plantarum* 06CC2 has been associated with the enhancement of the Th1 response, and resulted in the alleviation of influenza virus infection in mice [31]. In a recent study by Zhu et al., Jasmin, found in the Chinese herbal medicine of jasmin tea, was found to have antiviral activity *in vitro* [32] and increased production of TNF-α from RAW 264.7 cells occurred at higher concentrations of jasmin, ultimately demonstrating antiviral activity against HSV-1 shown via a plaque reduction assay [32]. *Panax ginseng* Meyer, or white ginseng, has the ability to increase IL-1β, IL-6, TNF-α, and IL-23, via activation of the MAPK kinase (MKK)4-c-Jun N-terminal kinase (JNK)-c-Jun signalling pathway [33,34]. This is important considering viral infections like that of the influenza virus decrease innate IL-23 and IL-12p70 concentrations, to ultimately decrease IL-17 and IFN- γ responses, making the

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host more susceptible to further infection [35]. Therefore, our data showing that SC232 and SC234 can specifically modulate these cytokines could suggest their application in supporting viral immunity.

The effects of SC212 and SC210 were different to those seen with the other fermentates. BMDC cells when activated with LOX and LPS, in the presence of SC212 and SC210, show enhanced levels of IL-10, and decreased secretion of pro-inflammatory cytokines IL-12p40, and IL-1β.

It is well established that increased pro-inflammatory cytokine secretion, including increased IL-1β, IL-12p40, IL-6, TNF-α, and IL-27 has been linked to chronic inflammation and disease progression, particularly in the case of UC and CD [36–43]. This over-activation of the immune response produces the increased levels of pro-inflammatory cytokines, resulting in the chronic inflammation and disease severity. IL-1β, IL-12p40, IL-6, TNF-α, and IL-27 are pro-inflammatory cytokines with key immunomodulatory effects and contribute to this chronic inflammation seen in UC, and CD. They have functions in modulating immune responses, immune activation, host resistance, and immune mediated inflammation. Specifically, blocking Treg and Th17 differentiation, restricting IL-2 production, promoting Th1 cells and amplifying CD8 responders inhibiting Th2 mucosal responses, leucocyte trafficking, immune complex clearance, activation of endothelial cells, B and T cell proliferation and differentiation, Ig production, T cell activation and differentiation, natural killer cell stimulation, cytokine production, and activating acute phase responses [21,23–27,44–59]. When increased to a larger degree, IL-10 plays an important immune regulatory role in resolution of inflammation, and inhibition of disease pathogenesis. IL-10 functions through increased CD4 produced Th2 cytokine responses, suppresses Th1 responses, downregulates antigen presentation capacities of antigen presenting cells (APCs), inhibits activation and effector function of T cells, monocytes, and macrophage, limits host immune response to invading pathogens, and ultimately prevents damage to the host from over activation of the pro-inflammatory molecules [28,60–62].

Previous research has demonstrated that modulation of these cytokines can have anti-inflammatory effects. In a mice model of UC and of CD, compound LASSBio-1524 and its three analogues LASSBio-1760, LASSBio- 1763, and LASSBio-1764 reduced the secretion of TNF-α, IL-1β, IL-6, IL-12, and IFN-γ and increased secretion of IL-10, leading to a reduction in measures of disease severity, including, colonic tissue damage, infiltration of inflammatory cells, and the production and expression of pro-inflammatory mediators, suggesting its role as a novel therapeutic for the treatment of UC and CD [63]. In another model of murine colitis, treatment with the isoflavone genistein led to the reduction in pro-inflammatory mediators IL-6, TNF-α, MCP-1 and IL-1β, as well as an increases in IL-10 from $CD4^+$T cells, and M2 macrophage, and an increased M2 and decreased M1 macrophage phenotype, leading to reduced inflammation and disease severity, mediating colitis severity [64]. *Saccharomyces boulardii* a widely used gastrointestinal treatment, has been shown to decrease levels of IL-1β, IL-6, and, TNF-α, to aid in suppressed colonic inflammation in a DSS induced murine model of UC [65]. In a rat model of colitis, curcumin was shown to decrease disease activity, with reduced colonic mucosa damage, through the decreased expression of IL-27, via inhibition of the TLR4/NF-κB signalling pathway [66]. Furthermore, curcumin has also been shown to improve UC symptoms and alleviate chronic inflammation in DSS induced colitis mice models via the reduction on pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-9, IL-17A, IL-27, TNF-α, C-C motif chemokine ligand 2 (CCL2), and promoting the anti-inflammatory cytokine IL-10 in colonic tissue [67–70]. Thus, it is clear from the literature that reducing the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, TNF-α, and IL-27 while increasing the secretion of anti-inflammatory cytokine IL-10, can be linked to improved disease prognosis for UC and CD. Therefore, fermentates SC212 and SC210 could be novel candidates for the management of UC and CD in humans due to their ability to reduce the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, TNF-α, and IL-27 while increasing the anti-inflammatory cytokine IL-10.

Our data demonstrating the differential effects of our fermentates on both viral immunity and inflammation is further supported by our additional findings that SC209 and SC229 had little or no bioactivity when subjected to the same immune stimuli LOX and LPS. Therefore, not all fermentates

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influence cytokine secretion by dendritic cells and those that do, differ in their effects demonstrating that the fermentates have unique specificity in their bioactivity, as opposed to a generalised bioactivity as a result of the fermentate presence.

Given the differences in bioactivity of the fermentates we examined whether there were any differences in the metabolites present. We chose SC232, as an immune boosting fermentate, SC212 as key anti-inflammatory fermentates, as well as additional fermentates, SC218, SC40 and SC215 from our database for contrast. The data clearly indicated a differential metabolite profile between the fermentates.

SC232 has been clearly identified as an immune boosting fermentate, while SC212 has a mixed profile. SC232 shows a higher concentration of succinate, as does SC212, while SC212 also has a higher concentration of desaminotyrosine. Studies showing the increased levels of desaminotyrosine suggest the role of this metabolite in viral immunity and gut health. Thus suggesting a role for SC212 as an anti-viral and an anti-inflammatory due to its high levels of desaminotyrosine in comparison to its RSM. Desaminotyrosine influences mucosal barrier function, is important in maintaining mucosal immune homeostasis protecting barrier integrity, has been found to attenuate DSS-induced mucosal inflammation, protects mice from bacterial endotoxin-induced septic shock, is associated with an enhanced viral immunity , while also having anti-inflammatory effects and effects on influenza, via the induction of human-associated gut microbe, *Clostridium orbiscindens,* to produce desaminotyrosine [71–74]. Succinate is an immunomodulator that regulates the function of immune cells in the intestine, increasing the abundance of tight junction proteins claudin-1, zona occluden (ZO)-1, and ZO-2, to aid in intestinal epithelial barrier function and improving mucosal barrier integrity and microbial dysbiosis for improved gut health [75–77]. Succinate as well as suppressing immune responses can support inflammation and promoting immune boosting mechanisms through promoting the expression of inflammatory cytokines interleukin (IL)-25, IL-10, IL-8, and IL-18 aiding in antiviral ability, reducing vesicular stomatitis virus, influenza virus infection [78,79]. However, in recent reviews, succinate has been elevated in patients with IBDs, suggesting the role of high levels of succinate in IBD-associated mucosal inflammation, and its potential detrimental effects to the gut microbiome [80,81].

SC212 has been identified as largely anti-inflammatory fermentates. This is evident with SC212, as well as SC218 have significantly higher levels of glutamate, and lactate. Of most note SC212 has the highest levels of glutamate, and lactate which as demonstrated in the literature are anti-inflammatory metabolites largely associated with gut health, thus high presence of such metabolites may aid in gut health and alleviation of IBD associated inflammation. Lactate is a distinct signalling molecule that acts as a metabolic feedback regulator, that regulates cells, receptors, mediators, microenvironment-specific effects that augment Th17 cells, macrophage specifically M2, tumour associated macrophage, and neutrophil function, all with significant effect in cancer, sepsis, autoimmunity, wound healing [82]. Lactate enhances intestinal epithelial cell migration to improve wound healing, via enhanced mitochondrial ATP production driving cell migration, and has anti-inflammatory effects in UC models, preventing histological damage, bacterial translocation, or increase of pro-inflammatory cytokine IL-6 concentrations in serum, as well as downregulating proinflammatory immune responses in macrophage and dendritic cells promoting the M2 phenotype alleviating inflammatory impairment [83–85]

Therefore not only does fermentate SC232 have the ability to increase pro-inflammatory cytokines related to enhanced viral immunity, it also contains higher concentration of antiviral associated metabolites. In contrast SC212 has the ability to enhance the secretion of anti-inflammatory cytokines to aid in chronic inflammation associated with IBDs, while also containing higher levels of metabolites associated with anti-inflammatory effect, and improved gut health. Similarly we hypothesis that SC234 with its ability to enhance proinflammatory cytokine secretion for viral immunity, may also have higher concentrations of anti-viral associated metabolites desaminotyrosine, and succinate. While SC210 with its anti-inflammatory cytokine secretion, may have higher concentrations of anti-inflammatory associated metabolites glutamate, and lactate. Overall however, while the above-mentioned metabolites may have specific roles in the fermentates

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described here, further studies are required to elucidate and confirm the precise mechanism of action of these fermentates from a metabolomics point of view but this is beyond the scope of this current study reported here..

5. Conclusions

As demonstrated from the current literature available on similar functional foods, and building on previous work in our research group, we suggest a role for fermentates SC232 and SC234 as potential novel candidates for defence against viral infection in humans. This is due to their ability to support the secretion of pro-inflammatory cytokines IL-6, TNF-α and IL-27 while increasing anti-inflammatory cytokine IL-10 to maintain immune-homeostasis, and prevent viral persistence and ultimate viral infection, as well as demonstrating the presence of high levels viral associated metabolites such as desaminotyrosine, and succinate. Furthermore, novel fermentates SC212 and SC210 have been highlighted as potential novel anti-inflammatory candidates which may be useful in the control, management , and treatment of the chronic inflammation that is often seen in UC and CD. This is due to their ability to support the secretion of anti-inflammatory cytokines IL-10, while decreasing pro-inflammatory cytokines IL-1β and IL-12p40, for an enhanced immune response for the resolution of chronic inflammation, as well as demonstrating the presence of high levels of anti-inflammatory gut health associated metabolites such as glutamate, and lactate. Bioactivity is unique and specific to different fermentates when challenged with different immune stimulus, thus suggesting their specific role in modulating the immune system.

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