Journal of Probiotics & Health
ISSN: 2329-8901

Davies et al., J Prob Health 2018, 6:1
DOI: 10.4172/2329-8901.1000195

Research Article Open Access

# *Lactobacillus* and *Bifidobacterium* Promote Antibacterial and Antiviral Immune Response in Human Macrophages

**Davies TS*, Plummer SF, Jack AA, Allen MD and Michael DR**

*Cultech Ltd., Unit 2, Christchurch Road, Baglan Industrial Park, Port Talbot, SA12 7BZ, United Kingdom*

***Corresponding author:** Davies TS, Cultech Ltd., Unit 2, Christchurch Road, Baglan Industrial Park, Port Talbot, SA12 7BZ, United Kingdom, Tel: +441639825100; E-mail: tomd@cultech.co.uk

**Received date:** April 10, 2018; **Accepted date:** April 20, 2018; **Published date:** April 27, 2018

**Copyright:** © 2018 Davies TS, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

## Abstract

**Objective:** The objective of this study was to identify the immunomodulatory potential of two consortia of lactic acid bacteria, "Lab4" and "Lab4b", using human macrophages as an *in vitro* model system.

**Methods:** THP-1 monocyte-derived macrophages were exposed to metabolites of Lab4 or Lab4b. RT-qPCR was performed on macrophages to determine the expression of M1 pro-inflammatory (IL-1β, IL-18 and CD80) or M2 anti-inflammatory (CD206) marker mRNA in addition to IL-1β protein and inflammasome (NLRP3, Caspase-1, NLRP1, NLRC4 and AIM2) mRNA expression. Bacterial (LPS and ATP) and viral (Poly I:C) challenge were simulated to determine the potential of these consortia to regulate IL-1β protein, inflammasome mRNA expression, and antiviral IL-12 mRNA expression and protein under inflammatory conditions. The ability of these consortia to modulate macrophage phagocytosis of *E. coli* was also assessed.

**Results:** Lab4 and Lab4b metabolites promoted an M1 phenotype in macrophages *in vitro* by increasing mRNA expression of IL-1β, IL-18, and CD80 and reducing mRNA expression of CD206. Induction of IL-1β protein suggested involvement of the inflammasome. mRNA expression of NLRP3, Caspase-1, NLRP1 and AIM2 was induced by Lab4 and mRNA expression of NLRP3 and Caspase-1 was induced by Lab4b suggesting different potential modes of action of the two consortia. Lab4 and Lab4b metabolites, in combination with this LPS and ATP challenge, enhanced IL-1β mRNA and protein expression further, accompanied by different mRNA expression profiles of inflammasome genes by the two consortia. Lab4 and Lab4b also induced expression of mRNA and protein of the antiviral response gene, IL-12. In combination with Poly I:C challenge, Lab4 induced IL-12 protein further, while Lab4b induced IL-12p25/IL-12p40 mRNA further highlighting potential differences. Both consortia were also able to induce phagocytosis of *E. coli* particles.

**Conclusion:** Data generated from this study suggest the potential for organism-dependent control of the immunoregulatory response seen in human macrophages.

**Keywords** Probiotic; Polarisation; Inflammasome, Poly I:C; Phagocytosis; Macrophage; Antibacterial; Antiviral

## Introduction

Macrophages are key modulators of immune response and are widely distributed throughout the body, particularly the gut [1]. These cells form a crucial line of defence between the environment and the host and provide a target for the investigation of host-microbe immunological interactions. Macrophages exhibit significant plasticity and can alter their phenotype to suit their environment and function [2]. Macrophage polarisation typically produces two phenotypes; a classical pathogen-clearing pro-inflammatory 'M1', or, an alternatively-activated anti-inflammatory 'M2' phenotype [3,4]. To enhance pathogen clearance, macrophages can utilize the IL-1β and IL-18-producing inflammasome- an intracellular multiprotein complex essential for innate immune response [5,6]. Numerous inflammasome complexes can form, each with distinct properties that allow the host cell to elicit antibacterial and antiviral responses accompanied by cytokine production [7-12]. Although the classical nucleotide-binding oligomerization domain-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome is typically implicated in driving IL-1β secretion, Caspase-1 is also required for maturation and secretion of IL-1β, in addition to non-NLRP3 inflammasomes that can form which can also regulate IL-1β [6].

The human gut houses the most densely populated bacterial ecosystem in the body with around $1 \times 10^{14}$ bacterial cells and these bacteria outnumber host human cells by over 10:1 [13,14]. These microorganisms can significantly impact metabolic diseases, inflammation and infection and modulation of the gut microbiota is being targeted for the prevention and/or treatment of gut dysbiosis with probiotics being widely used to restore imbalance [15-21]. Probiotics are classified as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' and a plethora of *in vivo* evidence now supports their beneficial immunomodulatory potential [22-28]. Recent emphasis has been placed on delineating these benefits at a mechanistic level and it is emerging that non-pathogenic commensal organisms can activate distinct inflammatory and inflammasome pathways in immune cells to impart health benefits [6,29,30].

The beneficial effects of the Lab4 and Lab4b probiotic consortia are well-reported [22,27], however, the potential mechanisms by which they have been able to reduce the incidence of upper respiratory tract infections (URTI) and atopic sensitization during childhood have remained unclear [22,27]. Therefore, the aim of this study was to assess the regulatory roles of Lab4 and Lab4b on the innate immune system using cultured macrophages.

## Materials and Methods

### Bacterial culture

All bacterial preparations were provided by Cultech Ltd. DeMan Rogosa Sharpe (MRS) broth (Oxoid) was inoculated with freeze-dried preparations of a consortium comprising of *Lactobacillus acidophilus* CUL21 (NCIMB 30156), *Lactobacillus acidophilus* CUL60 (NCIMB 30157), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis subsp. lactis* CUL34 (NCIMB 30172) (Lab4), or, a consortium comprising of *Lactobacillus salivarius* CUL61 (NCIMB 30211), *Lactobacillus paracasei* CUL08 (NCIMB 30154), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis subsp. lactis* CUL34 (NCIMB 30172) (Lab4b) and was incubated anaerobically (10% carbon dioxide, 10% hydrogen and 80% nitrogen) at 37°C for 18 hours, diluted to $1 \times 10^9$ CFU/mL and harvested by centrifugation at 2,500 g for 10 minutes. Bacterial pellets were then resuspended in phosphate-buffered saline (PBS), centrifuged, and resuspended in RPMI-1640 medium containing no serum or antibiotics. Cell suspensions were incubated for 5 hours under anaerobic conditions at 37°C, centrifuged, and supernatants were passed through a 0.22 µm polyethylene syringe filter. The pH of the supernatant was then adjusted to 7.4 and supplemented with 50 µg/mL penicillin and 50 U/mL streptomycin. Cell-free supernatants containing either Lab4 or Lab4b metabolites were applied directly to cultured macrophages during experiments. Hereafter, unless stated otherwise, Lab4 and Lab4b cell-free supernatants containing metabolites will be referred to as just Lab4 and Lab4b.

### Macrophage cell culture

THP-1 monocytes were maintained in RPMI-1640 supplemented with 10% (v/v) FCS, 50 µg/mL streptomycin and 50 U/mL penicillin at 37°C in 5% (v/v) $CO_2$ (reagents from Life Technologies, UK). THP-1 monocytes were plated at $1 \times 10^6$ cells per well in 24-well plates and differentiated into macrophages by incubation with 160 nM phorbol 12-myristate 13-acetate (PMA) for 24 hours [31]. PMA, poly I:C, LPS and ATP were all prepared in PBS, pH was adjusted where appropriate to 7.4 and PBS was used as a vehicle control. For bacterial challenge studies, THP-1 monocyte-derived macrophages were primed with 1 µg/mL LPS for 4 hours, before also stimulating with 5 mM ATP for 30 minutes while co-incubating with control, Lab4 or Lab4b cell-free supernatant. For the assessment of IL-12 expression, either metabolites, poly I:C viral challenge (50 µg/mL) or a combination of the two were incubated with cells for 4 hours. Viability of THP-1 monocyte-derived macrophages was maintained in all conditions tested (Supplementary Figure S1B). All reagents and cells, unless otherwise stated, were purchased from Sigma Aldrich, UK.

### RT-qPCR

RNA was extracted using RiboZol (VWR, UK) following manufacturer's instructions and was quantified using QuBit Fluorometric Quantification (Thermo Fisher, UK). cDNA (500 ng) was generated using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, UK). For RT-qPCR, 10 ng cDNA was used in each 25 µL SYBR green (BioRad) reaction. Primer sets were designed, or, primer sets were obtained from the literature (for sequences and PCR cycling conditions, see Supplementary Table S1). Negative control RNA samples not treated with reverse transcriptase were also analysed alongside RT-qPCR samples to monitor primer-specificity and ensure reactions were contaminant-free. Custom primers were designed in-house using BLAST® (NCBI) with primer storage and oligonucleotide analysis performed using oliGO (https://oligo.co). Quantification was carried out on a CFX96 (Bio-Rad) and data are expressed as fold changes according to the $2^{-(\Delta Ct1-\Delta Ct2)}$ method. Primers used were: For M1 and M2 polarisation mRNA expression analysis (Figure 1): Interleukin (IL)-1β, IL-18, cluster of differentiation (CD)-80 and CD206. For inflammasome mRNA expression analysis (Figures 2 and 3): NLRP3, Caspase-1, NLRP1, NLR family caspase activation and recruitment domain (CARD) domain-containing protein 4 (NLRC4) and absent in melanoma-2 (AIM2). For antiviral response mRNA expression analysis (Figures 4 and 5): IL-12p35 and IL-12p40.

### IL-1β and IL-12 quantification by ELISA

Cell culture supernatants were centrifuged at 5,000 g for 5 minutes and the resultant cell-free supernatant was assayed for IL-1β or IL-12 concentration using human IL-1β or IL-12 standard 3,3',5,5'-Tetramethylbenzidine (TMB) ELISA development kits (Peprotech), respectively, in accordance with manufacturer's instructions.

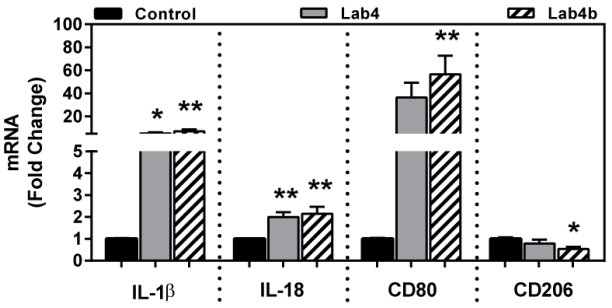


**Figure 1:** Lab4 and Lab4b drive the mRNA expression of M1 markers in human macrophages. The mRNA expression levels of IL-1β, IL-18, CD80 and CD206 in human THP-1 macrophages incubated with control medium (black bars) or treated with Lab4 (grey bars) or Lab4b (hashed bars) for 4 hours. Data (mean ± SEM) are from five independent experiments expressed as fold changes from the control arbitrarily set to 1.0. Statistics: One-way ANOVA with Tukey's multiple comparisons. Significance: *=$P \leq 0.05$, **=$P \leq 0.01$.

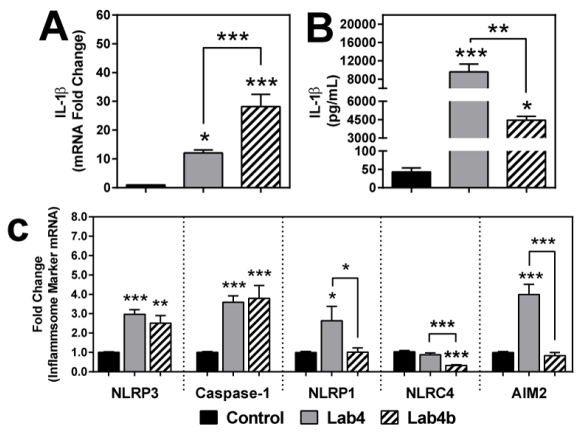


**Figure 2:** Lab4 and Lab4b activate the inflammasome in human macrophages. (A) The mRNA expression levels of IL-1β, and (B) IL-1β protein secretion, and (C) mRNA expression levels of NLRP3, Caspase-1, NLRP1, NLRC4 and AIM2 were determined in human THP-1 monocyte-derived macrophages that were treated with control medium (black bars), Lab4 (grey bars) or Lab4b (hashed bars) over 4 hours. Data (mean ± SEM) are from four independent experiments expressed as fold changes from the control arbitrarily set to 1.0. Statistics: One-way ANOVA with Tukey's multiple comparisons. Significance: *=$P \leq 0.05$, **=$P \leq 0.01$, ***=$P \leq 0.001$.

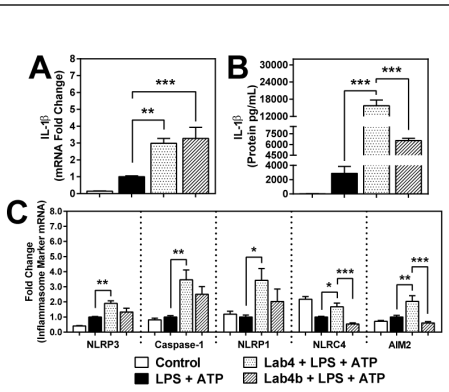


**Figure 3:** Lab4 and Lab4b enhance inflammasome activation in LPS- and ATP-challenged human macrophages. (A) The mRNA expression of IL-1β, and (B) protein secretion of IL-1β, and (C) mRNA expression of NLRP3, Caspase-1, NLRP1, NLRC4 and AIM2 were determined in human THP-1 monocyte-derived macrophages. Conditions are: Control medium-treated cells (white bars) with a PBS vehicle control for 4 hours 30 minutes (duration of experimental period), LPS (1 μg/mL, 4 hours) followed by ATP (5 mM, 30 minutes)-treated cells (black bars), Lab4-treated cells with the same LPS and ATP conditions (dotted bars), Lab4b-treated cells with the same LPS and ATP conditions (hashed bars). Data (mean ± SEM) are from four independent experiments expressed as fold change compared to the LPS- and ATP-treated cells arbitrarily set to 1.0. Statistics: One-way ANOVA with Tukey's multiple comparisons. Significance: *=$P \leq 0.05$, **=$P \leq 0.01$, ***=$P \leq 0.001$.

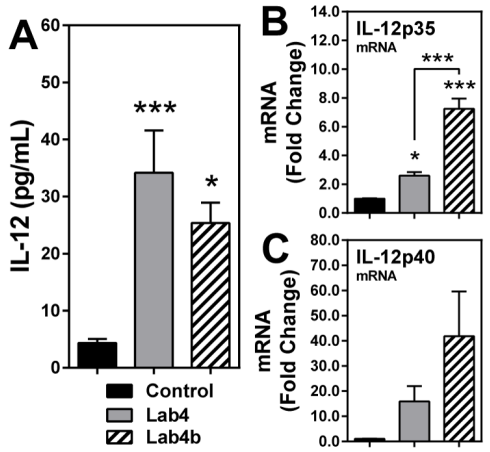


**Figure 4:** Lab4 and Lab4b promote IL-12 production in human macrophages. (A) The secreted protein levels of IL-12, and (B) mRNA expression of IL-12p35, and (C) mRNA expression of IL-12p40 were determined in human THP-1 monocyte-derived macrophages. Conditions are: Control medium-treated cells (black bars), Lab4-treated cells (grey bars), Lab4b-treated cells (hashed bars). Data (mean ± SEM) are from four independent experiments. Statistics: One-way ANOVA with Tukey's multiple comparisons. Significance: *=$P \leq 0.05$, ***=$P \leq 0.001$.

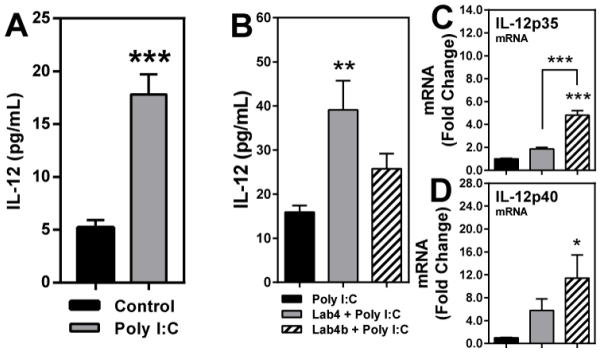


**Figure 5:** Lab4 and Lab4b promote IL-12 production in poly I:C-challenged human macrophages. (A) The secreted protein levels of IL-12 in response to 50 μg/mL poly I:C over 4 hours. Conditions are: Control medium with a PBS vehicle-treated cells (black bars) and poly I:C treated cells (grey bars). (B) The secreted protein levels of IL-12, and (C) mRNA expression of IL-12p35, and (D) mRNA expression of IL-12p40 in human THP-1 monocyte-derived macrophages. Conditions are: control medium-treated cells co-incubated with poly I:C (black bars), Lab4-treated cells co-incubated with poly I:C (grey bars) and Lab4b-treated cells co-incubated with poly I:C (hashed bars). Data (mean ± SEM) are from four independent experiments. Statistics: One-way ANOVA with Tukey's multiple comparisons. Significance: *=$P \leq 0.05$, **=$P \leq 0.01$, ***=$P \leq 0.001$.

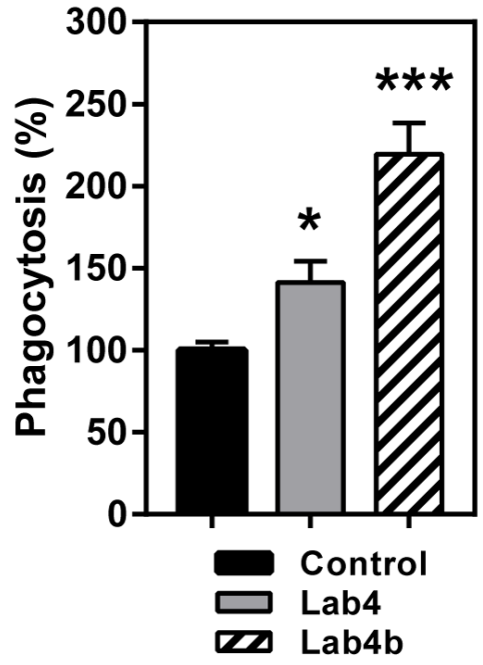


**Figure 6:** Lab4 and Lab4b promote phagocytosis in human macrophages. THP-1 macrophages were differentiated as described and incubated with control medium (black bars) or treated with Lab4 (grey bars) or Lab4b (hashed bars) for 4 hours. After this time, cells were washed briefly before the addition of Vybrant® fluorescently-labelled bioparticles for 2 hours in 1 X HBSS. Data (mean ± SEM) are from five independent experiments and expressed as percentage change from the control arbitrarily set to 100%. Statistics: One-way ANOVA with Tukey's multiple comparisons. Significance: *=$P \leq 0.05$, ***=$P \leq 0.001$.

### Phagocytosis assay

Phagocytosis assays were performed using the Vybrant® assay kit (Thermo Fisher, UK). $1 \times 10^5$ THP-1 macrophages per well were plated in 96-well plates and incubated in the presence of either control medium or Lab4 or Lab4b cell-free supernatant for 4 hours. These supernatants were diluted 1:10 to be compatible with the kit. Cells were washed with PBS before incubating with FITC-labelled *E. coli* bioparticles for 2 hours and the plate was read with excitation/emission at 480/520 nm.

### Statistical analysis

Prior to statistical significance testing, normality of the data was assessed using D'Agostino-Pearson and Shapiro-Wilk tests. For multiple comparisons, values of *P* were determined using one-way ANOVA with Tukey's post-hoc analysis when data were normally distributed. For data not normally distributed, non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc analysis was performed. For single comparisons between two groups, values of *P* were calculated using a two-tailed unpaired student's t-test. Statistical outliers were excluded from analysis. All statistical testing was performed using GraphPad 7.0 (Prism) and data are presented as the mean ± SEM of the defined number of experiments. Significance was defined when $P<0.05$ and *P* values are stated unless they fall below $P<0.001$.

## Results

### Promotion of the M1 phenotype in human macrophages

The presence of Lab4 or Lab4b induced M1 marker mRNA expression in THP-1 macrophages of: IL-1β (5.2-fold, $P=0.022$ and 7.2-fold, $P=0.0014$), IL-18 (2.0-fold, $P=0.0082$ and 2.1-fold, $P=0.0021$) and CD80 (36.3-fold, $P=0.081$ and 56.7-fold, $P=0.0050$) for Lab4 and Lab4b, respectively, compared to the control. Lab4 and Lab4b reduced the mRNA expression of M2 marker CD206 (0.79-fold, $P=0.37$ and 0.31-fold, $P=0.022$, respectively) compared to the control.

### IL-1β activation in human macrophages

The effects of Lab4 and Lab4b on inducing IL-1β and IL-18 mRNA expression (Figure 1) suggested the involvement of the inflammasome [32]. In separate experiments, IL-1β mRNA expression was induced by Lab4 (12.1-fold, $P=0.013$) and Lab4b (28.2-fold, $P<0.001$). Lab4b also induced IL-1β mRNA expression significantly more than Lab4 (28.2-fold vs. 12.1-fold, $P<0.001$, Figure 2A). IL-1β protein secretion was 9,586 pg/mL for Lab4 and 4,459 pg/mL for Lab4b compared to a control of 43.0 pg/mL ($P<0.001$ and $P=0.022$, respectively) with Lab4 producing more of a response than Lab4b ($P=0.0060$, Figure 2B).

### Inflammasome activation in human macrophages

Inflammasome activation was determined by measuring mRNA expression of five different inflammasome pathway components: NLRP3, Caspase-1, NLRP1, NLRC4 and AIM2. Both Lab4 and Lab4b induced the expression of NLRP3 (3.0-fold, $P<0.001$ and 2.5-fold $P=0.0010$, respectively) and Caspase-1 (3.6-fold, $P<0.001$ and 3.8-fold, $P<0.001$, respectively) compared to the control. Lab4 induced mRNA expression of NLRP1 (2.6-fold, $P=0.040$) and AIM2 (4.0-fold, $P<0.001$). Lab4b attenuated mRNA expression of NLRC4 (0.3-fold, $P<0.001$, Figure 2C). Lab4 induced significantly more NLRP1 mRNA expression compared to Lab4b (2.6-fold vs. 1.0-fold, respectively, $P=0.040$). Lab4b attenuated significantly more NLRC4 mRNA expression compared to Lab4 (0.7-fold less vs. 0.1-fold less, respectively, $P<0.001$, from the control arbitrarily set to 1.0). Lab4 induced significantly more AIM2 mRNA expression compared to Lab4b (4.0-fold vs. 0.8-fold, respectively, $P<0.001$).

### Enhanced IL-1β activation in challenged human macrophages

Bacterial challenge was simulated by the addition of LPS and ATP to macrophages as described in the methods. LPS and ATP were able to significantly induce IL-1β mRNA and protein (Supplementary Figure 2). With the co-incubation of Lab4 or Lab4b with challenge, IL-1β mRNA (2.9-fold, $P=0.0030$ and 3.3-fold, $P<0.001$, respectively) and IL-1β protein (15,756 pg/mL and 6,546 pg/mL vs. 2,873 pg/mL LPS and ATP challenge, $P<0.001$, Figure 3B) expression was enhanced further by both consortia. Differences between Lab4 and Lab4b were observed where Lab4 was able to induce more IL-1β protein than Lab4b (15,756 pg/mL vs. 6,546 pg/mL, $P<0.001$ Figure 3B).

### Enhanced inflammasome activation in challenged human macrophages

In the presence of LPS and ATP challenge, NLRP3 and AIM2 mRNA expression was significantly induced, while NLRC4 mRNA expression was attenuated compared to the control (Supplementary Figure 2). Using these challenged conditions as a baseline, co-incubation of Lab4 or Lab4b was also able to further upregulate inflammasome mRNA expression. With challenge co-incubation, Lab4 was able to induce mRNA expression of NLRP3 (1.9-fold, $P$=0.0020), Caspase-1 (3.5-fold, $P$=0.0010), NLRP1 (3.4-fold, $P$=0.030), NLRC4 (1.7-fold, $P$=0.030) and AIM2 (2.0-fold, $P$=0.0050) when compared to challenge alone (Figure 3C). There were trends for Lab4b co-incubation with challenge to upregulate NLRP3, Caspase-1, NLRP1 and downregulate NLRC4 and AIM2 mRNA expression compared to challenge alone which were not significant (Figure 3C). The two consortia also demonstrated differential effects on inflammasome gene expression. Lab4 and Lab4b regulation of NLRC4 (1.7-fold vs. 0.5-fold, respectively, $P$<0.001) and AIM2 (2.0-fold vs. 0.6-fold, respectively, $P$<0.001) mRNA expression were significantly different.

### Induction of an antiviral response in human macrophages

Viral infection triggers host innate immune responses indicated by cytokine production (such as IL-12) and inflammasome activation [33]. Both Lab4 and Lab4b induced the macrophage secretion of IL-12 protein. For Lab4, 34.2 pg/mL ($P$<0.001) and, for Lab4b 25.4 pg/mL ($P$=0.010) compared to 4.3 pg/mL detected in the control (Figure 4A). IL-12p35 mRNA expression was induced 2.6-fold ($P$=0.040) and 7.2-fold ($P$<0.001) in the presence of Lab4 and Lab4b, respectively (Lab4b induction significantly higher than Lab4 for IL-12p35, $P$<0.001, Figure 4B). Expression of IL-12p40 was not significantly different for either consortia, though there was a trend towards induction (Figure 4C).

### Enhanced IL-12 activation in challenged human macrophages

Viral challenge was simulated by the viral mimic, poly I:C [34], and this challenge induced the mRNA expression of IL-12p35 and IL-12p40 compared to the control (Supplementary Figure 3). IL-12 protein expression was induced by stimulation with poly I:C (17.8 pg/mL for challenge vs. 5.21 pg/mL control, $P$<0.001, Figure 5A). Co-incubation of poly I:C with Lab4 enhanced IL-12 protein production compared to poly I:C challenge alone (39.1 pg/mL vs. 15.9 pg/mL, $P$<0.0020, Figure 5B). There was a trend for Lab4b to induce IL-12 protein which was not significant but did further highlight the different potential of these two consortia (Figure 5B).

IL-12p35 (4.8-fold, $P$<0.001, Figure 5C) and IL-12p40 (11.4-fold, $P$=0.030, Figure 5D) mRNA expression was induced in the presence of challenge and Lab4b. There was no significant induction of IL-12p35 or IL-12p40 by Lab4 with poly I:C challenge, however, Lab4b was able to induce more IL-12p35 mRNA compared to Lab4 (4.8-fold vs. 1.8-fold, $P$<0.001, Figure 5C).

### Induction of phagocytosis by human macrophages

Macrophages were incubated with Lab4 or Lab4b prior to incubation with FITC-labelled *E. coli* bioparticles. Both Lab4 and Lab4b significantly induced phagocytosis of FITC-labelled bioparticles by the macrophages (141.2%, $P$=0.033, and 219.4%, $P$<0.001, respectively vs. 100% control, Figure 6).

## Discussion

Metabolites generated by the Lab4 and Lab4b consortia of probiotics, *in vitro*, are able to polarise cultured macrophages towards a classical M1 pro-inflammatory phenotype. Under conditions chosen to simulate bacterial and viral challenge *in vitro*, the Lab4 and Lab4b consortia enhanced the macrophage M1 pro-inflammatory responsiveness. mRNA expression data indicated that both Lab4 and Lab4b upregulated mRNA expression of IL-1β, IL-18 and CD80 with a greater response observed for Lab4b. mRNA expression of the M2 marker CD206 was dampened by both Lab4 and Lab4b (Figure 1). Lab4b has been found to reduce atopic eczema sensitization [22] and the ability of this consortium to skew towards the M1 phenotype could help explain this outcome.

Miettinen et al. have previously shown that both live and heat-killed *Lactobacillus rhamnosus* GG are capable of activating NLRP3, Caspase-1, and IL-1β [6]. However, to our knowledge, the role of numerous alternatively non-NLRP3 activated inflammasomes by commensal bacteria (such as *Lactobacillus* and *Bifidobacterium*) has not yet been demonstrated. The induction of both IL-1β mRNA and protein by Lab4 and Lab4b suggested the involvement of the inflammasome. This IL-1β response was potentially regulated by the NLRP3 inflammasome, as indicated by induction of NLRP3 and Caspase-1 mRNA (Figure 2). However, in this study we show that the consortia can also differentially regulate NLRP1, NLRP3, NLRC4 and AIM2 mRNA expression (Figure 2). The Lab4 consortium reduced the incidence and duration of upper respiratory tract infection (URTI) in children attending preschool and the activation of different inflammasome pathways could provide a possible link [27]. The Lab4b strain, *Lactobacillus paracasei* CUL08, has recently been shown to dampen IL-1β production *via* NF-κB inhibition in THP-1 macrophages [30]. In contrast, we show that Lab4b is able to induce an inflammatory response alone, and, is less pro-inflammatory when compared to Lab4. This could potentially be explained by Lab4b demonstrating a more specific response, regulating just NLRP3 and NLRC4, while still inducing IL-1β mRNA and protein when compared to Lab4. These data highlight the potential of these two consortia to regulate immune function in a species-specific manner and suggests the potential benefits of using different consortia.

To ascertain whether Lab4 and Lab4b were able to assist or enhance macrophage activation and immune response during bacterial challenge, LPS was used, in addition to ATP. It is well-documented that LPS provides the initial 'priming' signal required for inflammasome activation, and ATP represents the second 'danger' signal which is required for full IL-1β maturation and secretion [34-36]. Challenge of Lab4- or Lab4b-treated macrophages with these agents was able to induce a greater inflammasome response (Figure 3). Lab4 and Lab4b were significantly different in their potential to regulate the inflammasome. These data suggest that Lab4 may regulate NLRP3, NLRP1, NLRC4 and AIM2 inflammasomes during challenge, while Lab4b regulates just NLRC4 and AIM2, highlighting potential species-specific effects.

Viruses are also a major cause of URTI [37] and so the antiviral potential of Lab4 and Lab4b was investigated. IL-12 is a key mediator in viral response that is activated in response to toll-like receptor 3 (TLR3) stimulation with double-stranded viral RNA [38-40]. Both Lab4 and Lab4b were able to induce expression of IL-12 mRNA and protein (Figure 4). Both consortia were also able to enhance the IL-12 response in the presence of the TLR3 viral mimic agonist, poly I:C (Figure 5), which is commonly used to simulate viral challenge in

macrophages [34], indicating the potential of Lab4 and Lab4b to prime macrophages towards antiviral responsiveness [40,41].

Phagocytosis is an essential clearance mechanism carried out by macrophages that ensures removal of bacteria, viruses and debris [42] and phagocytosis assays were performed to assess the functionality of Lab4 and Lab4b. Significant increases in phagocytosis by THP-1 macrophages were observed in response to exposure of Lab4 or Lab4b treatment of *E. coli* bioparticles indicating antipathogenic activity by these cells (Figure 6).

These studies indicate both antibacterial and antiviral responses of macrophages to both Lab4 and Lab4b- a characteristic recently observed in other commensal bacteria and supports the findings of reduced incidence of URTI and reduced atopic eczema sensitization [22,27].

## Conclusion

To our knowledge, we show for the first time that different consortia of *Lactobacillus* and *Bifidobacteria* probiotic bacteria have differential effects on macrophage activation, phenotype, inflammasome and viral activation in cultured macrophages. These *in vitro* data suggest that the Lab4 and Lab4b consortia can (i) polarise macrophages towards an 'M1' phenotype, (ii) induce IL-1β secretion in human macrophages, (iii) enhance their response to bacterial challenge by differentially regulating a number of inflammasome components, (iv) induce IL-12 secretion by human macrophages and enhance their response to viral challenge, and (v) enhance the phagocytic activity of human macrophages. These *in vitro* findings provide novel insights into the potential immunomodulatory mechanisms underlying the actions of different strains of *Lactobacillus* and *Bifidobacteria*. This study provides a foundation and valuable basis for future investigation in primary cells, animal models and clinical trials.

## Author Contributions

TSD, DRM and SFP were responsible for the design of the study, Experiments were performed by TSD, DRM, AAJ and MDA. Data Analysis was performed by TSD and DRM. TSD prepared the manuscript and all authors contributed to the review of the manuscript.

## Acknowledgments

We thankfully acknowledge the contributions of Professor Dipak P. Ramji at Cardiff University for reading the manuscript and for his helpful comments, together with Joe. W. E. Moss and Victoria O'Morain for their input.

## Competing Financial Interest

This study was supported by Cultech Ltd., Port Talbot, UK. TSD, SFP, AAJ, MDA and DRM are employees of Cultech Ltd.

## References

1. Lee SH, Starkey PM, Gordon S (1985) Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80. The Journal of Experimental Medicine 161: 475-489.
2. Davis MJ, Tsang TM, Qiu Y, Dayrit JK, Freij JB, et al. (2013) Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in Cryptococcus neoformans infection. MBio 18: e00264-13.
3. Chen Y, Zhang X (2017) Pivotal regulators of tissue homeostasis and cancer: Macrophages. Experimental Hematology & Oncology 6: 23.
4. Deng H, Li Z, Tan Y, Guo Z, Liu Y, et al. (2016) A novel strain of Bacteroides fragilis enhances phagocytosis and polarises M1 acrophages. Scientific Reports 6: 29401.
5. Chen GY (2017) Regulation of the gut microbiome by inflammasomes. Free Radical Biology and Medicine 105: 35-40.
6. Miettinen M, Pietilä TE, Kekkonen RA, Kankainen M, Latvala S, et al. (2012) Nonpathogenic Lactobacillus rhamnosus activates the inflammasome and antiviral responses in human macrophages. Gut Microbes 3: 510-522.
7. Gimenez F, Bhela S, Dogra P, Harvey L, Varanasi SK (2016) The inflammasome NLRP3 plays a protective role against a viral immunopathological lesion. Journal of Leukocyte Biology 99: 647-657.
8. Hanamsagar R, Aldrich A, Kielian T (2014) Critical role for the AIM2 inflammasome during acute central nervous system bacterial infection. Journal of Neurochemistry 129: 704-711.
9. Jiang S, Zhang Y, Zheng JH, Li X, Yao YL, et al. (2016) Potentiation of hepatic stellate cell activation by extracellular ATP is dependent on P2X7R-mediated NLRP3 inflammasome activation. Pharmacological Research 117: 82-93.
10. Kim JJ, Jo EK (2013) NLRP3 inflammasome and host protection against bacterial infection. Journal of Korean Medical Science 28: 1415-1423.
11. Kopitar-Jerala N (2017) The role of interferons in inflammation and inflammasome activation. Frontiers in Immunology 8: 873.
12. Schroder K, Tschopp J (2010) The inflammasomes. Cell 140: 821-832.
13. Belkaid Y, Naik S (2013) Compartmentalized and systemic control of tissue immunity by commensals. Nature Immunology 14: 646-653.
14. Simon GL, Gorbach SL (1984) Intestinal flora in health and disease. Gastroenterology 86: 174-193.
15. Ejtahed HS, Mohtadi-Nia J, Homayouni-Rad A, Niafar M, Asghari-Jafarabadi M, et al. (2012) Probiotic yogurt improves antioxidant status in type 2 diabetic patients. Nutrition 28: 539-543.
16. Jones ML, Martoni CJ, Prakash S (2012) Cholesterol lowering and inhibition of sterol absorption by Lactobacillus reuteri NCIMB 30242: a randomized controlled trial. European Journal of Clinical Nutrition 66: 1234-1241.
17. Michael DR, Moss JW, Calvente DL, Garaiova I, Plummer SF, et al. (2016) Lactobacillus plantarum CUL66 can impact cholesterol homeostasis in Caco-2 enterocytes. Beneficial Microbes 7: 443-451.
18. Michael DR, Davies TS, Moss JWE, Calvente DL, Ramji DP, et al. (2017) The anti-cholesterolaemic effect of a consortium of probiotics: An acute study in C57BL/6J mice. Scientific Reports 6: 2883.
19. Mitchell CM, Davy BM, Halliday TM, Hulver MW, Neilson AP, et al. (2015) The effect of prebiotic supplementation with inulin on cardiometabolic health: Rationale, design, and methods of a controlled feeding efficacy trial in adults at risk of type 2 diabetes. Contemporary Clinical Trials 45: 328-337.
20. Rebello CJ, Burton J, Heiman M, Greenway FL (2015) Gastrointestinal microbiome modulator improves glucose tolerance in overweight and obese subjects: A randomized controlled pilot trial. Journal of Diabetes Complications 29: 1272-1276.
21. Yamashita T (2017) Intestinal immunity and gut microbiota in atherogenesis. Journal of Atherosclerosis and Thrombosis 24: 110-119.
22. Allen SJ, Jordan S, Storey M, Thornton CA, Gravenor M (2014) Probiotics in the prevention of eczema: A randomised controlled trial. Archives of Disease in Childhood 99: 1014-1019.
23. Lucas C, Barnich N, Nguyen HTT (2017) Microbiota, inflammation and colorectal cancer. International Journal of Molecular Sciences 18: 1310.
24. Allen SJ, Wareham K, Bradley C, Harris W, Dhar A (2012) A multicentre randomised controlled trial evaluating lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea in older people

admitted to hospital: the PLACIDE study protocol. BMC Infectious Diseases 6: 108.

25. d'Ettorre G, Ceccarelli G, Giustini N, Serafino S, Calantone N, et al. (2015) Probiotics reduce inflammation in antiretroviral treated, HIV-infected individuals: results of the "Probio-HIV" clinical trial. PLoS One 10: e0137200.
26. Di Pierro F, Colombo M, Zanvit A, Rottoli AS (2016) Positive clinical outcomes derived from using Streptococcus salivarius K12 to prevent streptococcal pharyngotonsillitis in children: a pilot investigation. Drug, Healthcare and Patient Safety 21: 77-81.
27. Garaiova I, Muchová J, Nagyová Z, Wang D, Li JV (2015) Probiotics and vitamin C for the prevention of respiratory tract infections in children attending preschool: A randomised controlled pilot study. European Journal of Clinical Nutrition 69: 373-379.
28. World Health Organization (2006) Probiotics in food, health and nutritional properties and guidelines for evaluation. Food and Nutritional Paper no. 85. FAO.
29. Chen K, Shanmugam NK, Pazos MA, Hurley BP, Cherayil BJ (2016) Commensal bacteria-induced inflammasome activation in mouse and human macrophages Is dependent on potassium efflux but does not require phagocytosis or bacterial viability. PLoS One 11: p.e0160937.
30. Sun KY, Xu DH, Xie C, Plummer S, Tang J, et al. (2017) Lactobacillus paracasei modulates LPS-induced inflammatory cytokine release by monocyte-macrophages *via* the up-regulation of negative regulators of NF-kappaB signaling in a TLR2-dependent manner. Cytokine 92: 1-11.
31. Moss JW, Davies TS, Garaiova I, Plummer SF, Michael DR (2016) A unique combination of nutritionally active ingredients can prevent several key processes associated with atherosclerosis *in vitro*. PLoS ONE 11: p.e0151057.
32. Man SM, Kanneganti TD (2015) Regulation of inflammasome activation. Immunological Reviews 265: 6-21.
33. Llewellyn A, Foey A (2017) Probiotic Modulation of Innate Cell Pathogen Sensing and Signaling Events. Nutrients 9: 1156.
34. Lyons C, Fernandes P, Fanning LJ, Houston A, Brint E (2015) Engagement of Fas on Macrophages Modulates Poly I:C Induced Cytokine Production with Specific Enhancement of IP-10. PLoS One 10: e0123635.
35. Pelegrin P, Barroso-Gutierrez C, Surprenant A (2008) P2X7 receptor differentially couples to distinct release pathways for IL-1beta in mouse macrophage. Journal of Immunology 180: 7147-7157.
36. Embry CA, Franchi L, Nuñez G, Mitchell TC (2011) Mechanism of impaired NLRP3 inflammasome priming by monophosphoryl lipid A. Science Signaling 4: ra28-ra28.
37. Raina MacIntyre C, Chughtai AA, Zhang Y, Seale H, Yang P, et al. (2017) Viral and bacterial upper respiratory tract infection in hospital health care workers over time and association with symptoms. BMC Infectious Diseases 17: 553.
38. Cui B, Liu W, Wang X, Chen Y, Du Q (2017) Brucella Omp25 upregulates miR-155, miR-21-5p, and miR-23b to inhibit interleukin-12 production *via* modulation of programmed Death-1 signaling in human monocyte/macrophages. Frontiers in Immunology 8: 708.
39. Liu J, Cao S, Herman LM, Max (2003) Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. The Journal of Experimental Medicine 198: 1265-1276.
40. Ma X, Yan W, Zheng H, Du Q, Zhang L (2015) Regulation of IL-10 and IL-12 production and function in macrophages and dendritic cells. F1000Res 4.
41. Onore CE, Schwartzer JJ, Careaga M, Berman RF, Ashwood P (2014) Maternal immune activation leads to activated inflammatory macrophages in offspring. Brain Behaviour and Immunity 38: 220-226.
42. Gordon S (2016) Phagocytosis: An Immunobiologic Process. Immunity 44: 463-475.