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# Interaction of mouse splenocytes and macrophages with bacterial strains *in vitro*: the effect of age in the immune response

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**RESEARCH ARTICLE**

## Abstract

Probiotics influence the immune system, both at the local and systemic level. Recent findings suggest the relation between microbiota and the immune system alters with age. Our objective was to address direct effects of six bacterial strains on immune cells from young and aged mice: *Lactobacillus plantarum* WCFS1, *Lactobacillus casei* BL23, *Lactococcus lactis* MG1363, *Bifidobacterium breve* ATCC15700, *Bifidobacterium infantis* ATCC15697, and *Akkermansia muciniphila* ATCC BAA-835. We used splenocytes and naïve or interferon-γ-stimulated bone marrow-derived macrophages (BMDM) as responder populations. All tested bacterial strains induced phenotypic and cytokine responses in splenocytes and BMDM. Based on magnitude of the cellular inflammatory response and cytokine profiles, two subgroups of bacteria were identified, i.e. *L. plantarum* and *L. casei* versus *B. breve*, *B. infantis,* and *A. muciniphila*. The latter group of bacteria induced high levels of cytokines produced under inflammatory conditions, including tumour necrosis factor (TNF), interleukin (IL)-6 and IL-10. Responses to *L. lactis* showed features of both subgroups. In addition, we compared responses by splenocytes and BMDM derived from young mice to those of aged mice, and found that splenocytes and BMDM derived from aged mice had an increased IL-10 production and dysregulated IL-6 and TNF production compared to young immune cells. Overall, our study shows differential inflammatory responses to distinct bacterial strains, and profound age-dependent effects. These findings, moreover, support the view that immune environment importantly influences bacterial immune effects.

**Keywords:** probiotics, bone marrow, spleen, aging, macrophage activation

## 1. Introduction

The human digestive tract is colonised by about $10^{14}$ commensal bacteria (Ley *et al.*, 2006; Luckey, 1972). Commensals digest fibres, inhibit pathogen growth, and modulate immunity (Ivanov and Littman, 2011). These commensals thus contribute to immune homeostasis in the gut, but also for instance in the bone marrow (BM) (Chu and Mazmanian, 2013). Live microorganisms that are present in foods and dietary supplements and that confer health benefits to the host are defined as probiotics (Borchers *et al.*, 2009). Probiotics affect the course of gastrointestinal inflammatory diseases like inflammatory bowel disease in a strain-dependent manner (Girardin and Seidman, 2011). Inflammatory conditions also characterise the aging process. The complex process of aging is accompanied, on the one hand, by decreased immune competence, and, on the other hand, by low-grade inflammation (Heintz and Mair, 2014; López-Otín *et al.*, 2013). Bacterial supplementations, therefore, might be beneficial to restore immune balance in aged individuals. Indeed, beneficial effects for health in elderly subjects were shown to be induced by several *Lactobacillus* and *Bifidobacterium* strains (Biagi *et al.*, 2012; Candore *et al.*, 2008; Lahtinen *et al.*, 2011; Tiihonen *et al.*, 2010). In addition, long-term supplementation with *Bifidobacterium animalis* subsp. *lactis* LKM512 extended lifespan and improved quality of life in mice (Matsumoto *et al.*, 2011).

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Bacterial supplementations can increase intestinal epithelial barrier function and compete with colonization of pathogens (Ivanov and Littman, 2011). One of the mechanisms underlying the crosstalk between bacteria and the host, is the presence of microbe-associated molecular patterns (MAMP) in bacteria. MAMP bind to pattern recognition receptors (PRR) present on host cells, among which mononuclear phagocytes, comprising macrophages and dendritic cells (DC), are prime responders. The activation of PRR results in cell maturation, cytokine secretion and upregulation of costimulatory molecules. By presenting antigens and secreting cytokines, macrophages and DC activate or suppress other immune cells and induce e.g. regulatory T cells in order to maintain gut homeostasis (Foligne *et al.*, 2007c; Karczewski *et al.*, 2010; Sherman *et al.*, 2009). Macrophages and DC are able to sample the gut lumen directly, in response to luminal bacteria (Chieppa *et al.*, 2006; Farache *et al.*, 2013; Rescigno *et al.*, 2001) and thus are thought to be important regulators of gut immunity.

The direct effects of probiotics on innate immunity in aging have not been studied. Our objective, therefore, was to address direct effects of different bacterial strains on immune cells derived from young and aged mice. We used splenocytes as a complex mix of immune cells (as a 'PBMC' collection) and macrophages as a versatile responder. To test the influence of exposure of the cells to immune stimuli, we included naïve (M0) and interferon-gamma (IFN-γ)-stimulated (M-IFN) macrophages (Murray *et al.*, 2014), generated from young and aged mice. Aged splenocytes and macrophages were included to gain insight in cell-intrinsic properties that alter in aging, affecting responses to bacterial supplementation.

We selected six bacterial strains, five of which have shown probiotic activity. *Lactobacillus plantarum* WCFS1 is a single colony isolate of strain NCIMB8826, isolated from human saliva (Vesa *et al.*, 2000). Proteins derived from *Lactobacillus casei* BL23 have been identified to be responsible for its beneficial health effects (Bäuerl *et al.*, 2010; Mazé *et al.*, 2010). *Lactococcus lactis* MG1363 is originating from cheese starter derivatives and is studied extensively for its use as prototype for bioactive molecule delivery in the gut (Foligne *et al.*, 2007b; Wegmann *et al.*, 2007). *Bifidobacterium breve* ATCC15700 is not widely studied. Other *B. breve* subspecies, however, induce anti-inflammatory responses after allergic sensitization (Hougee *et al.*, 2009). *Bifidobacterium infantis* ATCC15697 increases epithelial barrier function in the gut, and attenuates induced colitis in mice (Ewaschuk *et al.*, 2008). Presence of *Akkermansia muciniphila* has been inversely correlated to acute appendicitis (Swidsinski *et al.*, 2011). This bacterium is known as an intestinal mucus degrader and it is abundant in the human digestive tract (Belzer and De Vos, 2012; Van Passel *et al.*, 2011). The important *in vivo* findings are summarised per strain in Table 1.

In this study, we addressed the effect of immune environment and age on the *in vitro* response by splenocytes and macrophages to bacterial supplementations.

**Table 1. Properties of tested bacterial strains.**

| Species | Strain | Origin | *In vivo* activities[1] | References |
|---|---|---|---|---|
| *Lactobacillus plantarum* | WCFS1; DSMZ20174; ATCC BAA-793 | human pharynx | anti-allergic (Betv1); pro-allergic (peanut); increased # splenic Tregs; reduced HFD-induced pathology; trend to protection in TNBS colitis | Daniel *et al.*, 2006; Derrien *et al.*, 2011; Ivanovic *et al.*, 2015; Kleerebezem *et al.*, 2003; Marco *et al.*, 2009; Meijerink *et al.*, 2012; Repa *et al.*, 2003; Snel *et al.*, 2011 |
| *Lactobacillus casei*[T] | BL23; plasmid-cured ATCC393 | dairy products | protective in TNBS colitis | Foligne *et al.*, 2007b; Mazé *et al.*, 2010 |
| *Lactococcus lactis* | MG1363 | dairy products | not reported | Foligne *et al.*, 2007a; Foligne *et al.*, 2007b; Smelt *et al.*, 2012 |
| *Bifidobacterium breve*[T] | ATCC15700; DSM20213 | infant intestine | no protection against *P. aeruginosa*[2] | Matsumoto *et al.*, 2008 |
| *Bifidobacterium infantis*[T] | ATCC15697; DSM20088 | infant intestine | [3] | |
| *Akkermansia muciniphila*[T] | ATCC BAA-835 | adult intestine | protection against obesity and T2D; increased # goblet cells and VAT Tregs | Derrien *et al.*, 2011; Everard *et al.*, 2013; Kang *et al.*, 2013; Shin *et al.*, 2014 |

[T] A type strain defines a species and is representative of that species. Literature findings referring to the same species but with other strain numbers are indicated with a number.

[1] HFD = high fat diet; T2D = type 2 diabetes; TNBS = 2,4,6-trinitrobenze sulfonic acid; Treg = regulatory T helper cell; VAT = visceral adipose tissue.

[2] Anti-allergic (Hougee *et al.*, 2009).

[3] Protection against Rotavirus and protection against dextran sulphate sodium induced colitis (Muñoz *et al.*, 2011; Osman *et al.*, 2006).

## 2. Materials and methods

### Bacterial cultures

*L. plantarum*, *L. casei*, *L. lactis*, *B. breve*, *B. infantis* and *A. muciniphila* were grown until stationary phase was reached. *L. lactis* was grown on M17 medium (Merck, Darmstadt, Germany), *A. muciniphila* was grown on mucin-based medium (Derrien *et al.*, 2004), whereas the other four strains were grown on De Man Rogosa and Sharpe medium (Merck). *B. breve*, *B. infantis*, and *A. muciniphila* were cultured under strictly anaerobic conditions. Viability and cfu were checked by plating and measuring the $OD_{600}$. The bacterial batches were freshly cultured for each individual experiment.

### Mice

Male 7-week-old C57Bl/6J mice were purchased from Harlan (Horst, the Netherlands). Male 18-month-old C57Bl/6J mice were purchased from Janvier (Saint-Berthevin, France) and housed for 7 months at the animal facility of Wageningen University. All animals were specific pathogen free, and had free access to water and feed. Mice were fed D12450B diet (Research Diet Services, Wijk bij Duurstede, the Netherlands). All experiments were performed with approval of the animal care and use committee of Wageningen University. Young mice were sacrificed between 8-12 weeks of age and aged mice at 25 months of age.

### Spleen cultures

Mice were sacrificed by anaesthesia with isoflurane. Spleen single cells suspensions were obtained by disrupting the organs and passing cells through a cell strainer. Lysis of erythrocytes was performed using RBC lysis buffer (eBioscience, San Diego, CA, USA). About $10^6$ fresh total spleen cells were cultured in 48-wells plates and stimulated with 200 ng/ml lipopolysaccharides (LPS) (*Escherichia coli* 055:B5, Sigma-Aldrich, Zwijndrecht, the Netherlands) + 50 ng/ml recombinant mouse IFN-γ (BioLegend, San Diego, CA, USA) or 0.1, 1 or 10 cfu of viable bacterial strain cultures per splenocyte in RPMI 1640 medium (Gibco, Breda, the Netherlands) supplemented with 10% foetal calf serum (Gibco), 100 U/ml penicillin-streptomycin (Gibco), and 50 μM β-mercaptoethanol (Sigma-Aldrich). IFN-γ was included to mimic an immune-activated state and to enhance the response by e.g. macrophages and T cells present in the culture. Supernatants were harvested after 24 h stimulation and stored maximally one month at -20 °C for cytokine analysis.

### Macrophage cultures

Femora were flushed to obtain BM cells, which were passed through a cell strainer. Half a million BM cells were cultured in 24-well plates in the presence of 10% Ladmac-conditioned medium (Sklar *et al.*, 1985) and 10 ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF) (CSF-1, eBioscience) to generate bone marrow-derived macrophages (BMDM, hereafter called M0 macrophages). After 6 days, BMDM were stimulated overnight with 200 ng/ml LPS as a positive control or with viable bacterial strains (1 cfu per 1 BMDM). To generate BMDM-IFN (hereafter called M-IFN macrophages), 50 ng/ml IFN-γ was added to the standard culture medium along with overnight (18 h) stimulation. Cells were harvested for flow cytometry and supernatants were stored frozen for later cytokine analysis.

### Flow cytometry

Flow cytometry was performed using standard procedures. Macrophages were stained with monoclonal antibodies for MHC-II-FITC (M5/114.152, eBioscience), F4/80-PerCP-Cy5.5 (BM8, eBioscience), CD11c-PE-Cy7 (N418, eBioscience), CD86-APC (GL1, eBioscience), CD11b-APC-Cy7 (M1/70, BD Biosciences, Erembodegem, Belgium), and CD54-PB (ICAM-1; YN1/1.7.4, BioLegend). Fluorescent signals were acquired using a BD FACSCanto II (BD Biosciences). Data were analysed with FlowJo vX.07 (Tree Star, Ashland, OR, USA) software.

### Cytokine measurements

Interleukin (IL)-12p70, tumour necrosis factor (TNF), IFN-γ, chemokine (C-C motif) ligand 2 (CCL2)/ monocyte chemotactic protein 1 (MCP-1), IL-10, and IL-6 concentrations in the supernatants of splenocyte and BMDM cultures were determined using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences), according to the manufacturer's instructions.

### Statistical analysis

Data are expressed as average ± standard error of the mean, unless otherwise stated. One-way ANOVA and subsequent Bonferroni post-hoc tests were performed to test differences between bacterial strains. GraphPad Prism version 5.0.3 (San Diego, CA, USA) was used to perform statistical tests.

## 3. Results

### Dose-response relation for bacterial stimulation on splenocytes

To determine the dose-response effects of bacterial strains on the immune cells studied, we used cultures of splenocytes, and after 24 h stimulation measured production of cytokines produced under inflammatory conditions. Splenocytes were incubated with three concentrations of bacteria (ratio bacteria:splenocytes of 1:10, 1:1, 10:1). A positive dose-response relation was observed for all bacterial strains (Figure 1). Both *Bifidobacterium* strains

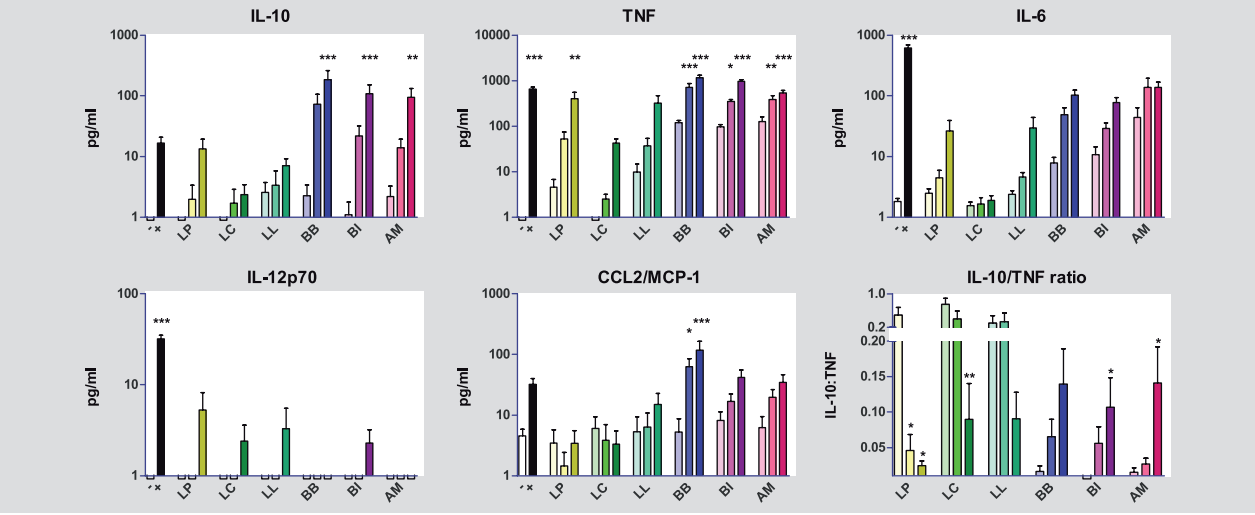


**Figure 1. Dose-response relation in cytokine responses of young splenocytes to different bacterial stimulations. Mean cytokine concentrations of the indicated splenocyte supernatants, as determined by cytometric bead array. For bacterial stimulations (LP – AM), each first bar of three represents the lowest dose of 1 cfu per 10 splenocytes, the middle bar 1 cfu per 1 splenocyte and each last bar of three represents the highest dose of 10 cfu per 1 splenocyte. The lower right panel represents the IL-10/TNF ratio for each condition. Significant differences compared with medium control or with lowest bacterial dose for the IL-10/TNF ratio are indicated: * = *P*<0.05; **= *P*<0.01; *** = *P*<0.001. – = medium control; + = LPS+IFN-γ; LP = *Lactobacillus plantarum*; LC = *Lactobacillus casei*; LL = *Lactococcus lactis*; BB = *Bifidobacterium breve*; BI = *Bifidobacterium infantis*; AM = *Akkermansia muciniphila*. Data represent the mean + standard error of the mean determined in three independent experiments, using splenocytes from a single mouse in each experiment.**

and *A. muciniphila* induced the highest levels of IL-10 (to 200 pg/ml), TNF (to 1000 pg/ml), IL-6 (to 100 pg/ml), as well as CCL2/MCP-1 (to 100 pg/ml). After applying the highest dose of some of the bacterial strains, or after applying positive control LPS+IFN-γ, only low levels of IL-12p70 were detected. These low levels of IL-12p70 were expected, as IFN-γ is necessary to induce IL-12p70 (instead of IL-12p40) production (Hayes *et al.*, 1995; Yi *et al.*, 2002).

We calculated the IL-10/TNF ratio for each bacterial dose as a measure of anti- and pro-inflammatory cytokine balance (Figure 1). The *Bifidobacterium* strains and *A. muciniphila* showed an increasing IL-10/TNF ratio with increasing dose, implicating that higher doses of those strains preferentially stimulated IL-10 production rather than TNF production. Both *Lactobacillus* strains and *L. lactis*, in contrast, showed a decreasing IL-10/TNF ratio with increasing bacterial dose.

These data indicate that two distinct subgroups of bacterial strains are identified, independent of the applied dose: *Lactobacillus* strains and *L. lactis*, inducing lower levels of inflammatory cytokines than the *Bifidobacterium* strains and *A. muciniphila*. These different profiles are also reflected in decreasing and increasing IL-10/TNF ratios, with increasing bacterial dose.

### Differential phenotypic response by macrophages on different bacterial strains

Next, we studied the effects of the distinct types of bacterial strains on naïve (M0) and IFN-γ-stimulated (M-IFN) BMDM in order to determine whether the immune environment of the macrophages is of influence on the responsiveness. As sentinel cells, macrophages are known to be fast and potent in their responses (as confirmed in our experiments). To detect differences between bacterial strains at the most sensitive level, we incubated the macrophages overnight with 1 cfu per macrophage, and assessed phenotypic changes by flow cytometry. Mature macrophages were defined as $F4/80^{+}CD11b^{+}$ (Figure 2A). These were gated for further analysis. Bacterial stimulation and IFN-γ stimulation did not affect CD11b expression of macrophages from young mice, allowing appropriate gating after all stimulations (Supplementary Figure S1). M0 macrophages responded phenotypically upon incubation with LPS or bacteria by increasing CD54 expression, and frequently up-regulating F4/80. In incidental cases MHC class II or CD86 expression was found to be increased in M0 macrophages (Figure 2B). Most marked changes were observed with *A. muciniphila* (CD54, CD86, and F4/80), *L. lactis* (CD54 and MHC-II; Figure 2B) and both *Bifidobacterium* strains (CD54 and F4/80). *L. plantarum* induced higher expression of only CD54, as compared with the control. *L. casei* stimulation caused no phenotypic changes in M0 macrophages. Priming of macrophages with

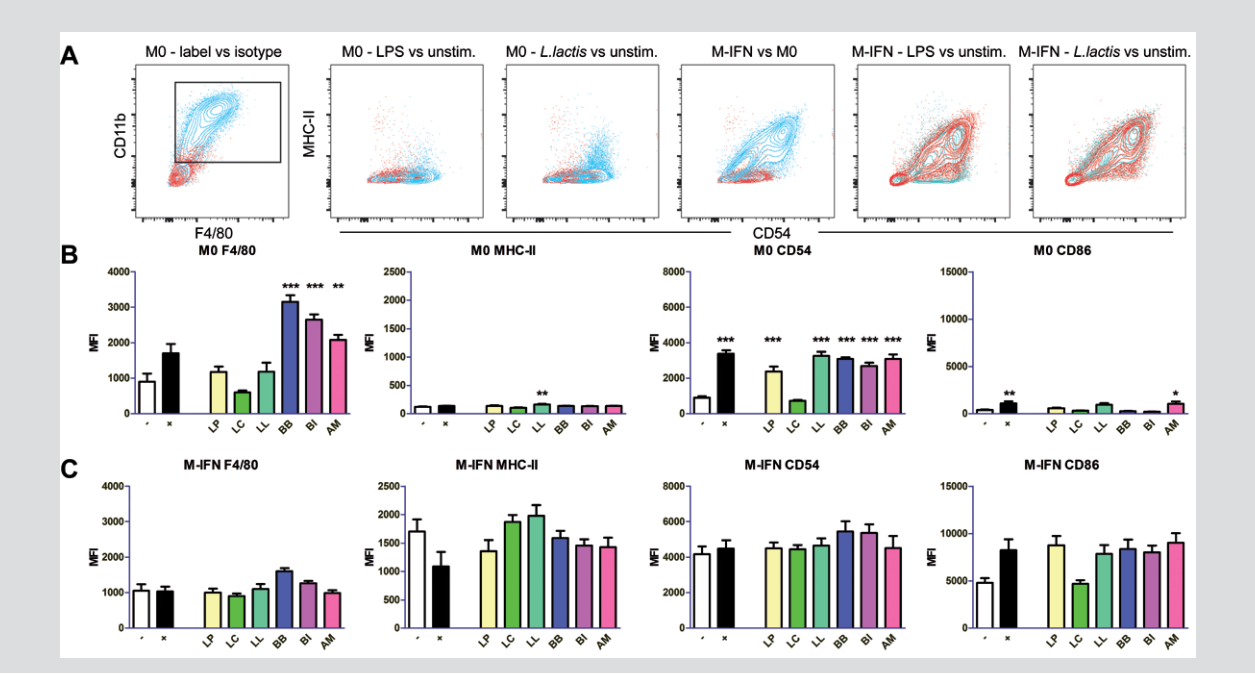


**Figure 2. Phenotypic analysis of M0 and M-IFN macrophages derived from young mice stimulated with different bacterial strains. (A) Mature macrophages were identified using gating of F4/80$^+$CD11b$^+$ M0 macrophages (blue, left panel; cf. isotype control in red). Subsequent panels show representative examples of CD54- and MHC-II expression by treated (blue) vs control (red) macrophages in different immune environments. (B) Marker expression (indicated as median fluorescence intensity (MFI)) by M0 macrophages stimulated overnight with different bacterial strains. (C) Marker expression by IFN-γ-primed macrophages, simultaneously stimulated with different bacterial strains. Significant differences relative to unstimulated medium control are indicated: * = *P*<0.05; ** = P<0.01; *** = P<0.001. x-axis legend as indicated in Figure 1. Data represent the mean + standard error of the mean determined in three independent experiments, using bone marrow from a single mouse in each experiment.**

IFN-γ caused a prominent increase in CD54, CD86, and MHC-II expression (Figure 2C). In general, simultaneous stimulation with bacteria and IFN-γ did not further change surface marker expression on M-IFN of CD11b, CD54, F4/80, and MHC-II (Figure 2C). One-way ANOVA for analysis of variance revealed differences in CD86 expression, comparing all conditions ($P$=0.002). A trend for increasing CD86 expression was observed for all bacterial stimulations except *L. casei*.

Together, these data demonstrate that M0 macrophages respond phenotypically to bacterial stimulation. IFN-γ stimulation exerts a prominent effect on macrophage phenotype, which is hardly influenced by simultaneous bacterial stimulation. Furthermore, a clear qualitative difference between bacterial strains is observed, with *L. casei* being the only bacterial strain not changing macrophage phenotype at all. Finally, based on changes in F4/80 expression induced in M0 macrophages, the same two subgroups of bacteria (*L. plantarum, L. casei,* and *L. lactis* versus *B. breve, B. infantis,* and *A. muciniphila*) were observed as identified before.

## Bacterial exposure differentially stimulates macrophage cytokine secretion

Upon activation, macrophages are capable of producing high amounts of cytokines. Therefore, we studied cytokine secretion by differentially stimulated macrophages. IL-10 secretion was only detectable when M0 macrophages were stimulated with LPS, the bifidobacteria or *A. muciniphila* (Figure 3). This secretion profile was similar in M-IFN macrophages with IL-12p70 instead of IL-10 secretion. The highest IL-10 production in M0 was observed upon stimulation with *A. muciniphila*. TNF and IL-6 production was observed in both M0 and M-IFN macrophages, in particular after incubation with the bifidobacteria and *A. muciniphila*. CCL2/MCP-1 production was increased upon *B. infantis* and *A. muciniphila* incubation, in M0 macrophages as well as in M-IFN macrophages. The IL-10/TNF ratio of M0 macrophages revealed that *A. muciniphila* mostly increased IL-10 production (compared to TNF production; Figure 4). Next to the IL-10/TNF ratio, we calculated a production index, by normalizing IL-10+TNF production upon bacterial stimulation against IL-10+TNF production upon LPS stimulation. The production index indicates a clear difference in induction of IL-10 and TNF production between the tested lactobacilli

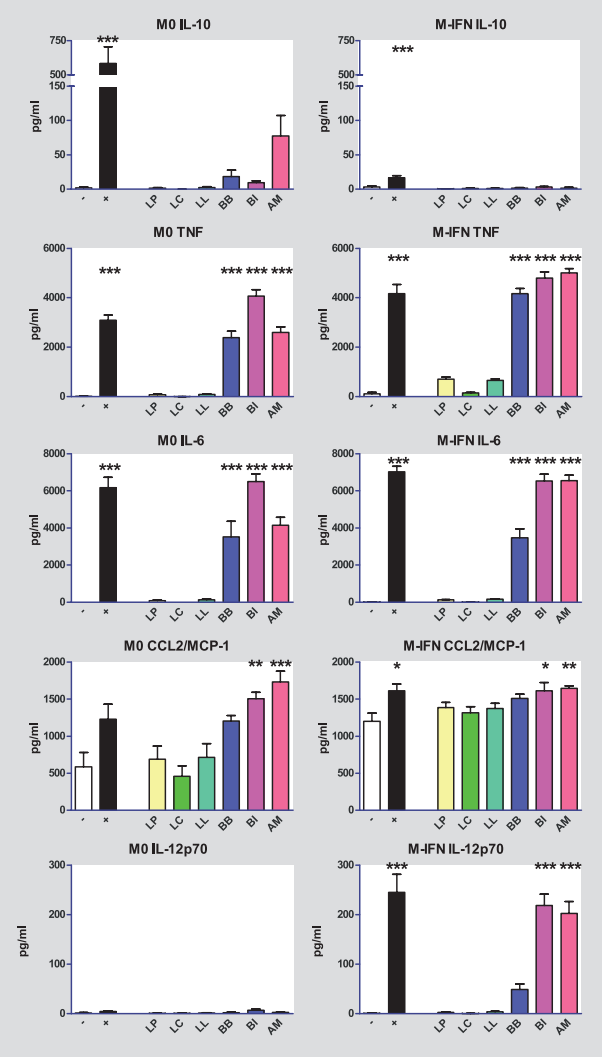


**Figure 3. Effect of bacterial strains on mean cytokine production in young M0 and M-IFN macrophages. Bars represent mean cytokine production by M0 (upper panels) and M-IFN macrophages (lower panels) upon bacterial stimulations, measured with cytometric bead array. Significant differences compared to medium control: * = *P*<0.05; ** = *P*<0.01; *** = *P*<0.001. x-axis legend as indicated in Figure 1. Data represent the mean + standard error of the mean determined in three independent experiments, using bone marrow from a single mouse in each experiment.**

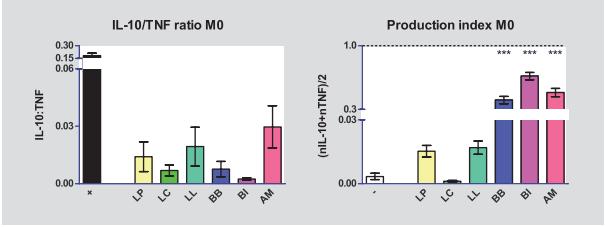


**Figure 4. IL-10/TNF ratio and cytokine production index of young M0 macrophages stimulated with different bacterial strains. The production index indicates normalised IL-10 + normalised TNF levels, divided by 2. Normalisation was performed against lipopolysaccharides (LPS; resulting in a value of 1 for LPS indicated by the dotted line). Significant differences: *** = *P*<0.001. x-axis legend as indicated in Figure 1. Error bars represent 95% confidence intervals.**

and *Lactococcus* on one side, and the bifidobacteria and *Akkermansia* on the other side. Application of IL-10/TNF ratio for M-IFN is impossible, because IFN-γ priming blocks IL-10 secretion nearly completely in most conditions.

These data show differential effects of bacterial strains on cytokine production by macrophages. As in splenocyte cytokine production and macrophage phenotype, a division between the *Lactobacillus* strains and *Lactococcus* on one hand, and the *Bifidobacterium strains* and *Akkermansia* on the other hand is indicated by the greater capacity of the latter to induce inflammatory cytokines, in particular TNF and IL-6.

### Age-dependent shift in cytokine production by splenocytes

To investigate whether the response to bacterial strains does change during aging, we isolated spleens from aged (25-months-old) mice, and compared the reactivity of splenocytes to that of young (8-12-weeks-old) mice. Splenocytes were incubated with bacteria in a 1:1 ratio. Aged splenocytes produced markedly higher levels of IL-10, as compared with young splenocytes, upon stimulation with virtually any of the bacterial strains (Figure 5, Supplementary Table S1). Upon LPS+IFN-γ stimulation, used as positive control for inflammatory cytokine induction, production of TNF, IL-6, IL-12p70, and CCL2/MCP-1 was lower in aged splenocytes compared with young splenocytes. This down-modulation was not generally observed upon stimulation with bacteria. In particular, TNF production was enhanced in aged splenocytes in response to bacteria (Supplementary Table S1). The response to *L. lactis*, the *Bifidobacterium* strains and *A. muciniphila* slightly changed with age. *B. breve*-stimulated splenocytes from aged mice in particular did not show changes in cytokine responses compared to young splenocytes; only CCL2/MCP-1 production was decreased. The IL-10/TNF ratio was greater in aged splenocytes than in young splenocytes for most of the conditions, except for *L. casei* (Figure 5). *L. casei* showed an increased cytokine induction in aged splenocytes, as compared with young splenocytes. The levels induced by *L. casei*, however, did not reach those induced by the *Bifidobacterium* strains or *Akkermansia* (Supplementary Table S1).

These data show an age-dependent shift of cytokine responses towards IL-10. The increase is stronger when comparing IL-10 to TNF, IL-6, and CCL2/MCP-1 production. It also indicates that certain bacterial strains exert a different effect on aged immune cells than on young immune cells. Particularly, *L. casei* showed increased induction of responses

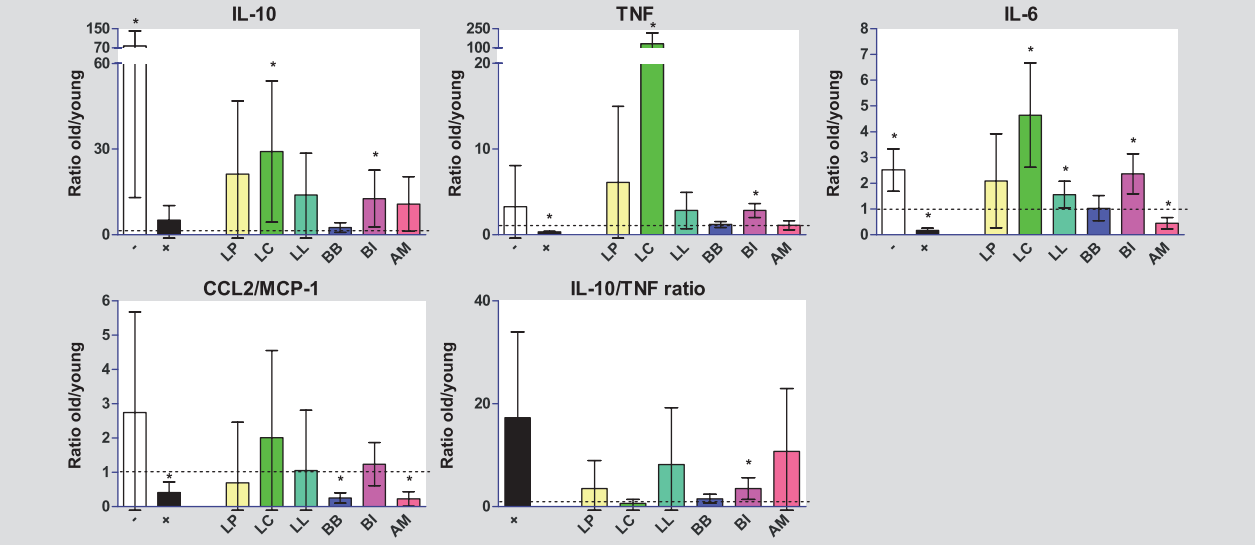


**Figure 5. Aging affects cytokine responses of splenocytes, in particular by increasing IL-10 levels. Cytokine levels measured in cultures of aged splenocytes stimulated with different bacterial strains are expressed as a ratio to cytokine levels from similarly stimulated young splenocytes. Significant differences are indicated by: * = *P*<0.05. X-axis legend as indicated in Figure 1. Data represent the means of six independent experiments. Error bars represent 95% confidence intervals. The dotted line shows the reference value (1) in young mice.**

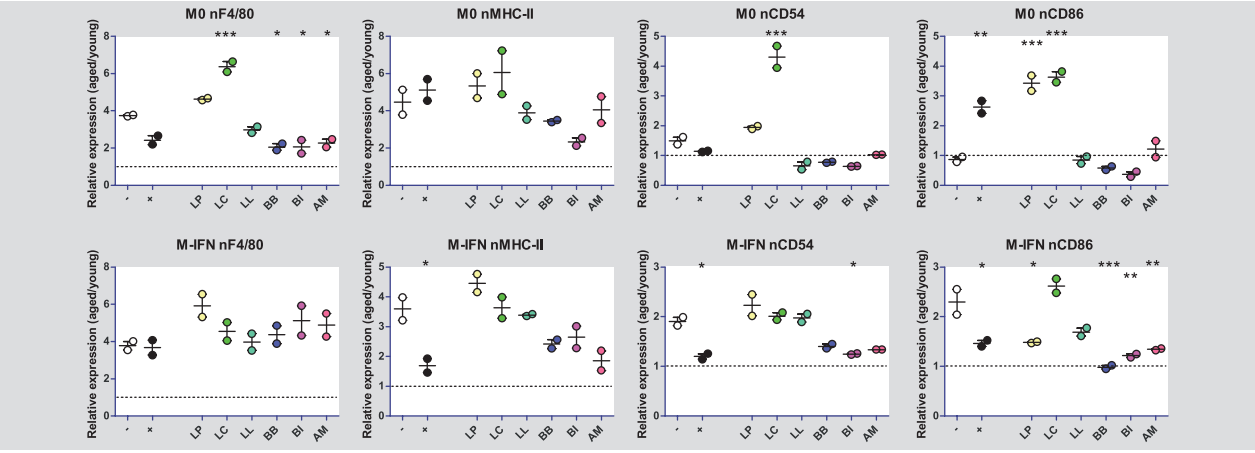


**Figure 6. Aging affects the phenotype of M0 and M-IFN macrophages. Phenotypic data are represented after normalisation (indicated as nF4/80, nMHC-II, etc.) against the stable surface marker CD11b, due to differences in absolute median fluorescence intensity induced by executed maintenance and calibration on the flow cytometer between experiments. Subsequently, normalized values from aged macrophages are divided by the average value from young macrophages. Each symbol represents a culture from an individual animal. Significant differences are indicated by: * = *P*<0.05. X-axis legend as indicated in Figure 1. The dotted line shows the reference value (1) in young mice.**

in aged splenocytes, while it was non-responsive in young immune cells.

### Aging affects macrophage phenotype and cytokine responses to bacteria

Next, we compared the effects of bacterial strains on macrophages from aged mice to those of young in surface marker expression and cytokine production. Due to altered signal acquisition in flow cytometry (caused by technical maintenance in between experiments), we were unable to compare values directly side-by-side, but approached this by normalizing MFI values for each surface marker against the stable surface marker CD11b. F4/80 and MHC-II were upregulated with age under all conditions (Figure 6). The division between the *Lactobacillus* strains and *L. lactis* stimulation on the one hand, and the *Bifidobacterium* strains and *A. muciniphila* stimulation on the other hand, was also observed in various aspects in aged macrophage phenotypic changes. Compared to unstimulated control,

stimulation with *L. lactis*, both *Bifidobacterium* strains, or *A. muciniphila* resulted in a relatively lower increase of F4/80 expression in aged M0 macrophages than in young M0 macrophages, and a lower increase of MHC-II, CD54, and CD86 expression in aged M-IFN macrophages than in young M-IFN macrophages (Figure 6). In contrast, stimulation with either of the *Lactobacillus* strains resulted in a relatively higher increase of F4/80 expression in aged M0 macrophages than in young M0 macrophages.

Changes for different stimuli were also observed in cytokine profiles when macrophages from young and aged mice were compared. IL-10 secretion by M0 macrophages increased with age in response to most bacterial stimulations (Figure 7, Supplementary Table S2). In response to LPS, however, IL-10, TNF, and IL-6 levels were lower in aged BMDM, whereas CCL2/MCP-1 levels were higher, as compared with young BMDM. Relative production of TNF was reduced in aged M0 macrophages upon exposure to the *Bifidobacterium* strains and *A. muciniphila* incubation, but not in *L. plantarum* and *L. casei*-stimulated M0 macrophages (Figure 7). *L. lactis*-induced cytokine responses by aged macrophages were without exception down or similar compared to young macrophages. Similarly, IL-6 production was reduced with aging in M0 and M-IFN macrophages upon some stimulations (except for *L. casei*-stimulated macrophages and *L. plantarum*-stimulated M-IFN macrophages; Figure 7). It is noteworthy that *L. casei* induced an increased cytokine response in macrophages derived from old mice compared to those from young mice. However, in absolute values, the differences are rather small (Supplementary Tables S2 and S3).

In general, the differential effects of the previously identified subgroups of bacteria are also evident in macrophages from aged mice in the relative increase (*Lactobacillus* strains) or decrease (*Bifidobacterium* strains and *Akkermansia*) of surface markers and cytokines. However, the difference between the various bacterial strains observed in young cultures (Figure 1-4), is less pronounced in aged cultures (Supplementary Tables S2, S3). These data indicate that aging impacts the response of splenocytes and macrophages to bacteria.

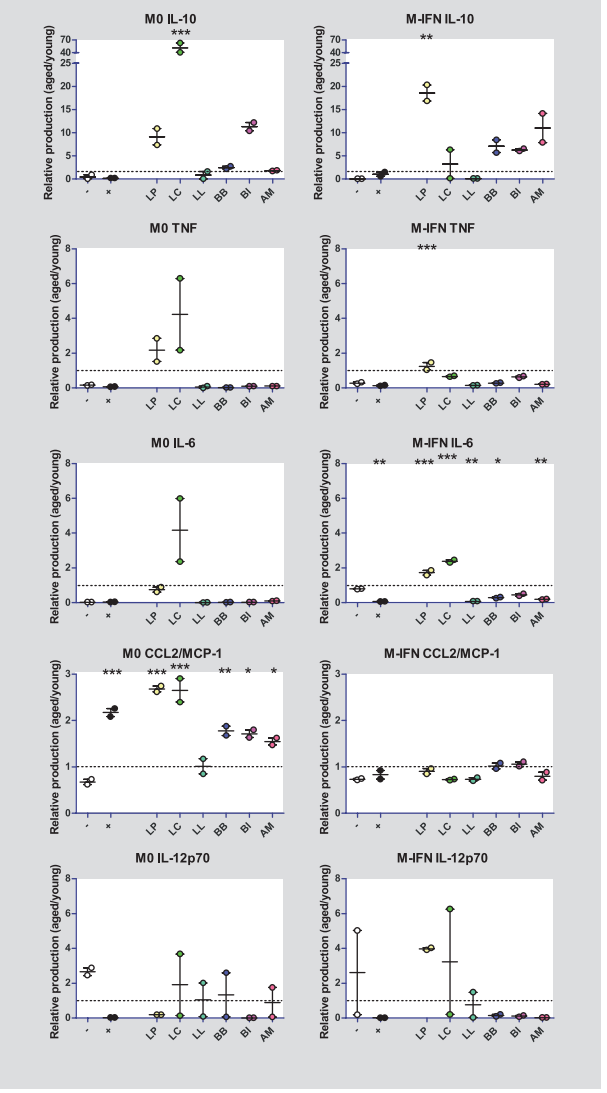


**Figure 7. Age-dependent changes in cytokine response by M0 macrophages to bacterial stimulations. Cytokine levels measured in cultures of aged macrophages stimulated with different bacterial strains are expressed as a ratio to cytokine levels from similarly stimulated young macrophages. Individual values are divided by the average value derived from young macrophages. Each symbol represents a culture from an individual animal. X-axis legend as indicated in Figure 1. The dotted line shows the reference value (1) in young mice.**

## 4. Discussion

In this study, we used splenocyte and BM-derived macrophage cultures to investigate the effect of immune environment and age on immune activation induced by bacteria potentially used as food supplement. Differential reactivity to two groups of bacterial strains was consistently observed in splenocyte and macrophage cultures, i.e. the *Lactobacillus plantarum* WCFS1 and *Lactobacillus casei* BL23 strains and *Lactococcus lactis* MG1363 inducing rather moderate responses compared to the positive control (LPS±IFN-γ), the *Bifidobacterium breve* ATCC15700 and *Bifidobacterium infantis* ATCC15697 strains, and *Akkermansia muciniphila* ATCC BAA-835. Aging alters the inflammatory response of immune cells to bacteria. The IL-10 secretion, in particular, by aged splenocytes was greater than the IL-10 secretion by young splenocytes. The same trend for greater IL-10 production was observed in BMDM, in particular when comparing IL-10 production to IL-6 and TNF production.

In probiotic research, PBMC and DC are commonly used to select immune-active bacterial strains (Foligne *et al.*,

2007b; Meijerink *et al.*, 2010; Van Hemert *et al.*, 2010). These studies did not take into account different immune environment and age of responder cells. The rationale in the current study was to compare the responsiveness of M0 and M-IFN, i.e. different immune environments. M0 macrophages, i.e. naïve or M-CSF-primed macrophages, reacted differently to the same bacterial strains as M-IFN macrophages. Our data therefore suggest to focus not only on one type of immune environment but on different types of mononuclear phagocytic cells to gain insight into the immune-potentiating effects of bacterial strains.

Many previous studies testing direct bacteria-immune interactions *in vitro* made use of human PBMC in which IL-10/IL-12 ratios were taken as a measure for pro- or anti-inflammatory capacity (Snel *et al.*, 2011; van Hemert *et al.*, 2010). In the current study, we used splenocytes as a PBMC collection. We could not apply IL-10/IL-12 ratios as IL-12p70 is produced in very low amounts in mice, and only induced in the presence of IFN-γ plus a simultaneous stimulation such as LPS. We considered measuring IL-12p40 values instead, but deemed such results difficult to interpret in inflammatory terms, since p40 subunits may occur either as single chains, as heterodimers with p35, p19 or other partners with pro- or unknown inflammatory activity, or even as inhibitory p80 dimers (Abdi *et al.*, 2014; Gee *et al.*, 2009). Therefore, we applied IL-10/TNF ratios. From a previous study, it is clear that TNF and IL-12p70 production by human monocyte-derived DC is closely correlated (Meijerink *et al.*, 2010), and also in our study we have an indication for such a correlation.

Despite the conceptual differences, comparing responses by freshly isolated spleen cells with those by BMDM, the results obtained in these culture systems generally were in close agreement. The bacterial strains that induced the greatest responses upon direct contact, responded similarly in splenocyte and in macrophage cultures. However, calculated per cell, splenocyte cultures produced much less cytokines than macrophage cultures. Therefore, higher doses might be needed to measure certain cytokines in splenocyte supernatants. Most cytokine levels were below or around detection limits when using the lowest dose of certain bacterial strains, but most of the cytokines could be detected when using a higher dose. This dose-response relation is important when applying bacteria *in vivo*. A limitation of this study is that we only determined the response of macrophages to a single bacterial dose, and thus could not establish whether the two groups of bacteria showed a similar decreasing and increasing IL-10/TNF ratio, respectively, upon stimulation with increasing bacterial doses. However, it is evident that BMDM are more potent in their response to bacteria compared to splenocytes. Therefore, the optimal dose for stimulating splenocyte cultures is probably much higher than for macrophages. In addition, measuring multiple time points after addition of bacterial stimulations would have given insight in the kinetics of the observed immune responses.

Based on macrophage phenotype, and splenocyte and macrophage cytokine responses, we identified two subgroups of bacteria (*L. plantarum* WCFS1 and *L. casei* BL23 versus *B. breve* ATCC15700, *B. infantis* ATCC15697 and *A. muciniphila* ATCC BAA-835). It is remarkable, that *A. muciniphila*, as the only tested gram-negative species, induces similar responses as the *Bifidobacterium* strains. Furthermore, the first group represents facultative anaerobic bacteria, whereas the second group includes strictly anaerobic bacteria. *L. lactis* MG1363 resides mostly in the first group, but sometimes induced different responses, depending on the culture system we used. It is the only included strain which is generally recognized as safe for human consumption, but non-probiotic (Foligne *et al.*, 2007a). We demonstrate that this strain is inducing a weak inflammatory response, which could support its widely studied function in bioactive molecule delivery in the gut (Wegmann *et al.*, 2007). With regard to the differences between bacterial strains, the choice for standardising the doses based on cfu might have influenced the response triggered by the different strains. For instance, the biomass per cfu will most likely differ between bacterial strains, which might have had an impact on the triggered response.

IL-10/IL-12 or IL-10/TNF ratios can be valuable to assess pro- or anti-inflammatory capacities of bacterial strains in order to predict *in vivo* responses (Foligne *et al.*, 2007b). They are, however, inappropriate when applying to e.g. aging individuals, considering the fact that Toll-like receptor (TLR)-mediated responses are affected by aging (Kollmann *et al.*, 2012). We also observed that the IL-10/TNF ratio in response to bacterial stimulations changes with age, which is probably mediated by age-acquired TLR signalling defects (Boehmer *et al.*, 2005; Chelvarajan *et al.*, 2006) and dysregulated expression of the negative feedback regulator of TLR signalling miR-146a (Jiang *et al.*, 2012). Besides, responsiveness to LPS is reduced, supporting the evidence for TLR4-signalling defects in aged immune cells (Sebastián *et al.*, 2005). In general, IL-10 production was markedly increased in splenocytes and macrophages. This was reported by several previous studies (Boehmer *et al.*, 2005; Kollmann *et al.*, 2012). Further research is necessary to investigate the underlying mechanisms. On the bacterial side, mutants could play an important role by elucidating the role of surface molecules or secreted factors (e.g. short-chain fatty acids) by bacteria (Górska *et al.*, 2014). On the host side, IL-10 blocking or IL-12p70 supplementation might be interesting treatments to restore the response to bacterial supplementation in aged cells. A technical note of caution related to the interpretation of age-related differences is that the supplier of aged mice was different from the supplier of young mice, which most likely resulted in a difference in the microbiota composition.

The microbiota composition has a crucial role in priming several immune cell types (Chu and Mazmanian, 2013). The fact that the obtained cells from young and aged mice could have been differently primed *in vivo*, might have influenced the outcome of this study.

Aging effects found in splenocytes (isolated as primary cells from aged mice) are similar to those observed in BM-derived macrophages. The latter are derived from aged BM precursor cells, which have undergone multiple cell cycles *in vitro*, up to 8-9, before being exposed to bacteria. The finding that similar age-related changes have been retained in these cells compared to freshly isolated splenocytes implies that differences, probably due to epigenetic changes during aging (Li *et al.*, 2011; Olivieri *et al.*, 2013; Warren and Rossi, 2009), are preserved during *in vitro* multiplication and differentiation of the cells. In accordance, it has been reported that hematopoietic stem cells are epigenetically dysregulated with age (Chambers *et al.*, 2007; Sun *et al.*, 2014). Another study, using a spermatogonial stem cell culture of over two years, demonstrated a remarkable imprinting potential and a resilience to epigenetic modifications *in vitro* (Kanatsu-Shinohara *et al.*, 2005).

Taking the differential effects of age on the immune response into account, it can be envisaged that probiotics that are beneficial in children, might have no favourable effect in elderly, and vice versa. For example, the reported beneficial effect of probiotic cheese containing *L. rhamnosus* HN001 and *L. acidophilus* NCFM in elderly (Lahtinen *et al.*, 2011), should be tested in children and adults to confirm its positive effect in different age classes. Acknowledging the generic adverse effects of age on functions of all body cells, including immune cells, we expect different effects of probiotics in children, adults, and elderly. A side-by-side study with children, adults, and elderly would thus provide insight into the *in vivo* interaction between probiotics and host, and the effect of age on this interaction.'

In conclusion, we observed that the response of splenocytes and macrophages on stimulation with different bacterial strains is altered with aging. It underlines the caution which is needed when translating findings in young immune cells or individuals to aged cells or elderly individuals.

## Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/BM2015.0094.

Figure S1. CD11b expression unaltered after stimulation of M0 and M-IFN macrophages derived from young mice with bacterial strains stimulated with different bacterial strains.

Table S1. Mean cytokine secretion by splenocytes from young and old mice.

Table S2. Mean cytokine secretion by M0 macrophages from young and old mice.

Table S3. Mean cytokine secretion by M-IFN macrophages from young and old mice.

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## References

Abdi, K., Singh, N.J., Spooner, E., Kessler, B.M., Radaev, S., Lantz, L., Xiao, T.S., Matzinger, P., Sun, P.D. and Ploegh, H.L., 2014. Free IL-12p40 monomer is a polyfunctional adaptor for generating novel IL-12-like heterodimers extracellularly. Journal of Immunology 192: 6028-6036.

Bäuerl, C., Pérez-Martínez, G., Yan, F., Polk, D.B. and Monedero, V., 2010. Functional analysis of the p40 and p75 proteins from *Lactobacillus casei* BL23. Journal of Molecular Microbiology and Biotechnology 19: 231-241.

Belzer, C. and De Vos, W.M., 2012. Microbes inside – from diversity to function: the case of *Akkermansia*. ISME Journal 6: 1449-1458.

Biagi, E., Candela, M., Fairweather-Tait, S., Franceschi, C. and Brigidi, P., 2012. Ageing of the human metaorganism: the microbial counterpart. Age 34: 247-267.

Boehmer, E.D., Meehan, M.J., Cutro, B.T. and Kovacs, E.J., 2005. Aging negatively skews macrophage TLR2-and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway. Mechanisms of Ageing and Development 126: 1305-1313.

Borchers, A.T., Selmi, C., Meyers, F.J., Keen, C.L. and Gershwin, M.E., 2009. Probiotics and immunity. Journal of Gastroenterology 44: 26-46.

Candore, G., Balistreri, C.R., Colonna-Romano, G., Grimaldi, M.P., Lio, D., Listi', F., Scola, L., Vasto, S. and Caruso, C., 2008. Immunosenescence and anti-immunosenescence therapies: the case of probiotics. Rejuvenation Research 11: 425-432.

Chambers, S.M., Shaw, C.A., Gatza, C., Fisk, C.J., Donehower, L.A. and Goodell, M.A., 2007. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS Biology 5: e201.

Chelvarajan, R.L., Liu, Y., Popa, D., Getchell, M.L., Getchell, T.V., Stromberg, A.J. and Bondada, S., 2006. Molecular basis of age-associated cytokine dysregulation in LPS-stimulated macrophages. Journal of Leukocyte Biology 79: 1314-1327.

Chieppa, M., Rescigno, M., Huang, A.Y. and Germain, R.N., 2006. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. Journal of Experimental Medicine 203: 2841-2852.

Chu, H. and Mazmanian, S.K., 2013. Innate immune recognition of the microbiota promotes host-microbial symbiosis. Nature Immunology 14: 668-675.

Daniel, C., Repa, A., Wild, C., Pollak, A., Pot, B., Breiteneder, H., Wiedermann, U. and Mercenier, A., 2006. Modulation of allergic immune responses by mucosal application of recombinant lactic acid bacteria producing the major birch pollen allergen Bet v 1. Allergy 61: 812-819.

Derrien, M., Van Baarlen, P., Hooiveld, G., Norin, E., Müller, M. and De Vos, W.M., 2011. Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader *Akkermansia muciniphila*. Frontiers in Microbiology 2: 1-14.

Derrien, M., Vaughan, E.E., Plugge, C.M. and De Vos, W.M., 2004. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. International Journal of Systematic and Evolutionary Microbiology 54: 1469-1476.

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J.P., Druart, C., Bindels, L.B., Guiot, Y., Derrien, M., Muccioli, G.G. and Delzenne, N.M., 2013. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proceedings of the National Academy of Sciences of the USA 110: 9066-9071.

Ewaschuk, J.B., Diaz, H., Meddings, L., Diederichs, B., Dmytrash, A., Backer, J., Looijer-van Langen, M. and Madsen, K.L., 2008. Secreted bioactive factors from *Bifidobacterium infantis* enhance epithelial cell barrier function. American Journal of Physiology – Gastrointestinal and Liver Physiology 295: G1025-G1034.

Farache, J., Koren, I., Milo, I., Gurevich, I., Kim, K.-W., Zigmond, E., Furtado, G.C., Lira, S.A. and Shakhar, G., 2013. Luminal bacteria recruit $CD103^+$ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity 38: 581-595.

Foligne, B., Dessein, R., Marceau, M., Poiret, S., Chamaillard, M., Pot, B., Simonet, M. and Daniel, C., 2007a. Prevention and treatment of colitis with *Lactococcus lactis* secreting the immunomodulatory *Yersinia* LcrV protein. Gastroenterology 133: 862-874.

Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., Dewulf, J., Brassart, D., Mercenier, A. and Pot, B., 2007b. Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World Journal of Gastroenterology 13: 236-243.

Foligne, B., Zoumpopoulou, G., Dewulf, J., Younes, A.B., Chareyre, F., Sirard, J.-C., Pot, B. and Grangette, C., 2007c. A key role of dendritic cells in probiotic functionality. PLoS One 2: e313.

Gee, K., Guzzo, C., Mat, C., Nor, F., Ma, W. and Kumar, A., 2009. The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. Inflammation and Allergy – Drug Targets 8: 40-52.

Girardin, M. and Seidman, E.G., 2011. Indications for the use of probiotics in gastrointestinal diseases. Digestive Diseases 29: 574-587.

Górska, S., Schwarzer, M., Jachymek, W., Srutkova, D., Brzozowska, E., Kozakova, H. and Gamian, A., 2014. Distinct immunomodulation of bone marrow-derived dendritic cell responses to *Lactobacillus plantarum* WCFS1 by two different polysaccharides isolated from *Lactobacillus rhamnosus* LOCK 0900. Applied and Environmental Microbiology 80: 6506-6516.

Hayes, M.P., Wang, J. and Norcross, M.A., 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. Blood 86: 646-650.

Heintz, C. and Mair, W., 2014. You are what you host: microbiome modulation of the aging process. Cell 156: 408-411.

Hougee, S., Vriesema, A., Wijering, S., Knippels, L., Folkerts, G., Nijkamp, F., Knol, J. and Garssen, J., 2009. Oral treatment with probiotics reduces allergic symptoms in ovalbumin-sensitized mice: a bacterial strain comparative study. International Archives of Allergy and Immunology 151: 107-117.

Ivanov, I.I. and Littman, D.R., 2011. Modulation of immune homeostasis by commensal bacteria. Current Opinion in Microbiology 14: 106-114.

Ivanovic, N., Minic, R., Dimitrijevic, L., Skodric, S.R., Zivkovic, I. and Djordjevic, B., 2015. *Lactobacillus rhamnosus* LA68 and *Lactobacillus plantarum* WCFS1 differently influence metabolic and immunological parameters in high fat diet-induced hypercholesterolemia and hepatic steatosis. Food and Function 6: 558-565.

Jiang, M., Xiang, Y., Wang, D., Gao, J., Liu, D., Liu, Y., Liu, S. and Zheng, D., 2012. Dysregulated expression of miR-146a contributes to age-related dysfunction of macrophages. Aging Cell 11: 29-40.

Kanatsu-Shinohara, M., Ogonuki, N., Iwano, T., Lee, J., Kazuki, Y., Inoue, K., Miki, H., Takehashi, M., Toyokuni, S. and Shinkai, Y., 2005. Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development 132: 4155-4163.

Kang, C.-s., Ban, M., Choi, E.-J., Moon, H.-G., Jeon, J.-S., Kim, D.-K., Park, S.-K., Jeon, S.G., Roh, T.-Y. and Myung, S.-J., 2013. Extracellular vesicles derived from gut microbiota, especially *Akkermansia muciniphila*, protect the progression of dextran sulfate sodium-induced colitis. PLoS One 8: e76520.

Karczewski, J., Troost, F.J., Konings, I., Dekker, J., Kleerebezem, M., Brummer, R.-J.M. and Wells, J.M., 2010. Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum in vivo* and protective effects on the epithelial barrier. American Journal of Physiology – Gastrointestinal and Liver Physiology 298: G851-G859.

Kleerebezem, M., Boekhorst, J., Van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M. and Fiers, M.W., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proceedings of the National Academy of Sciences of the USA 100: 1990-1995.

Kollmann, T.R., Levy, O., Montgomery, R.R. and Goriely, S., 2012. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. Immunity 37: 771-783.

Lahtinen, S.J., Forssten, S., Aakko, J., Granlund, L., Rautonen, N., Salminen, S., Viitanen, M. and Ouwehand, A.C., 2011. Probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM® modifies subpopulations of fecal lactobacilli and *Clostridium difficile* in the elderly. Age 34: 133-143.

Ley, R.E., Peterson, D.A. and Gordon, J.I., 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124: 837-848.

Li, Z., Liu, C., Xie, Z., Song, P., Zhao, R.C., Guo, L., Liu, Z. and Wu, Y., 2011. Epigenetic dysregulation in mesenchymal stem cell aging and spontaneous differentiation. PLoS One 6: e20526.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M. and Kroemer, G., 2013. The hallmarks of aging. Cell 153: 1194-1217.

Luckey, T., 1972. Introduction to intestinal microecology. American Journal of Clinical Nutrition 25: 1292-1294.

Marco, M.L., Peters, T.H., Bongers, R.S., Molenaar, D., Van Hemert, S., Sonnenburg, J.L., Gordon, J.I. and Kleerebezem, M., 2009. Lifestyle of *Lactobacillus plantarum* in the mouse caecum. Environmental Microbiology 11: 2747-2757.

Matsumoto, M., Kurihara, S., Kibe, R., Ashida, H. and Benno, Y., 2011. Longevity in mice is promoted by probiotic-induced suppression of colonic senescence dependent on upregulation of gut bacterial polyamine production. PLoS One 6: e23652.

Matsumoto, T., Ishikawa, H., Tateda, K., Yaeshima, T., Ishibashi, N. and Yamaguchi, K., 2008. Oral administration of *Bifidobacterium longum* prevents gut-derived *Pseudomonas aeruginosa* sepsis in mice. Journal of Applied Microbiology 104: 672-680.

Mazé, A., Boël, G., Zúñiga, M., Bourand, A., Loux, V., Yebra, M.J., Monedero, V., Correia, K., Jacques, N. and Beaufils, S., 2010. Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. Journal of Bacteriology 192: 2647-2648.

Meijerink, M., Van Hemert, S., Taverne, N., Wels, M., De Vos, P., Bron, P.A., Savelkoul, H.F., Van Bilsen, J., Kleerebezem, M. and Wells, J.M., 2010. Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One 5: e10632.

Meijerink, M., Wells, J.M., Taverne, N., Zeeuw Brouwer, M.L., Hilhorst, B., Venema, K. and Bilsen, J., 2012. Immunomodulatory effects of potential probiotics in a mouse peanut sensitization model. FEMS Immunology & Medical Microbiology 65: 488-496.

Muñoz, J.A.M., Chenoll, E., Casinos, B., Bataller, E., Ramón, D., Genovés, S., Montava, R., Ribes, J.M., Buesa, J. and Fàbrega, J., 2011. Novel probiotic *Bifidobacterium longum* subsp. infantis CECT 7210 strain active against rotavirus infections. Applied and Environmental Microbiology 77: 8775-8783.

Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdt, S., Gordon, S., Hamilton, J.A., Ivashkiv, L.B. and Lawrence, T., 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41: 14-20.

Olivieri, F., Rippo, M.R., Prattichizzo, F., Babini, L., Graciotti, L., Recchioni, R. and Procopio, A.D., 2013. Toll-like receptor signaling in 'inflammaging': microRNA as new players. Immunity and Ageing 10: 11.

Osman, N., Adawi, D., Molin, G., Ahrne, S., Berggren, A. and Jeppsson, B., 2006. *Bifidobacterium infantis* strains with and without a combination of oligofructose and inulin (OFI) attenuate inflammation in DSS-induced colitis in rats. Bmc Gastroenterology 6: 31.

Repa, A., Grangette, C., Daniel, C., Hochreiter, R., Hoffmann-Sommergruber, K., Thalhamer, J., Kraft, D., Breiteneder, H., Mercenier, A. and Wiedermann, U., 2003. Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy. Vaccine 22: 87-95.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.-P. and Ricciardi-Castagnoli, P., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nature Immunology 2: 361-367.

Sebastián, C., Espia, M., Serra, M., Celada, A. and Lloberas, J., 2005. MacrophAging: a cellular and molecular review. Immunobiology 210: 121-126.

Sherman, P.M., Ossa, J.C. and Johnson-Henry, K., 2009. Unraveling mechanisms of action of probiotics. Nutrition in Clinical Practice 24: 10-14.

Shin, N.-R., Lee, J.-C., Lee, H.-Y., Kim, M.-S., Whon, T.W., Lee, M.-S. and Bae, J.-W., 2014. An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut 63: 727-735.

Sklar, M.D., Tereba, A., Chen, B.D.M. and Walker, W.S., 1985. Transformation of mouse bone marrow cells by transfection with a human oncogene related to c-myc is associated with the endogenous production of macrophage colony stimulating factor 1. Journal of Cellular Physiology 125: 403-412.

Smelt, M.J., De Haan, B.J., Bron, P.A., Van Swam, I., Meijerink, M., Wells, J.M., Faas, M.M. and De Vos, P., 2012. *L. plantarum*, *L. salivarius*, and *L. lactis* attenuate Th2 responses and increase Treg frequencies in healthy mice in a strain dependent manner. PLoS One 7: e47244.

Snel, J., Vissers, Y., Smit, B., Jongen, J., Van der Meulen, E., Zwijsen, R., Ruinemans-Koerts, J., Jansen, A., Kleerebezem, M. and Savelkoul, H., 2011. Strain-specific immunomodulatory effects of *Lactobacillus plantarum* strains on birch-pollen-allergic subjects out of season. Clinical and Experimental Allergy 41: 232-242.

Sun, D., Luo, M., Jeong, M., Rodriguez, B., Xia, Z., Hannah, R., Wang, H., Le, T., Faull, K.F. and Chen, R., 2014. Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal. Cell Stem Cell 14: 673-688.

Swidsinski, A., Dörffel, Y., Loening-Baucke, V., Theissig, F., Rückert, J.C., Ismail, M., Rau, W.A., Gaschler, D., Weizenegger, M. and Kühn, S., 2011. Acute appendicitis is characterized by local invasion with *Fusobacterium nucleatum/necrophorum*. Gut 60: 34-40.

Tiihonen, K., Ouwehand, A.C. and Rautonen, N., 2010. Human intestinal microbiota and healthy ageing. Ageing Research Reviews 9: 107-116.

Van Hemert, S., Meijerink, M., Molenaar, D., Bron, P.A., De Vos, P., Kleerebezem, M., Wells, J.M. and Marco, M.L., 2010. Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells. BMC Microbiology 10: 293.

Van Passel, M.W., Kant, R., Zoetendal, E.G., Plugge, C.M., Derrien, M., Malfatti, S.A., Chain, P.S., Woyke, T., Palva, A. and De Vos, W.M., 2011. The genome of *Akkermansia muciniphila*, a dedicated intestinal mucin degrader, and its use in exploring intestinal metagenomes. PLoS One 6: e16876.

Vesa, T., Pochart, P. and Marteau, P., 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Alimentary Pharmacology and Therapeutics 14: 823-828.

Warren, L.A. and Rossi, D.J., 2009. Stem cells and aging in the hematopoietic system. Mechanisms of Ageing and Development 130: 46-53.

Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M.J. and Kuipers, O.P., 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. Journal of Bacteriology 189: 3256-3270.

Yi, A.-K., Yoon, J.-G., Yeo, S.-J., Hong, S.-C., English, B.K. and Krieg, A.M., 2002. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. Journal of Immunology 168: 4711-4720.