



Research article

Sustaining exposure to high concentrations of bifidobacteria inhibits gene expression of Mouse's mucosal immunity

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ABSTRACT

Numerous dietary products are supplemented with probiotics that may be beneficial for human health. Recently, bifidobacteria have received increasing attention as a genus of probiotic bacteria with high efficiency and few side effects. To examine potential effects of different bifidobacteria concentrations on the mucosal immune response, we fed mice with (a) 10^8 colony-forming units (CFU) of bifidobacteria (group 10^8B), and (b) with 10^{12} CFU of bifidobacteria (group $10^{12}B$) over 42 days and assessed gene expression in intestinal mucosa and immune marker concentrations in serum samples; ten untreated female mice were used as a control. Continuous exposure to 10^8 CFU of bifidobacteria activated both macrophages and T_{reg} immune cells through significantly increasing the expression of mucosal TLR2 and IL10-mRNA genes, but inhibited Th1 and Th2 cells via significant down-regulation of IL4 and IFN γ gene expression, compared to untreated mice. Interestingly, group $10^{12}B$ showed down-regulated expression of TLR2, IL10, and IL4 genes but up-regulated expression of IFN γ , compared to group 10^8B and to the control. Also, polyclonal immunoglobulins IgG, IgM, and IgA showed a significant increase in all treated mice compared to the control. We conclude that high concentrations of bifidobacteria reduced innate immune functions. Furthermore, adaptive immunity seemed to be enhanced by increasing stimulation of T and B lymphocytes, suggesting aberration of the immune system following intestinal inflammation due to constant exposure to high concentrations of bifidobacteria. Both experimental bifidobacteria concentrations increased the total levels of circulating Igs, particularly of IgA.

1. Introduction

The human intestinal microbiome consists of a highly diverse microbial community (Eckburg, 2005; Turrone et al., 2008). These microbes can interact with their host in a symbiotic relationship where the microbiome exerts beneficial effect on human health, and the host provides a suitable habitat for the microbes. Furthermore, the gut microbiome is considered a barrier against exogenous pathogenic bacteria by occupying adhesion sites on the mucosa, competing for nutrients, and producing antimicrobial compounds (Shanahan, 2002; Hao and Lee, 2004; Blaut and Clavel, 2007). The human gut microbiome comprises numerous taxa of bacteria and yeast that may have probiotic properties, including *Lactobacillus* sp., *Bifidobacterium* spp., *Saccharomyces boulardii*,

Escherichia coli, and *Enterococcus* sp., which are also used as probiotic supplements in products (Fijan, 2014). The intestinal microbiome of an adult person comprises approximately 100 species of bacteria which affect a variety of physiological functions (Haller et al., 2000).

Bifidobacteria are anaerobic gram-positive bacteria, and their cell walls are composed of peptidoglycan and polysaccharide compounds (Lee and O'Sullivan, 2010; Chapot-Chartier and Kulakauskas, 2014). Bifidobacteria are the most common species of bacteria within a normal human intestinal community and are particularly common in newborns (Uemura and Matsumoto, 2014). Several strains of the genus *Bifidobacterium* are considered probiotics because of their numerous beneficial effects on human health, including the prevention of infections with enteric pathogens, regulation of digestion, inhibition of colon cancer

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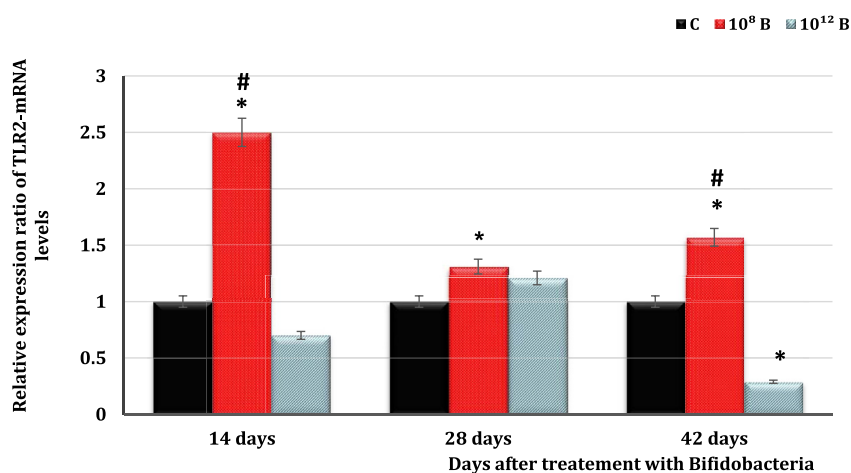


Figure 1. Regulation of TLR2 gene expression in untreated and treated mice groups. Where (C) represents untreated mice group (10^8 B) represent mice group treated with 10^8 CFU Bifidobacteria, and (10^{12} B) represent mice group treated with 10^{12} CFU Bifidobacteria. Total mRNA was isolated from the intestine of the indicated mouse strain and subjected to TLR2 qRT-PCR. (*) Significant at $P < 0.05$ as determined by analysis of variance, comparison was performed using One-factor ANOVA test. (*) Comparison between control and treated groups. (#) Comparison between the treated groups.

development, modulation of the immune system, anti-allergic properties, and protective effects against acute diarrhea (Liepke et al., 2002; Jia et al., 2010). Diet, age, and the environment are known to affect colonization of the intestinal tract by bifidobacteria (Srikanth and McCormick, 2008).

Various probiotic supplements are widely applied in food products such as yogurt, fermented milk products, fermented juices, and freeze-dried supplements (Parvez et al., 2006). A previous study reported that probiotics did not enhance immune responses or affect general health and well-being (Praharaj et al., 2015); however, a different study showed that probiotics in doses of up to 10^9 and 2×10^9 colony-forming units (CFU) can decrease infection risk (Ouwehand, 2017). Probiotics can stimulate the release of numerous kinds of immune mediators from different types of immune cells (Savan and Sakai, 2006), which affects both the innate and the adaptive branch of the immune system (Foligné et al., 2010). Cell wall components of probiotics and particularly those of bifidobacteria can stimulate nitric oxide synthase, which plays an important role in pathogen-infected cell death mechanisms elicited by macrophages through pro-inflammatory cytokine secretion (Schwandner et al., 1999). A previous study showed that probiotics increase gut barrier functions through activating B cells followed by an effect on cytokine production, which stimulates the host's adaptive immune responses (Abul Kalam Azad et al., 2018). Few studies have so far assessed potential effects of high doses of probiotic bacteria on systemic and mucosal immune responses. The objective of the current study was to assess the effects of two concentrations of bifidobacteria on immune responses in mice, assessed by gene expression of some mucosal immune factors and

by measuring the levels of polyclonal immunoglobulins in serum samples.

2. Results

2.1. Regulation of TLR2, IL4, IL10, and IFN γ gene expression following administration of different concentrations of bifidobacteria

TLR2, IFN γ , IL4, and IL10-mRNA expression profiles were compared between the treatment groups 10^8 B and 10^{12} B and the control. A substantial and significant up-regulation of TLR2 gene expression was observed in mucosal immune cells of 10^8 B mice after 14, 28, and 42 days, compared to the control ($P = 0.001$, $P = 0.0222$, and $P = 0.001$, respectively). TLR2-mRNA showed an early down-regulation in mice of group 10^{12} B after 14 days, which was, however, not statistically significant ($P = 0.48$); a non-significant elevation of this gene product was observed in the same treatment group on day 28, compared to the control ($P = 0.094$). TLR2 receptor gene expression was significantly down regulated in group 10^{12} B, compared to the control ($P = 0.0004$) on day 42. TLR2 expression in mucosal immune cells after 14 and 42 days was also significantly up-regulated in group 10^8 B compared to group 10^{12} B ($P < 0.001$); however, this increase in group 10^8 B did not differ significantly from that in group 10^{12} B on day 28 ($P = 0.396$; Figure 1).

Group 10^8 B showed non-significant down-regulation in IFN γ -mRNA expression in the mucosa compared to the control group after 14 days ($P = 0.748$); however, IFN γ expression was significantly down-regulated in group 10^8 B after 28 and 42 days, compared to the control ($P = 0.020$ and

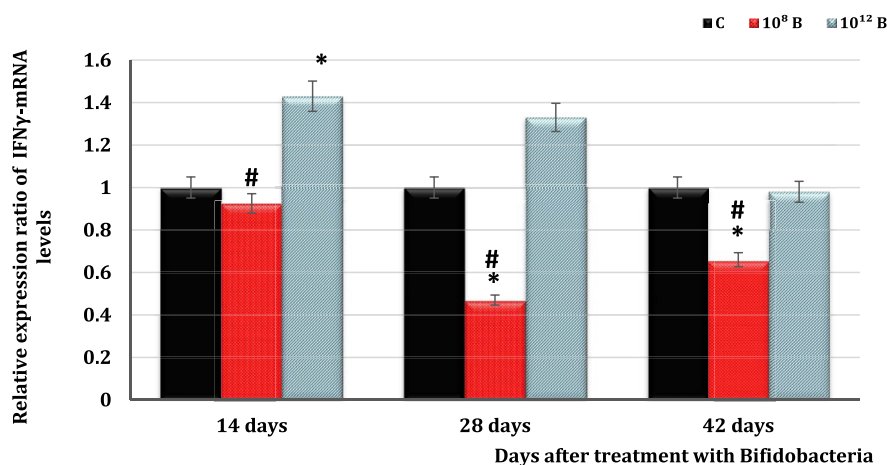


Figure 2. Regulation of IFN γ gene expression in untreated and treated mice groups. Where (C) represent untreated mice group (10^8 B) represent mice group treated with 10^8 CFU Bifidobacteria, and (10^{12} B) represent mice group treated with 10^{12} CFU Bifidobacteria. Total mRNA was isolated from the intestine of the indicated mouse strain and subjected to IFN γ qRT-PCR. (*) Significant at $P < 0.05$ as determined by analysis of variance, comparison was performed using One-factor ANOVA test. (*) Comparison between controls and treated groups. (#) Comparison between the treated groups.

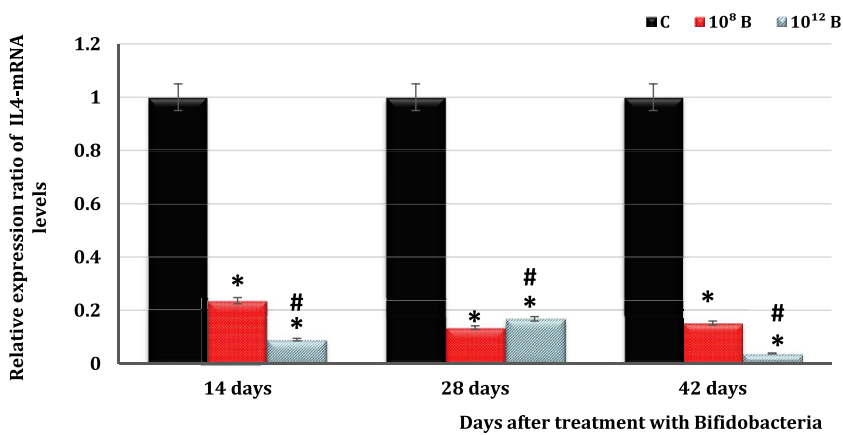


Figure 3. Regulation of IL4 gene expression in untreated and treated mice groups. Where (C) represent untreated mice group (10^8B) represent mice group treated with 10^8 CFU Bifidobacteria, and ($10^{12}B$) represent mice group treated with 10^{12} CFU Bifidobacteria. Total mRNA was isolated from the intestine of the indicated mouse strain and subjected to IL4 qRT-PCR. (*) Significant at $P < 0.05$ as determined by analysis of variance, comparison was performed using One-factor ANOVA test. (*) Comparison between controls and treated groups. (#) Comparison between the treated groups.

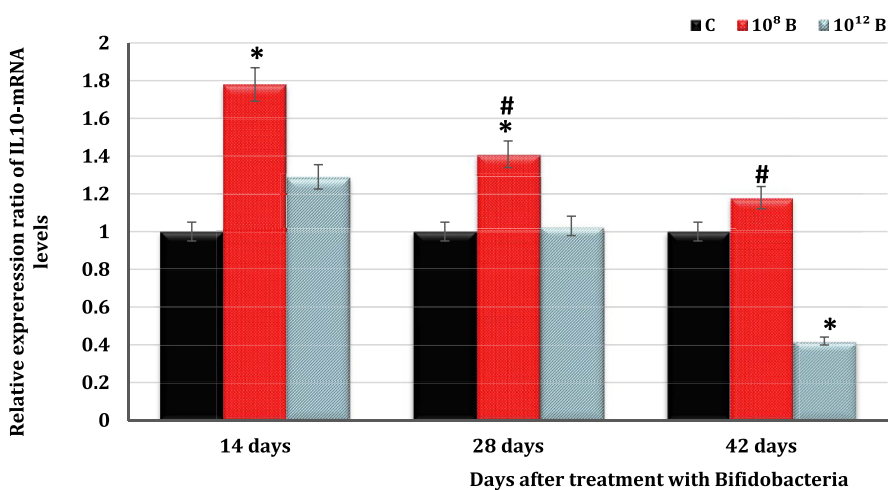


Figure 4. Regulation of IL10 gene expression in untreated and treated mice groups. Where (C) represent untreated mice group (10^8B) represent mice group treated with 10^8 CFU Bifidobacteria, and ($10^{12}B$) represent mice group treated with 10^{12} CFU Bifidobacteria. Total mRNA was isolated from the intestine of the indicated mouse strain and subjected to IL10 qRT-PCR. (*) Significant at $P < 0.05$ as determined by analysis of variance, comparison was performed using One-factor ANOVA test. (*) Comparison between controls and treated groups. (#) Comparison between the treated groups.

$P = 0.024$, respectively). IFN γ -mRNA showed an earlier but non-significant increase in group $10^{12}B$ compared to the control after 14 and 28 days ($P = 0.08$ and $P = 0.108$, respectively). After 42 days, IFN γ -mRNA expression in group $10^{12}B$ was similar to that in the control ($P = 0.876$). The levels of IFN γ -mRNA expression showed a significant downregulation in group 10^8B after 14, 28, and 42 days compared to those in group $10^{12}B$ ($P = 0.045$, $P = 0.0013$, and $P = 0.031$, respectively; Figure 2).

IL4 gene expression was significantly down-regulated in mucosal immune cells of both group 10^8B and $10^{12}B$ after 14, 28, and 42 days, compared to the control ($P < 0.001$); IL4 was also significantly down-regulated in group $10^{12}B$ after 14 and 42 days, compared to group 10^8B ($P = 0.002$ and $P < 0.001$, respectively), but it was significantly up-regulated in group $10^{12}B$ relative to group 10^8B , after 28 days ($P = 0.030$; Figure 3).

IL10-mRNA in group 10^8B showed a highly significant increase after 14 and 28 days ($P = 0.0092$ and $P = 0.0058$, respectively) and a non-significant increase after 42 days ($P = 0.4705$), compared to the control; furthermore, expression of this cytokine gene indicated a non-significant increase in intestinal cells of group $10^{12}B$ after 14 and 28 days, compared to the control ($P = 0.241$ and $P = 0.28$, respectively). IL10-mRNA expression in $10^{12}B$ mice showed a significant decrease after 42 days, compared to the control ($P = 0.042$), and it increased non-significantly in group 10^8B compared to group $10^{12}B$ ($P = 0.070$) after 14 days; however, IL10-mRNA expression was significantly increased in mucosal cells of group 10^8B compared to that in group $10^{12}B$ after 28 and 42 days ($P = 0.009$ and $P = 0.012$, respectively; Figure 4).

2.2. Levels of polyclonal IgG, IgM, and IgA after exposure to different concentrations of bifidobacteria

Systemic IgG, IgM, and IgA concentrations were compared between the treatment groups. Group 10^8B showed a highly significant elevation in total IgG after 14 and 28 days, whereas after 42 days, the increase was non-significant, compared to the control ($P = 0.000$ and $P = 0.152$, respectively). Mice of group $10^{12}B$ showed a non-significant increase in IgG after 14 and 28 days, compared to the control ($P = 0.47$), however, this increase was significant on day 42 ($P = 0.006$). Polyclonal IgG concentrations were significantly higher in group 10^8B than in group $10^{12}B$ on days 14 and 28 ($P < 0.001$) but were lower in 10^8B than in $10^{12}B$ after 42 days exposure to bifidobacteria, which was, however, not significant ($P = 0.0791$; Figure 5a).

Figure 5 b shows early significant decreases in circulating polyclonal IgM concentrations in group 10^8B on day 14 ($P = 0.0007$); non-significant IgM changes were recorded after 28 and 42 days in group 10^8B , compared to the control ($P = 0.24$ and $P = 0.131$, respectively). IgM showed a highly significant increase in group $10^{12}B$ after 14 and 28 days ($P = 0.0026$ and $P = 0.017$, respectively), however, the observed decrease on day 42 was non-significant, compared to the control ($P = 0.424$). Total IgM increased significantly in group $10^{12}B$ after 14 and 28 days compared to group 10^8B ($P = 0.000$ and $P = 0.043$, respectively), whereas IgM levels in group 10^8B did not differ from those in group $10^{12}B$ after 42 days ($P = 0.307$).

IgA showed an early significant increase in groups 10^8B and $10^{12}B$ throughout the experiment, compared to the control ($P = 0.000$). Moreover, total IgA showed a highly significant increase in group $10^{12}B$

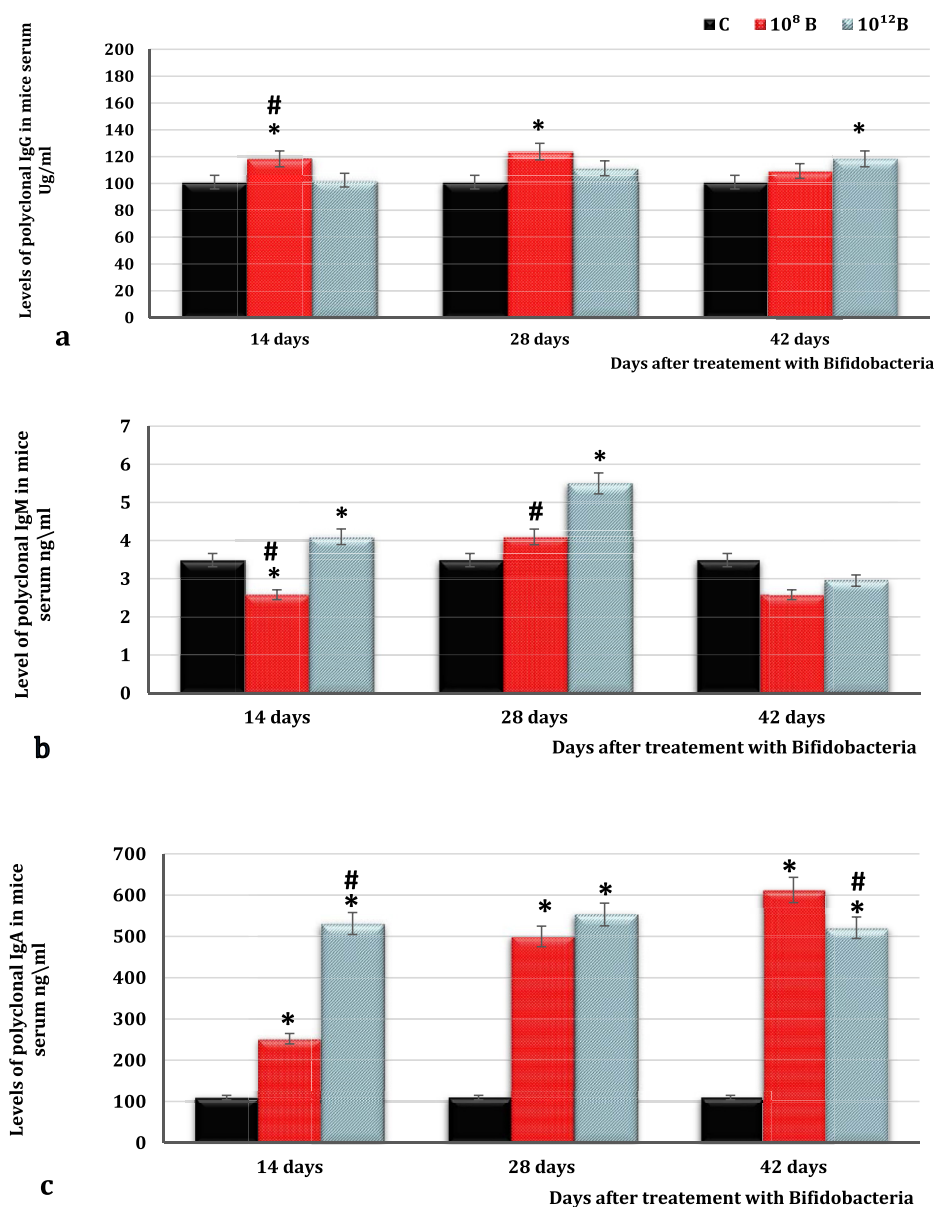


Figure 5. Profile of total immunoglobulin in mice serum of treated and untreated groups. (a) represent polyclonal IgG, (b) represent polyclonal IgM, and (c) represent polyclonal IgA. Where C represent the sera collected from untreated mice group, 10⁸B represent serum of mice collected post orally administration of 10⁸ CFU Bifidobacteria, and 10¹²B serum of mice collected post orally administration of 10¹² CFU Bifidobacteria. Significant at $p < 0.05$ as determined by analysis of variance, comparison was performed using One-factor ANOVA test. (*) Comparison between controls and treated groups. (#) Comparison between the treated groups.

compared to group 10⁸B after 14 days ($P = 0.000$), whereas no significant differences in IgA were observed between the two treatment groups after 28 and 42 days ($P = 0.258$ and $P = 0.133$, respectively; Figure 5c).

3. Discussion

Bifidobacteria were recognized recently as potent probiotic agents with few side effects. Bacteria of this genus are necessary to maintain and improve health of normal intestinal epithelial cells, and they have been used to cure diarrhea and other digestive disorders (Sivan et al., 2015; Saez-Lara et al., 2015; Moratalla et al., 2016). Moreover, numerous probiotics electro-spun within various fiber compounds have been applied in different fields of biomedicine (Akbar et al., 2018). Notably, probiotics exert inhibitory effects on the germination of oral pathogenic bacteria and have been applied in treatments of mild to moderate periodontitis (Cantore et al., 2018; Inchingolo et al., 2018). This led to the question whether different bifidobacteria concentrations would affect immune responses in the intestinal mucosa of mice. TLR2 is considered one of the pattern-recognition receptors that facilitate recognition of gram-positive bacterial peptidoglycan (Zeuthen et al., 2008; Beutler,

2009; Chang et al., 2012). Bifidobacteria act as immune-stimulators and immune-modulators through interactions with TLR2 (Mohammadzadeh et al., 2005; Zeuthen et al., 2008). In the current study, TLR2-mRNA expression was significantly up-regulated in mucosal immune cells of group 10⁸B compared to group 10¹²B and to the control. Furthermore, an earlier but non-significant difference in TLR2-mRNA receptor expression was observed between group 10¹²B and the control, followed by a significant downregulation at day 42, relative to the control. A previous study showed that probiotic bacteria or their antigenic particles can be internalized by dendritic cells or macrophages, which elicits signals to increase the number of TLR2 receptors (Chengcheng et al., 2016). Therefore, it seems possible that the effect of probiotic bacteria on immune cells involves TLR2, which may improve epithelial cell activation (Galdeano and Perdigón, 2004; Vinderola et al., 2005). In contrast, high concentrations of bifidobacteria such as 10¹² CFU can exceed host mucosal immunity and therefore reduce beneficial effects of bifidobacteria, and unsuccessful internalized by dendritic cells or macrophages prevents signals that would otherwise increase the number of TLR2 receptors (Galdeano and Perdigón, 2006; Vinderola et al., 2005). Antigens administrated at low concentrations seemed more appropriate to achieve

beneficial effects, which appeared to resemble the mechanisms of naturally occurring antigen inoculation more closely (Durudas et al., 2011). In the present study, changes in the expression of several fundamental immune modulator genes such as IFN γ , IL4, and IL10 were observed after treatment with 10⁸ CFU of bifidobacteria. For example, no up-regulation of IFN γ -mRNA gene expression was observed in mice treated with 10⁸ CFU of bifidobacteria, compared to untreated mice, which was in line with the results of Sánchez et al. (2015) and confirmed similar observations made at a low concentration of bifidobacteria (Dong et al., 2010). This down-regulation in IFN γ -mRNA may indicate inhibition of T-lymphocyte proliferation by probiotics such as bifidobacteria (Saez-Lara et al., 2015). Down-regulation of IFN γ -mRNA expression can be associated with a decrease in inflammatory processes, and bifidobacteria are considered more suitable in the context of inflammatory diseases based on their lower inflammatory potential as indicated by poor induction of Th1 responses and IFN γ -mRNA expression (Matsuoka et al., 2004; Neurath et al., 2002). Substantial early up-regulation of IFN γ expression was observed in group 10¹²B, compared to group 10⁸B and to the control. This observation suggests that continuous exposure to high concentrations of bifidobacteria increased Th1 activation which led to an induction of IFN γ -mRNA generation and changes in immune responses towards inflammatory processes (Trautmann et al., 2001; Mühl and Pfeilschifter, 2003; Saez-Lara et al., 2015). A previous study found that oral treatment with probiotics increased Th1 cytokines but inhibited Th2 cytokines in some atopic children (Oberge et al., 2011). Similarly, we observed significant downregulation of IL4 gene expression in groups 10⁸B or 10¹²B, compared to untreated mice; however, the highest concentration treatment (10¹² CFU) produced a more significant down-regulation in IL4-mRNA expression compared to mice treated with 10⁸ CFU, regardless of treatment time. In line with our results, *Lactobacillus* probiotic stimulation reduced concentrations of pro- and anti-inflammatory cytokines including IL2, TNF α , and IL4, and entirely reduced IL12 and TGF β in cultured human peripheral blood mononuclear cells after 96 h of incubation (Ashraf et al., 2014). IL10 is produced by numerous different immune cell populations such as macrophages, monocytes, DCs, B, Th2, Th1, and T_{reg} cells (Maloy and Powrie, 2001; Kamanaka et al., 2006; Maynard et al., 2009; Ouyang et al., 2011), however, also non-immune effector cell types such as epithelial cells and keratinocytes can produce IL10 in response to infection, tissue damage, or tumor genesis (Jung et al., 2004). In the current study, substantial up-regulation of IL10 expression was observed in group 10⁸B, compared to group 10¹²B and to the control, whereas no difference was observed between group 10¹²B and the control. Similarly, different species of the genus *Lactobacillus* primed monocyte-derived dendritic cells to maturation of T_{reg} cells, which augmented levels of IL10 and prevented proliferation of T cells in an IL10 concentration-dependent manner (Smits et al., 2005). IL10 gene expression observed in the present study may be due to a stimulation of intestinal CD103 + dendritic cells to produce IL10 and IL27 via the TLR2/MyD88 pathway in the large intestine after administration of bifidobacteria, particularly regarding *B. breve*. This species has been used to prevent intestinal inflammation through the induction of intestinal IL10 producing Tr1 cell (Jeon et al., 2012). However, down-regulation of TLR2 gene expression in the current study directly affected IL10 production due to high concentrations of bifidobacteria (10¹² CFU; Galdeano and Perdígón, 2006; Vinderola et al., 2005).

Increased levels of total antibodies, particularly IgM and IgG, may be interpreted as an activation of the specific immune response, mainly caused by the activation of T and B lymphocytes (Elbanna et al., 2018; Galdeano and Perdígón, 2004). Moreover, *Lactobacillus* strains influence the production of several types of immunoglobulins, particularly of IgG and IgA (Elbanna et al., 2018). In the present study, mice exposed to 10⁸ CFU of bifidobacteria showed an early and highly significant elevation in polyclonal IgG concentrations, compared to group 10¹²B and to the control; however, total IgM levels in group 10⁸B did not differ significantly from those in untreated mice, throughout the experiment.

Although group 10¹²B showed a significant increase in total IgG at a late point in time (day 42), compared to untreated mice, a highly significant increase in IgM levels was observed in this group compared to group 10⁸B and to the control. Remarkably, probiotic bacteria consolidate the systemic antibody response, depending on the type of antigen, where some antigens activated this immune response whereas others did not (Haghighi et al., 2006). Natural serum IgM is generated by B cells and exerts a wide range of functions; these antibodies commonly bind with low affinity to bacterial antigens, including lipopolysaccharides and capsular polysaccharides (Berland and Wortis, 2002; Bunker et al., 2015), also because natural IgM antibodies stimulate the induction of IgG specific to bacterial and viral antigens (Yilmaz et al., 2014; Bunker et al., 2015). Interestingly, in the present study, polyclonal IgA increased significantly in both treatment groups after 7 days compared to the control, throughout the experiment. Interestingly, in the present study, an earlier significant increase in the polyclonal IgA was detected in both treatment groups compared to the control, where the total IgA was also significantly increased in the high-concentration treatment (10¹² CFU), compared to the low-concentration treatment (10⁸ CFU). Numerous previous studies confirmed a significant increase in polyclonal IgA after exposure to various probiotics, particularly bifidobacteria, due to an increasing number of IgA-producing cells, which depended on the bacterial strain (Ibnou-Zekri et al., 2003; Vieira et al., 2013). This may be related to a concomitant increase in total IgG and IgM levels (Trautmann et al., 2001), and bifidobacteria and lipopolysaccharides can non-specifically enhance B lymphocyte proliferation and increase antibody synthesis from lymphocytes by activating the stimulation IL5 cytokine, which in turn stimulates an increase in IgA (Lazarenko et al., 2012). Our study revealed that probiotics can improve the defense function of epithelial cells by inducing secretion of cytokines and production of immunoglobulins and antimicrobial compounds (Boirivant and Strober, 2007).

4. Conclusion

To our knowledge, this is the first comprehensive study to assess the effects of high concentrations of bifidobacteria on mucosal immune responses. We found that administration of high doses of bifidobacteria (10¹² CFU) elicits undesirable activation of both innate and adaptive immunity, in contrast to 10⁸ CFU bifidobacteria treatments. Overall, substantial differences were observed between the treatments, and exposure to 10¹² CFU of bifidobacteria down-regulated gene expression of TLR2 and IL10 but significantly up-regulated IFN γ -mRNA. Our findings suggest a drift of mucosal immune responses towards inflammatory processes; however, both applied dosages of bifidobacteria can stimulate the production of total immunoglobulins, particularly of IgA.

5. Materials and methods

5.1. Bifidobacteria strains

Five strains of bifidobacteria (*B. lactis*, *B. longum*, *B. bifidum*, *B. infantis*, and *B. breve*) were obtained in powdered form from Custom Probiotics Inc, USA and the bacteria were prepared according to the manufacturer's instructions (Babar et al., 2012; Rajkumar et al., 2014).

5.2. Study animals and experimental design

Forty female Swiss mice were recruited at an age of eight weeks and were kept under standard laboratory conditions at the Center of King Fahad for Medical Research of the King Abdul-Aziz University in Jeddah, Saudi Arabia. Individuals weighed about 30–35 g. The mice were assigned to three treatment groups: group 10⁸B comprised 15 mice that were treated with 0.5 ml medium containing 10⁸ CFU of bifidobacteria, three times per week; group 10¹²B comprised 15 mice that received 0.5 ml medium containing 10¹² CFU of bifidobacteria, three times per week; a control group of ten individuals. Bacteria were administered by oral

Table 1. Primers used in the evaluation of TLR2, IFN γ , IL4, and IL10 genes expression extracted from mice mucosal intestine.

Gene	Polarity	Primer sequence (5'–3')	Primer length	Nucleotide positions	Reference Gene
β Actin	Sense	TATTGGCAACGAGCGG	16	856–871	NM_007393
	Antisense	CGGATGTCAACGTCAC	16	978–963	
TLR2	Sense	AACCTCAGACAAAGCGTCAAATC	23	797–816	NM_011905
	Antisense	ACCAAGATCCAGAAGAGCCAAA	22	993–974	
IFN γ	Sense	GGCCATCAGCAACAACATAAGCGT	24	321–344	NM_008337
	Antisense	TGGGTGTGTGACCTCAAACITGGC	24	415–438	
IL4	Sense	AGATGGATGTGCCAAACGTCCTCA	24	205–228	NM_021283
	Antisense	AATATGCCGAAGCACCTTGAAGCC	24	269–292	
IL10	Sense	AGAGAAGCATGGCCAGAAATC	22	336–357	NM_010548
	Antisense	TCATGGCCTTGATAGACACCTTG	22	521–542	

gavage. Five mice of each group were sacrificed 14, 28, and 42 days after the start of the experiment. Intestinal tissue and serum samples were stored at -80°C until laboratory analyses.

Animal experiments were approved by the Scientific Research Ethical Committee of the Faculty of Science, King Abdulaziz University and King Abdulaziz City for Science and Technology (KACST), Jeddah, KSA.

5.3. TLR2, IFN γ , IL4, and IL10 gene expression

Samples of intestinal tissue were submerged in RNAlater (QIAGEN, Cat NO.76106) to prevent RNA degradation; after this, total RNA was isolated using the RNeasy Maxi kit (QIAGEN, Cat No. 75162), according to the manufacturer's instructions. RNA was eluted using RNase-free water, and RNA samples were aliquoted and stored at -80°C until analyses. RNA was quantified and subsequently amplified using Verso SYBR Green 1-Step qRT-PCR Kit Master Mix reagents (Thermo Scientific, Cat No. AB-4108/C). Each reaction mixture for qRT-PCR was performed in one individual tube, according to the manufacturer's instructions. Primer sequences are shown in Table 1; each set of primers was added to a one-step RT-PCR mixture for each target gene. The total PCR reaction mixture was adjusted to 25 μl and contained 12.5 μl Verso SYBER Green master mix, 0.25 μl Verso enzyme, 0.25 mM of each primer, and 400–500 ng RNA; cDNA of the target genes was transcribed at 50°C for 30 min, followed by 40 cycles of 95°C for 15 s, 58 – 62°C for 30 s, and 72°C for 30 s. A commercially available real-time PCR detection system (CFX Connect™ Real-Time PCR, Bio-Rad) was used to quantify the amplified products based on continuously increasing fluorescence emission during PCR amplification. Relative mRNA quantification was calculated according to the $2^{-\Delta\Delta\text{Ct}}$ method. The same calibrator sample was employed for all measurements (Livak, and Schmittgen. 2001; Moucari et al., 2009).

5.4. Measurement of total IgA, IgM, and IgG in serum using a capture enzyme-linked immunosorbent assay (ELISA)

Polyclonal IgG, IgM, and IgA concentrations were measured in serum samples using capture ELISA quantitation kits (Abcam, United Kingdom, Cat. nos. ab157717, ab133047, and ab157719, respectively) according to the manufacturer's instructions. Absorbance was measured at 450 nm, and antibody concentrations were then determined according to standard curves produced by assaying standards of the respective immunoglobulin. Color intensity of the assay is proportional to the amount of specific antibody, and total serum immunoglobulin concentration was calculated as the sum of the respective IgA, IgM, and IgG concentrations.

5.5. Statistical analyses

The statistical analysis was accomplished between the untreated as well as two treated groups using MegaStat software version 10.1, and the

one-way ANOVA parametric test. Statistical significance is reported at $P < 0.05$.

Declarations

Author contribution statement

Sahar EL Hadad: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ayeshah Zakareya: Performed the experiments.

Ahmed Al-Hejin, Alia Aldahlawi, Mona Alharbi: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

- Abul Kalam Azad, M.d., Manobendro, S., Dan, W., 2018. Immunomodulatory effects of probiotics on cytokine profiles. *BioMed Res. Int.* 2018 (10).
- Akbar, Z., Zahoor, T., Huma, N., Jamil, A., Aysha, H., Kumar Irudayaraj, J.M., 2018. Electrospun probiotics: an alternative for encapsulation. *J. Biol. Regul. Homeost. Agents* 32, 1551–1556.
- Ashraf, R., Vasiljevic, T., Smith, S.C., Donkor, O.N., 2014. Effect of cell-surface components and metabolites of lactic acid bacteria and probiotic organisms on cytokine production and induction of CD25 expression in human peripheral mononuclear cells. *J. Dairy Sci.* 97, 2542–2558.
- Babar, V., Thomas, R., Bhaskar, M., 2012. Immunomodulatory activity of *Lactobacillus Sporigenes*. *Int. J. Ther. Appl.* 3, 32–38.
- Berland, R., Wortis, H.H., 2002. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol.* 20, 253–300.
- Beutler, B.A., 2009. TLRs and innate immunity. *Blood* 113, 1399–1407.
- Blaut, M., Clavel, T., 2007. Metabolic diversity of the intestinal microbiota: implications for health and disease. *Nutr. J.* 137, 751–755.
- Boirivant, M., Strober, W., 2007. The mechanism of action of probiotics. *Curr. Opin. Gastroenterol.* 23, 679–692.
- Bunker, J.J., Flynn, T.M., Koval, J.C., Shaw, D.G., Meisel, M., McDonald, B.D., Ishizuka, I.E., Dent, A.L., Wilson, P.C., Jabri, B., Antonopoulos, D.A., Bendelac, A., 2015. Innate and adaptive humoral responses coat distinct commensal bacteria with immunoglobulin A. *Immunity* 43, 541–553.
- Cantore, S., Ballini, A., De Vito, D., Abbinante, A., Altini, V., Dipalma, G., Inchingolo, F., Saini, R., 2018. Clinical results of improvement in periodontal condition by administration of oral probiotics. *J. Biol. Regul. Homeost. Agents* 32, 1329–1334.
- Chang, J.H., McCluskey, P.J., Wakefield, D., 2012. Recent advances in Toll-like receptors and anterior uveitis. *Clin. Exp. Ophthalmol.* 40, 821–828.
- Chapot-Chartier, M.-P., Kulakauskas, S., 2014. Cell wall structure and function in lactic acid bacteria. *Microb. Cell Factories* 13, 1–9.

- Chengcheng, R., Qiuxiang, Z., Bart, J., Hao, Z., 2016. Identification of TLR2/TLR6 signalling lactic acid bacteria for supporting immune regulation. *Sci. Rep.* 6, 34561.
- Dong, P., Yang, Y., Wang, W., 2010. The role of intestinal Bifidobacteria on immune system development of young rats. *Early Hum. Dev.* 86, 51–58.
- Durudas, A., Chen, H.L., Gasper, M.A., Sundaravaran, V., Milush, J.M., Silvestri, G., Johnson, W., Giavedoni, L.D., Sodora, D.L., 2011. Differential innate immune responses to low or high dose oral challenge in rhesus macaques. *Curr. HIV Res.* 9, 276–288.
- Eckburg, P.B., 2005. Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638.
- Elbanna, K., El Hadad, S., Assaeedi, A., Aldahlawi, A., Khider, M., Alheshi, A., 2018. In vitro and in vivo evidences for innate immune stimulators lactic acid bacterial starters isolated from fermented camel dairy products. *Sci. Rep.* 8, 12553.
- Fijan, S., 2014. Microorganisms with claimed probiotic properties: an overview of recent literature. *Int. J. Environ. Res. Public Health* 11, 4745–4767.
- Foligné, B., Dewulf, J., Breton, J., Claisse, O., Lonvaud-Funel, A., Pot, B., 2010. Probiotic properties of non-conventional lactic acid bacteria: immunomodulation by *Oenococcus oeni*. *Int. J. Food Microbiol.* 140, 136–145.
- Galdeano, C.M., Perdígón, G., 2006. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clin. Vaccine Immunol.* 13, 219–226.
- Galdeano, C.M., Perdígón, G., 2004. Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *J. Appl. Microbiol.* 97, 673–681.
- Haghighi, R.H., Gong, J., Carlton, L.G., Hayes, M.A., Zhou, H.B., Chambers, J.R., Sharif, S., 2006. Probiotics stimulate production of natural antibodies in chickens. *Clin. Vaccine Immunol.* 13, 975–980.
- Haller, D., Bode, C., Hammes, W.P., Pfeifer, A.M., Schiffrin, E.J., Blum, S., 2000. Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut* 47, 79–87.
- Hao, W.L., Lee, Y.K., 2004. Microflora of the gastrointestinal tract. *Methods Mol. Biol.* 268, 491–502.
- Ibnou-Zekri, N., Blum, S., Schiffrin, E.J., von der Weid, T., 2003. Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties *in vitro*. *Infect. Immun.* 71, 428–436.
- Inchingolo, F., Divalpa, G., Cirulli, N., Cantore, S., Saini, R.S., Altini, V., Santacroce, L., Ballini, A., aini, R., 2018. Microbiological results of improvement in periodontal condition by administration of oral probiotics. *J. Bio. Regul. Homeost. Agents.* 32, 1323–1328.
- Jeon, S.G., Kayama, H., Ueda, Y., Takahashi, T., Asahara, T., Tsuji, H., Tsuji, N.M., Kiyono, H., Ma, J.S., Kusu, T., Okumura, R., Hara, H., Yoshida, H., Yamamoto, M., Nomoto, K., Takeda, K., 2012. Probiotic Bifidobacterium breve induces IL-10-producing Tr1 cells in the colon. *PLoS Pathog.* 8, e1002714.
- Jia, L., Shigwedha, N., Mwandemele, O.D., 2010. Use of *D_{acid}*⁻, *D_{bile}*⁻, *z_{acid}*⁻ and *z_{bile}*⁻ values in evaluating bifidobacteria with regard to stomach pH and bile salt sensitivity. *J. Food Sci.* 75, 14–18.
- Jung, M., Sabat, R., Krätzschar, J., Seidel, H., Wolk, K., Schönbein, C., Schütt, S., Friedrich, M., Döcke, W.D., Asadullah, K., Volk, H.D., Grütz, G., 2004. Expression profiling of IL-10-regulated genes in human monocytes and peripheral blood mononuclear cells from psoriatic patients during IL-10 therapy. *Eur. J. Immunol.* 34, 481–493.
- Kamanaka, M., Kim, S.T., Wan, Y.Y., Sutterwala, S.F., Lara-Tejero, M., Galán, J.E., Harhaj, E., Flavell, R.A., 2006. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity* 25, 941–952.
- Lazarenko, L., Lidiia, B., Sichel, L.S., Pidgorskyi, V., 2012. Antagonistic action of lactobacilli and bifidobacteria in relation to *Staphylococcus aureus* and their influence on the immune response in cases of intravaginal staphylococcosis in mice. *Probiotics Antimicro. Proteins* 4, 78–89.
- Lee, J.H., O'Sullivan, D.J., 2010. Genomic insights into bifidobacteria. *Microbiol. Mol. Biol. Rev.* 74, 378–416.
- Liepke, C., Adermann, K., Raida, M., Magert, H.J., Forssmann, W.G., Zucht, H.D., 2002. Human milk provides peptides highly stimulating the growth of bifidobacteria. *Eur. J. Biochem.* 269, 712–718.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 25, 402–408.
- Maloy, K.J., Powrie, F., 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol.* 2, 816–822.
- Matsuoka, K., Inoue, N., Sato, T., Okamoto, S., Hisamatsu, T., Kishi, Y., Sakuraba, A., Hitotsumatsu, O., Ogata, H., Koganei, K., Fukushima, T., Kanai, T., Watanabe, M., Ishii, H., Hibi, T., 2004. T-bet upregulation and subsequent interleukin 12 stimulation are essential for induction of Th1 mediated immunopathology in Crohn's disease. *Gut* 53, 1303–1308.
- Maynard, C.L., Hatton, R.D., Helms, W.S., Oliver, J.R., 2009. Contrasting roles for all-trans retinoic acid in TGF- β -mediated induction of Foxp3 and Il10 genes in developing regulatory T cells. *J. Exp. Med.* 206, 343–357.
- Mohammadzadeh, A., Farhat, A., Esmaily, H., 2005. The effect of breast milk and lanolin on sore nipples. *Saudi Med. J.* 26, 1231–1234.
- Moratalla, A., Caparrós, E., Juanola, O., Portune, K., Puig-Kröger, A., Estrada-Capetillo, L., Bellot, P., Gómez-Hurtado, I., Piñero, P., Zapater, P., González-Navajas, J.M., Such, J., Sanz, Y., Francés, R., 2016. Bifidobacteria pseudocatenuatum CECT7765 induces an M2 anti-inflammatory transition in macrophages from patients with cirrhosis. *J. Hepatol.* 64, 135–145.
- Moucari, R., Martinot-Peignoux, M., Mackiewicz, V., Boyer, N., Ripault, M.P., Castelnau, C., Leclere, L., Dauvergne, A., Valla, D., Vidaud, M., Nicolas-Chanoine, M.H., Marcellin, P., 2009. Influence of genotype on hepatitis B surface antigen kinetics in hepatitis B e antigen-negative patients treated with pegylated interferon-alpha2a. *Antivir. Ther.* 14, 1183–1188.
- Mühl, H., Pfeilschifter, J., 2003. Anti-inflammatory properties of pro-inflammatory interferon- γ . *Int. Immunopharmacol.* 3, 1247–1255.
- Neurath, M.F., Weigmann, B., Finotto, S., Glickman, J., Nieuwenhuis, E., Iijima, H., Mizoguchi, A., Mizoguchi, E., Mudter, J., Galle, P.R., Bhan, A., Autschbach, F., Sullivan, B.M., Szabo, S.J., Glimcher, L.H., Blumberg, R.S., 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J. Exp. Med.* 195, 1129–1143.
- Oberg, H.H., Juricke, M., Kabelitz, D., Wesch, D., 2011. Regulation of T cell activation by TLR ligands. *Eur. J. Cell Biol.* 9, 582–592.
- Ouwehand, A.C., 2017. A review of dose responses of probiotics in human studies. *Benef. Microbes* 8, 143–151.
- Ouyang, W., Rutz, S., Crellin, N.K., Valdez, P.A., Hymowitz, S.G., 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu. Rev. Immunol.* 29, 71–109.
- Parvez, S., Malik, K.A., Ah Kang, S., Kim, H.Y., 2006. Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.* 100, 1171–1185.
- Praharaj, I., John, S.M., Bandyopadhyay, R., Kang, G., 2015. Probiotics, antibiotics and the immune responses to vaccines. *Philos. Trans. R. Soc. Biol. Sci.* 370–1671, 20140144.
- Rajkumar, H., Mahmood, N., Kumar, M., Varikuti, S.R., Challa, H.R., Myakala, S.P., 2014. Effect of probiotic (VSL#3) and omega-3 on lipid profile, insulin sensitivity, inflammatory markers, and gut colonization in overweight Adults: a randomized, controlled trial. *Mediat. Inflamm.* 2014 (8).
- Saez-Lara, M.J., Gomez-Llorente, C., Plaza-Diaz, J., Gil, A., 2015. The role of probiotic lactic acid bacteria and Bifidobacteria in the prevention and treatment of inflammatory bowel disease and other related diseases: a systematic review of randomized human clinical trials. *BioMed Res. Int.* 2015, 505878.
- Sánchez, B., González-Rodríguez, I., Arbolea, S., López, P., 2015. The effects of Bifidobacterium breve on immune mediators and proteome of HT29 cells monolayers. *BioMed Res. Int.* 2015, 479140.
- Savan, R., Sakai, M., 2006. Genomics of fish cytokines. *Comp. Biochem. Physiol. Genom. Proteonom.* 1, 89–101.
- Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., Kirschning, C.J., 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem.* 274, 17406–17409.
- Shanahan, F., 2002. The host-microbe interface within the gut. *Best Pract. Res. Clin. Gastroenterol.* 16, 915–931.
- Sivan, A., Corrales, L., Hubert, N., Williams, J.B., Aquino-Michaels, K., Earley, Z.M., Benyamin, F.W., Lei, Y.M., Jabri, B., Alegre, M.L., Chang, E.B., Gajewski, T.F., 2015. Commensal *Bifidobacterium* promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* 350, 1084–1089.
- Smits, H.H., Engering, A., van der Kleij, D., de Jong, E.C., Schipper, K., van Capel, T.M., Zaai, B.A., Yazdanbakhsh, M., Wieringa, E.A., van Kooyk, Y., Kapsenberg, M.L., 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J. Allergy Clin. Immunol.* 115, 1260–1267.
- Srikanth, C.V., McCormick, B.A., 2008. Interactions of the intestinal epithelial with the pathogen and the indigenous microbiota: a three-way crosstalk. *Interdiscip. Perspect. Infect. Dis.* 2008, 14, 626827.
- Trautmann, A., Akdis, M., Schmid-Grendelmeier, P., Disch, R., Bröcker, E.B., Blaser, K., Akdis, C.A., 2001. Targeting keratinocyte apoptosis in the treatment of atopic dermatitis and allergic contact dermatitis. *J. Allergy Clin. Immunol.* 108, 839–846.
- Turroni, F., Ribbera, A., Foroni, E., van Sinderen, D., Ventura, M., 2008. Human gut microbiota and Bifidobacteria: from composition to functionality. *Antonie Leeuwenhoek* 94, 35–50.
- Uemura, Y., Matsumoto, M., 2014. Chemical structure of the cell wall-associated polysaccharide of Bifidobacterium animalis subsp. *lactis* LKM512. *Glycoconj. J.* 31, 555–561.
- Vieira, A.T., Teixeira, Mauro M., Martins, F.S., 2013. The role of probiotics and prebiotics in inducing gut immunity. *Front. Immunol.* 4, 445.
- Vinderola, G., Matar, C., Perdígón, G., 2005. Role of the epithelial cells in the immune effects mediated by gram-positive probiotic bacteria. Involvement of Toll-like receptors. *Clin. Vaccine Immunol.* 12, 1075–1084.
- Yilmaz, A., Jennbacken, K., Fogelstrand, L., 2014. Reduced IgM levels and elevated IgG levels against oxidized low-density lipoproteins in HIV-1 infection. *BMC Infect. Dis.* 14, 143.
- Zeuthen, L.H., Fink, L.N., Frøkiær, H., 2008. Toll-like receptor 2 and nucleotide-binding oligomerization domain-2 play divergent roles in the recognition of gut-derived *lactobacilli* and Bifidobacteria in dendritic cells. *Immunology* 124, 489–502.