

ORIGINAL ARTICLE

***Bifidobacterium animalis* ssp. *lactis* Bb12 induces IL-10 through cell membrane-associated components via TLR2 in swine**M. Arenas-Padilla, J.L. Duarte-Gutiérrez and V. Mata-Haro 

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Abstract**Aim:** To investigate the role of Toll-like receptor 2 (TLR2) in interleukin-10 (IL-10) production induced by *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12) in swine immune cells.**Methods and Results:** Blood-monocytes and cells from mesenteric lymph nodes were obtained from pigs and cultured with live Bb12 for 4 and 12 h. Transcript levels of IL-10 and TLR2 were analysed. Furthermore, TLR2 was blocked to determine its participation in IL-10 production. TLR2 blockade was achieved with neutralizing antibodies, followed by stimulation with Bb12. Bifidobacteria induced IL-10 production in both swine monocytes and mesenteric cells. Monocytes with TLR2 blockade had a decrease in IL-10 transcripts, while mesenteric cells did not. Bacterial cell wall components were responsible for Bb12-induced IL-10 production since no IL-10 was detected in the culture supernatant.**Conclusions:** We demonstrated that IL-10 production is largely mediated through the recognition of Bb12 structures by TLR2, as bacterial metabolites in the culture supernatant failed to induce IL-10 expression.**Significance and Impact of the Study:** The present study provides evidence for the potential use of Bb12 in the swine industry; these bacteria can also be used as additional method to treat intestinal inflammation and enhance intestinal health in pigs.**Introduction**

Bifidobacteria are considered one of the key genera in the intestinal tracts of animals and humans; these bacteria represent approximately 3% of the microbiota in the intestines of healthy adult humans (Solano-Aguilar *et al.* 2008). *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12), the world's most documented probiotic, has been used in infant formula, dietary supplements and fermented milk products worldwide. Furthermore, Bb12 improves bowel function and reduces the side effects of antibiotic treatment, such as antibiotic-associated diarrhoea (Jungersen *et al.* 2014). Another benefits of Bb12 is the modulation of the immune system; however, few studies have focused on its immune function.

In the swine industry, diarrhoea is the most common disease in suckling piglets. In some outbreaks, this condition is responsible for high morbidity and mortality,

causing great economic losses. During the course of an intestinal infection, several inflammatory molecules are released to restrain the infection, and in consequence, some damage to the epithelial tissue may occur by the infectious agent, treatment with antibiotics or the host immune response (Fasano and Shea-Donohue 2005). Therefore, it is important to maintain a balance between the pro- and anti-inflammatory immune responses to eliminate the infection without presenting an exacerbated pro-inflammatory response (Garrett *et al.* 2010).

Interleukin (IL)-10 plays an important role in the maintenance of gut homeostasis, due to its anti-inflammatory functions. During infection, IL-10 inhibits the activity of Th1 cells, NK cells and macrophages, which are not only required for optimal pathogen clearance but also contribute to tissue damage. Consequently, IL-10 can both impede pathogen clearance and ameliorate immunopathology (Couper *et al.* 2008).

The *Bifidobacterium* genus has a high capacity to induce IL-10 production in monocyte-derived dendritic cells (DCs) and human peripheral blood mononuclear cells (PBMC) (López *et al.* 2010; Dong *et al.* 2011). Nevertheless, the precise mechanism by which these immunological benefits are exerted is not fully understood. One mechanism is by the activation of diverse pattern recognition receptors located on the membranes of immune system cells, such as Toll-like receptor (TLR) family. TLR activation leads to differential cascades in signalling pathways, transcription factors expression and cytokine production to induce the appropriate immune response (Akira and Takeda 2004). Specifically, TLR2 recognizes peptidoglycan and lipoteichoic acid on Gram-positive bacteria (Akira and Takeda 2004), such as lactobacilli and bifidobacteria (Zeuthen *et al.* 2008). Moreover, interest in the porcine immune system has been growing for its similarity with humans in terms of genetics, anatomy and physiology. Therefore, studies on the pig immune system could provide relevant information about the human immune response (Fairbairn *et al.* 2011; Bailey *et al.* 2013; Mair *et al.* 2014). Thus, the results of the present study can contribute not only to veterinary development, but also for potential future applications in medicine. In the present study, we demonstrate that Bb12 induces IL-10 production during the first hours of stimulation in both, swine monocytes and mesenteric lymph node (MLN) cells through bifidobacteria—TLR2 interactions.

Materials and methods

Bacteria

Bb12 was grown on Man Rogosa Sharpe broth (BD Difco, Franklin Lakes, NJ); supplemented with 0.05% cysteine (Sigma-Aldrich, Saint Louis, MO) at 37°C for 48 h under anaerobic conditions. Bacterial cultures were centrifuged at 3000 *g* for 10 min (Centrifuge Sorvall ST16; ThermoFisher Scientific, Waltham, MA). The bacterial pellet was washed with phosphate-buffered saline (PBS), and the bacterial concentration was standardized at 2.0 on the McFarland scale. For some experiments, the culture supernatant (Bb12SN) was collected, neutralized to pH 7.4, and passed through a 0.22 μm pore size filter unit (Millex-GP; Millipore Corp., Ireland).

Animals

Conventional female pigs (2–4 months old) were obtained from a farm that was free of porcine reproductive and respiratory virus, influenza virus and swine enteric coronavirus diseases. The animals were housed at the animal facility of the Centro de Investigación en Alimentación y Desarrollo (CIAD)

with *ad libitum* access to water and food. The animals were euthanized according to the ethical standards of the Mexican regulation NOM-033-ZOO-1995. Animal experimentation was approved by the Ethics Committee of CIAD.

Tissue collection and cell harvesting

After euthanasia, peripheral blood and MLN were collected. Peripheral blood was collected in tubes containing 1.5 mg ml⁻¹ ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. PBMCs were isolated by density centrifugation using Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Piscataway, NJ). PBMCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Co., Grand Island, NY) supplemented with 50 μg ml⁻¹ gentamicin, 5000 IU ml⁻¹ penicillin, 5 μg ml⁻¹ streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). Cells (5×10^7) were placed in 75-cm² flasks (Corning Inc., Corning, NY) and incubated at 37°C and 5% CO₂ for 90 min to allow the adherence of monocytes. The flasks were washed four times with PBS, and the adhered monocytes were incubated for 10 min with trypsin-EDTA solution (Sigma-Aldrich), harvested and washed with DMEM.

Mesenteric lymph nodes were placed in tubes containing cold PBS (137 mmol l⁻¹ NaCl, 2.7 mmol l⁻¹ KCl, 10 mmol l⁻¹ Na₂HPO₄, and 4.2 mmol l⁻¹ KH₂PO₄). MLN cell suspension was obtained following the protocol described by Weigmann *et al.* (2007), with modifications. Mesenteric fat tissue was removed with a scalpel, and the MLN cells were cut into small pieces (≈ 0.2 – 0.5 cm length), placed in a sterile Erlenmeyer flask with 50 ml of DMEM supplemented with antibiotics, 10% FBS and 100 U ml⁻¹ type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ). The cells were incubated for 2 h at 37°C with slow rotation. The cell suspension was filtered through a 100 μm cell strainer (BD), and the remaining pieces were macerated. The suspension was washed with DMEM.

Cell stimulation

A total of 2×10^5 monocytes or MLN cells were added to 48-well plates and incubated with either culture medium (negative control), Bb12 (at a ratio of 1 : 100 cell/bacteria) or bacterial supernatant (Bb12SN, 10% v/v) for 2 or 4 h. For TLR2 blocking experiments, 40 μg ml⁻¹ of purified anti-mouse/human CD282 (TLR2) antibody (Biolegend, San Diego, CA) was added for 1 h prior to bacterial stimulation.

RNA extraction and quantitative reverse transcriptase PCR

Total RNA was extracted using the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) according to the

manufacturer's instructions. Quantitative reverse transcriptase PCR (RT-qPCR) was performed on a QuantStudio 6 Flex thermocycler (Applied Biosystems, Carlsbad, CA). The primers and hydrolysis probe sequences used in this study are shown in Table 1. For amplifications, 50 ng of RNA per reaction was added to a mixture containing 12.5 μ l of Brilliant II QRT-PCR 1-Step Master Mix (Agilent Technologies, Cedar Creek, TX), 0.75 μ l of each primer (10 μ mol) and 0.5 μ l of probe (10 μ mol, FAM) (Integrated DNA Technologies; IDT, Coralville, IA). The cycling conditions were one cycle of 50°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. At the end of the amplification, the cycle threshold (Ct) values were obtained. Transcripts of porcine peptidylprolyl isomerase A (PPIA, cyclophilin A) were also examined to normalize the amount of input RNA. Relative transcript levels were quantified by the $2^{-\Delta\Delta CT}$ method.

ELISA

The cytokine concentration was measured in cell-free supernatants of stimulation experiments by ELISA with an antibody pair kit using specific for IL-10 swine (Invitrogen) according to the manufacturer's instructions.

Statistical analysis

The data were analysed using one-way analysis of variance or unpaired *t*-test with Welch's correction. The pairwise multiple comparison procedures were determined by Holm–Sidak's method, and *P* values less than 0.05 were considered significant. Analyses were performed with Prism 6.0 software (GraphPad, San Diego, CA).

Results

Bb12 induces IL-10 in swine monocytes and MLN cells

The anti-inflammatory response is greatly controlled by IL-10; thus it was important to determine whether Bb12 induces IL-10 in swine cells. First, we examined for IL-10

transcript expression at 4 h after stimulation. We found a similar increase in IL-10 levels correlated with Bb12 stimuli in monocytes and MLN cells (Fig. 1a). The cytokine quantification in both types of cells stimulated for 12 h also yielded significant differences compared to unstimulated cells (Fig. 1b). Notably, unstimulated MLN cells showed some basal IL-10 production compared to unstimulated blood monocytes; this effect could be due to the source, since MLN cells are somehow primed by stimuli, such as food or commensal bacteria, and subsequently migrate to MLN, unlike peripheral blood monocytes, which are non-primed cells. This difference in IL-10 production can also be an effect of the cell composition; since blood-derived monocytes are 100% antigen presenting cells (Fig. S1) while in the MLN comprise about 30% of the cell population (Fig. S2). However, regardless of the cell type, there was an increase in IL-10 production in both populations induced by *Bifidobacterium*, which could direct the anti-inflammatory response. The overexpression of TLR2 in monocytes has been associated with pro-inflammatory processes (Lacerte *et al.* 2016), and the expression level is transiently increased in the first hours upon stimulation (Johnson and Tapping 2007).

Bb12-induced IL-10 is mediated by TLR2

Hence, we tested the transcript level expression of TLR2 by RT-qPCR after 4 h of stimulation with Bb12; the increased production of IL-10 was not due to an increase in TLR2 mRNA since there were no significant differences in TLR2 expression in either monocytes or MLN cells (Fig. 1c), consistent with previous reports on TLR2 expression in porcine leukocytes (Álvarez *et al.* 2008). To test the participation of TLR2 in IL-10 production, an anti-TLR2 blocking antibody was used prior to bacterial stimulation, and the expression of IL-10 was quantified in blood monocytes stimulated with Bb12 for 2 and 4 h. A significant increase in IL-10 was observed after 4 h of stimulation, while no increase in IL-10 mRNA was observed with 2 h of stimulation. The IL-10 transcript level was significantly decreased when TLR2 was blocked ($P < 0.001$) (Fig. 2a). These results suggest that the IL-10

Table 1 Primers and hydrolysis probe sequences used in the present study

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Hydrolysis probe sequence (5'–3')
PPIA*	GCCATGGAGCGCTTTGG	TTATTAGATTTGCCACAGTCAGCAAT	6-FAM TM -TGATCTTCTTGCTGGTCTTGCCATTCCT-MGB Eclipse [®]
IL-10*	TGAGAACAGCTGCATCCACTTC	TCTGGTCCTTCGTTTGAAGAAA	6-FAM TM -CAACCAGCCTGCCCCACATGC-MGB Eclipse [®]
NF- κ B*	CTGGCAGCTCTCCTCAAAGC	CACGAGTCATCCAGGTCATACAG	6-FAM TM -CTCAAAGTTCTCCACCAGGGGA-MGB Eclipse [®]
TLR2	TTCAGGCCAAGGATTTCCAG†	TCACTGTGCTGGGTTTCATTG†	6-FAM TM -TCTTCACATGAGGCGGCC-BHQ [®] -1‡

*Sequences from porcine immunology and Nutrition database (<http://www.ars.usda.gov/services/docs.htm?docid=6065>).

†Tohno *et al.* (2006).

‡Lab-designed.

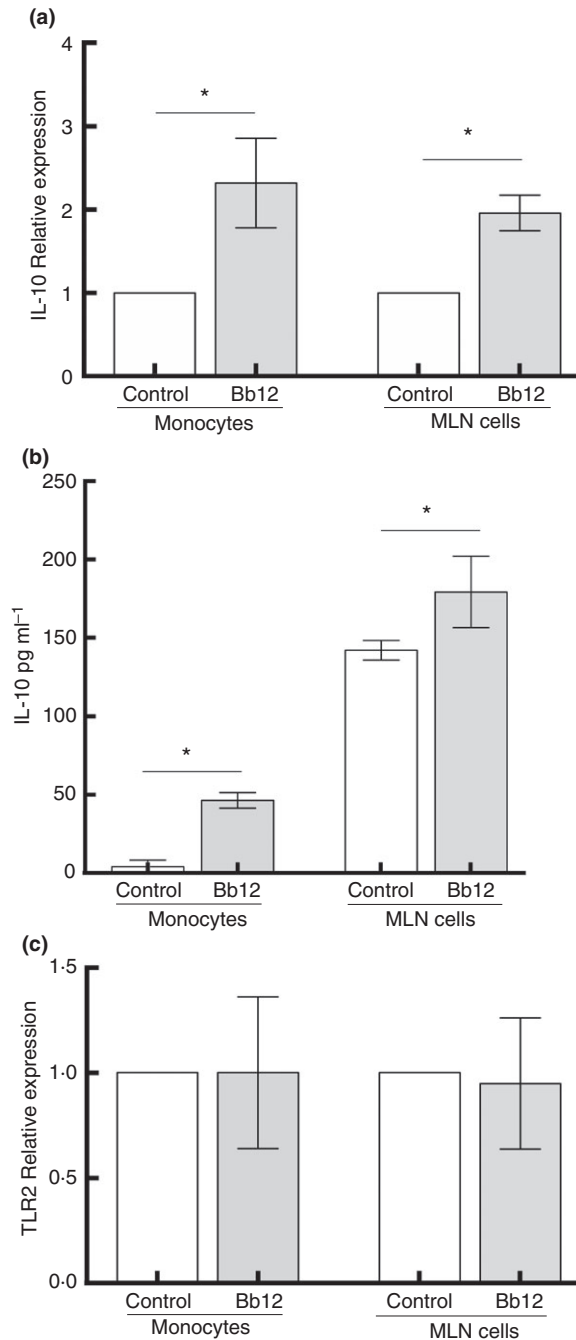


Figure 1 Bb12 induces IL-10 expression in swine cells. Monocytes and mesenteric lymph node (MLN) cells were cultured in presence of Bb12 for 4 h, and the transcript levels of IL-10 (a), or for 12 h and IL-10 production were measured in culture supernatants (b). Swine monocytes and MLN cells were cultured in the presence of Bb12 for 4 h, and the relative expression of TLR2 was measured (c). RT-qPCR results were normalized using expression level of swine PPIA gen. The data are shown as the means \pm SD of three independent experiments ($*P < 0.05$).

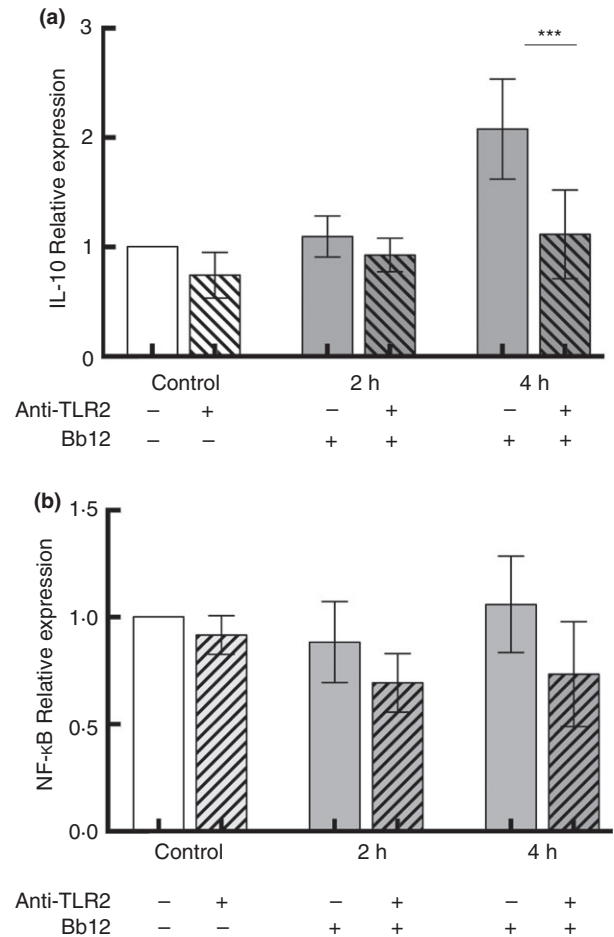


Figure 2 Bb12 induced IL-10 is mediated by TLR2. Monocytes were treated with an anti-TLR2 neutralizing antibody ($40 \mu\text{g ml}^{-1}$) prior to Bb12 stimulation for 2 and 4 h, and the transcript level of IL-10 (a) and NF- κ B (b) was measured by RT-qPCR. The results were normalized using expression level of swine PPIA gen. The data are shown as the means \pm SD of three independent experiments ($***P < 0.001$).

production induced by Bb12 occurs through the interaction of the receptor TLR2 with the bacteria.

We evaluated the participation of the transcription factor NF- κ B in IL-10 production in swine monocytes stimulated by Bb12 with or without anti-TLR2 antibody blocking prior to bacterial stimulation for 2 and 4 h. No significant changes of NF- κ B transcript level were observed under any of the conditions examined (Fig. 2b).

TLR2 and IL-10 expression is mediated through the interactions of bacterial cell wall components with swine monocytes

Previous studies have shown that bifidobacteria culture supernatant could activate TLR signaling. To examine the

capacity of bacterial metabolites to induce IL-10 and TLR2, we used culture supernatant to stimulate monocytes for 4 h. No differences in IL-10 expression were observed, whether the cells were stimulated with bacteria-free culture supernatant or not (Fig. 3a). In contrast, TLR2 expression decreased in the first hours of stimulation with Bb12 supernatant (Fig. 3b), which could indicate an anti-inflammatory mechanism induced by bacterial metabolites.

Discussion

In the pig industry, probiotics have been used as a food supplement for animal improvement purposes. Although not yet consistent in the literature; most reports have demonstrated that the application of probiotic strains, either separately or in combination, significantly contributes to health development. In this regard, the commercialized strain *B. animalis* ssp. *lactis* Bb12 (Bb12) has shown health-promoting benefits with respect to modulation of intestinal microbiota and immune response, and prevention and reduction of diarrhoea (Chattha *et al.* 2013; Kandasamy *et al.* 2014; Barba-Vidal *et al.* 2017); however, few studies have evaluated the effect of Bb12 or other bifidobacteria in swine immune cells, hence, we aimed to determine how Bb12 modulate the immune cells to attain its benefits.

There is a considerable interest in the use of specific probiotics strains for immune modulation. However, contrasting results have been reported when using specific strains; for instance, bifidobacteria and some lactobacilli polarize DCs towards a regulatory phenotype, whereas other lactobacilli promote Th1-inducing cytokine production in DCs (Christensen *et al.* 2002; Zeuthen *et al.* 2006). Over the past years, a substantial amount of *in vitro* and *in vivo* evidence has confirmed the potential capability of some bifidobacteria strains to modulate the immune response, specifically their ability to polarize to anti-inflammatory (Imaoka *et al.* 2008; Mortaz *et al.* 2015) and regulatory (Jeon *et al.* 2012) responses. Citar *et al.* (2015) found an increase in the expression of IL-10 and the repression of IL-6 and IL-12 β induced by several bifidobacteria strains on the monocytic THP-1 human cell line in the presence or absence of LPS (Citar *et al.* 2015). In a porcine intestinal epithelial cell line (PIE), *Bifidobacterium breve* MCC-117 shows anti-inflammatory effects against enterotoxigenic *Escherichia coli* (ETEC) though induction of IL-10 in CD4⁺ C25^{High} Treg lymphocytes (Fujie *et al.* 2011). In another report, pretreatment with either *B. longum* BB536 and *B. breve* M-16V reduce the expression of IL-6, IL-8, and MCP-1 induced by ETEC in PIE cells; blockade of TLR2 abolished the reduction of cytokines levels showing that TLR2 is required for the effect (Tomosada *et al.* 2013). Our

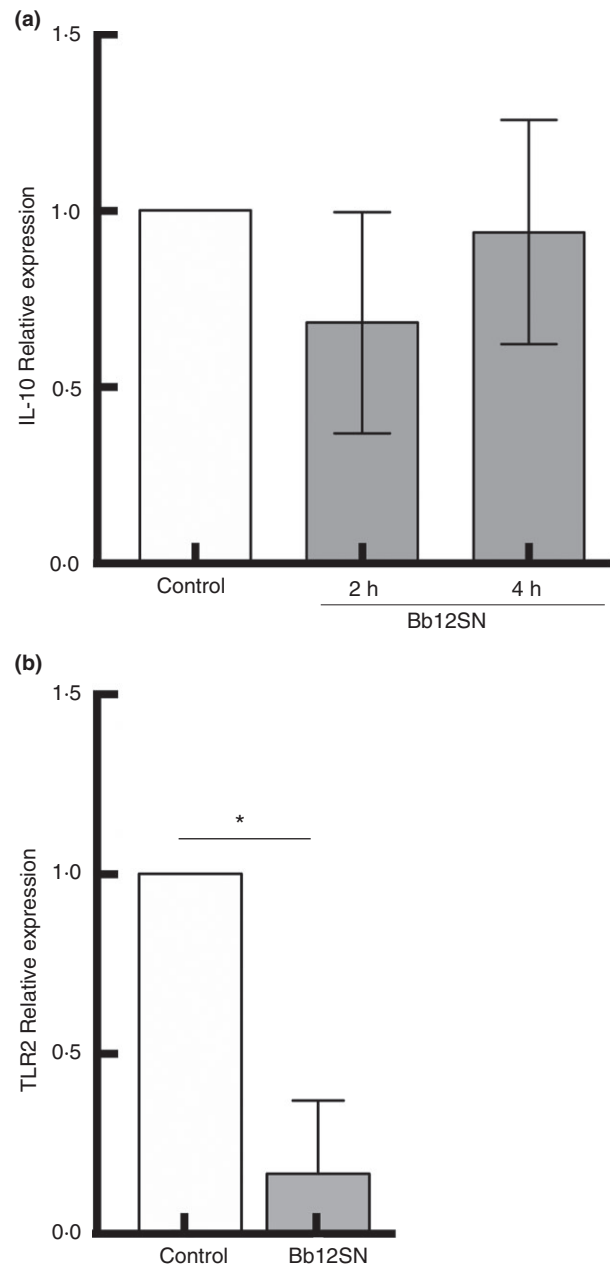


Figure 3 IL-10 production is mediated through the interaction of bacterial cell wall components with swine monocytes. Monocytes were stimulated with Bb12 culture supernatant for 4 h, and the transcript levels of IL-10 (a) and TLR2 (b) were measured. RT-qPCR results were normalized using expression level of swine PPIA gen. The data are shown as the means \pm SD of three independent experiments (* $P < 0.05$).

results show that Bb12 can stimulate swine immune cells to produce IL-10, hence inducing an anti-inflammatory response. The fact that Bb12 is capable to induce IL-10 expression in swine monocytes is suitable, considering that the probiotic can interact with recruited monocytes

during infection and inflammation. In a recent report, rotavirus-vaccinated gnotobiotic pigs co-colonized with *Lactobacillus rhamnosus* strain GG and *B. lactis* Bb12 had higher levels of ileal IL-10 compared to vaccinated pigs only, which may enhance intestinal specific IgA response (Kandasamy *et al.* 2014).

Immune cells produce varying amounts of IL-10 upon TLR2 ligation (Jang *et al.* 2004; Netea *et al.* 2004), which depends on the cell type. For instance, IL-10 can be produced by T regulatory cells and myeloid DCs, while plasmacytoid DCs are poor IL-10 producers (Boonstra *et al.* 2006; Maynard *et al.* 2009). Our results indicate that *in vitro* stimulation of swine cells with Bb12, increase the IL-10 mRNA levels in monocyte-derived peripheral blood cells and MLN (Fig. 1a); however, the production of the cytokine was only observed in monocytes but not in MLN (Fig. 1b). We ruled out that the increase in IL-10 transcript was due to an increase in TLR2, since TLR2 levels were similar in all the conditions evaluated whether stimulated or not (Fig. 1c). Contradictory results for the mRNA levels of TLRs after stimulation with bacteria have been reported. For instance, the oral administration of *Lactobacillus casei* CRL 431 increases the expression of TLR2, TLR4, TLR5 and TLR9 to activate an innate immune response in cells from the lamina propria (Castillo *et al.* 2011). However, only an increase of TLR9 expression but not significant changes in mRNA expression for either TLR2 or TLR4 was observed in the proximal colon of piglets fed with lyophilized *Bifidobacterium* Bb12 born from sows fed with *Bifidobacterium* Bb12 during gestation (Solano-Aguilar *et al.* 2008). These findings suggested a selective induction of TLR9 by higher levels of Bb12 compared to piglets born from sows that did not receive Bb12 or mothers treated only with Bb12 during the last trimester of pregnancy. Similar results were obtained in weaning piglets fed daily with 10^7 , 10^9 – 10^{11} CFU per ml of *B. animalis* RA-18 for 2 weeks. Moreover, TLR2 was increased in mononuclear cells isolated from the ileum of gnotobiotic pigs fed a combination of *L. rhamnosus* GG+*B. animalis* Bb12. In contrast, TLR9 levels were low in all tissues (Vlasova *et al.* 2013). Cells of the MLN and ileal Peyer's patches of suckling piglets produce cytokines after *in vitro* stimulation with heat-killed lactic acid-forming bacteria (Tohno *et al.* 2006) via TLR2 and TLR9. However, the results from the present study suggest that the IL-10 expression in swine monocytes stimulated with live bacteria of *Bifidobacterium* Bb12 is mediated through TLR2 since the bacterial DNA was unlikely available for recognition due to the short stimulation period (4 h) (Fig. 2a), although we did not test for TLR9 expression. Each probiotic strain shows specific immunomodulatory characteristics in a dose-dependent manner (Latvala *et al.* 2008), suggesting that the

differences in IL-10 activity or receptor recognition expression depends on the amount of bacteria. In addition, it is also important to consider the bacteria condition—that is, whether these organisms were used live, lyophilized, UV-killed or heat-killed. In the present study, bacteria were used live, but unable to proliferate given the composition of the cell culture media and growth conditions, which are not favourable for Bb12 growth.

Furthermore, no significant changes were observed in the NF- κ B transcript levels in any of the conditions tested (Fig. 2b). These results are consistent with the findings of a study in which THP-1-derived macrophages were stimulated with *Lactobacillus* sp. or *Bifidobacterium* sp., and no change in NF- κ B basal expression was observed (Mortaz *et al.* 2015). These results suggest that when a stimulus causes excessive inflammation, these bacteria act in a regulatory manner. NF- κ B-deficient macrophages have lower levels of IL-10 expression than NF- κ B-sufficient macrophages this fact is explained by several studies with cells deficient for tumour progression locus 2 (TPL2) or NF- κ B1, which support the role of extracellular signal-regulated kinase (ERK) in the induction of IL-10. TPL2 is an upstream activator of ERK; following TLR stimulation, TPL2 becomes dissociated from the TPL2-NF- κ B1 complex and activates ERK (Banerjee *et al.* 2006). *Bifidobacterium animalis* AHC7 attenuates NF- κ B and protects against *Salmonella typhimurium* infection in mice (O'Mahony *et al.* 2010). The expression of IL-10 can be induced by TLR or non-TLR signaling in myeloid cells. The activation of TLR results in the activation of NF- κ B, p38 and ERK pathways. Activation of these pathways results in the induction of IL-10 production and pro-inflammatory cytokines (Saraiva and O'garra 2010). The results of the present study suggest that *Bifidobacterium* Bb12 does not activate the NF- κ B pathway during the first 4 h of stimulation in swine monocytes. Probiotics can alter the NF- κ B pathway at many different levels: degradation and ubiquitination and inhibition of proteasome function (Petrof *et al.* 2004).

We further tested if the immunomodulatory properties were due to a metabolite produced by Bb12, hence the culture supernatant was used to stimulate mononuclear cells, but no IL-10 production was detected (Fig. 3a). Interestingly, in our study, Bb12 culture supernatant decreases the TLR2 transcript level (Fig. 3b). Hoarau *et al.* (2006), demonstrated that *B. breve* C50 (BbC50) supernatant induced prolonged DC survival, maturation and high IL-10 production through TLR2. *Bifidobacterium breve* BbC50 secretes extracellular components comprising a lipoprotein putatively associated with glucose moieties and acting in an aggregating form as an agonist of TLR2/TLR6 (Scutto *et al.* 2014). Additionally,

probiotic culture media may use TLR4 through IRAK-2 and via AP-1 to prevent IL-1 β -induced IL-6 induction in immature enterocytes (Meng *et al.* 2016). However, the involvement of TLR2 in this process remains unclear, although an immunoregulatory role for TLR2 in the recognition of probiotic strains has been described (Hoarau *et al.* 2006; Zeuthen *et al.* 2008; Kaji *et al.* 2010).

The probiotic Bb12 could be used in swine industry as diet supplement to enhance intestinal health in pig as an additional or postinfection treatment method, even contemplate its therapeutic use considering its ability to induce IL-10 in swine cells, which can contribute to the prevention of tissue damage. The dosage at which Bb12 could be added in feed, ranges from around 10⁹ to 10¹¹ CFU per day, this dosage range has been used as feeding supplementation with beneficial effects on swine immunity (Trevisi *et al.* 2008; Lahteinen *et al.* 2014; Zhu *et al.* 2014; Hou *et al.* 2015).

In summary, we present findings on how *B. animalis* ssp. *lactis* Bb12 exerts one of its many beneficial effects. In swine immune cells, Bb12 induce the production of IL-10, and the mechanism involves recruiting of TLR2. We also showed that NF- κ B was not differentially expressed with Bb12 stimulation, which indicates that other transcription factors are involved in IL-10 production.

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Conflict of Interest

The authors declare that there is no conflict of interest.

References

- Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. *Nat Rev Immunol* **4**, 499–511.
- Álvarez, B., Revilla, C., Doménech, N., Pérez, C., Martínez, P., Alonso, F., Ezquerro, A. and Domínguez, J. (2008) Expression of toll-like receptor 2 (TLR2) in porcine leukocyte subsets and tissues. *Vet Res* **39**, 13.
- Bailey, M., Christoforidou, Z. and Lewis, M.C. (2013) The evolutionary basis for differences between the immune systems of man, mouse, pig and ruminants. *Vet Immunol Immunopathol* **152**, 13–19.
- Banerjee, A., Gugasyan, R., McMahon, M. and Gerondakis, S. (2006) Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. *Proc Natl Acad Sci U S A* **103**, 3274–3279.
- Barba-Vidal, E., Castillejos, L., Roll, V.F.B., Cifuentes-Orjuela, G., Moreno Munoz, J.A. and Martin-Orue, S.M. (2017) The probiotic combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Bifidobacterium animalis* subsp. *lactis* BPL6 reduces pathogen loads and improves gut health of weaned piglets orally challenged with *Salmonella* Typhimurium. *Front Microbiol* **8**, 1570.
- Boonstra, A., Rajsbaum, R., Holman, M., Marques, R., Asselin-Paturel, C., Pereira, J.P., Bates, E.E.M., Akira, S. *et al.* (2006) Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J Immunol* **177**, 7551–7558.
- Castillo, N.A., Perdigón, G. and de LeBlanc, A.d.M. (2011) Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar Typhimurium infection in mice. *BMC Microbiol* **11**, 177.
- Chattha, K.S., Vlasova, A.N., Kandasamy, S., Rajashekar, G. and Saif, L.J. (2013) Divergent immunomodulating effects of probiotics on T cell responses to oral attenuated human rotavirus vaccine and virulent human rotavirus infection in a neonatal gnotobiotic piglet disease model. *J Immunol* **191**, 2446–2456.
- Christensen, H.R., Frøkiær, H. and Pestka, J.J. (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J Immunol* **168**, 171–178.
- Citar, M., Hacin, B., Tompa, G., Stempelj, M., Rogelj, I., Dolinsek, J., Narat, M. and Matijasic, B.B. (2015) Human intestinal mucosa-associated *Lactobacillus* and *Bifidobacterium* strains with probiotic properties modulate IL-10, IL-6 and IL-12 gene expression in THP-1 cells. *Benef Microbes* **6**, 325–336.
- Couper, K.N., Blount, D.G. and Riley, E.M. (2008) IL-10: the master regulator of immunity to infection. *J Immunol* **180**, 5771–5777.
- Dong, H., Rowland, I. and Yaqoob, P. (2011) Comparative effects of six probiotic strains on immune function in vitro. *Br J Nutr* **108**, 459–470.
- Fairbairn, L., Kapetanovic, R., Sester, D.P. and Hume, D.A. (2011) The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. *J Leukoc Biol* **89**, 855–871.
- Fasano, A. and Shea-Donohue, T. (2005) Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nat Clin Pract Gastroenterol Hepatol* **2**, 416–422.
- Fujie, H., Villena, J., Tohno, M., Morie, K., Shimazu, T., Aso, H., Suda, Y., Shimosato, T. *et al.* (2011) Toll-like receptor-2-activating bifidobacteria strains differentially regulate inflammatory cytokines in the porcine intestinal epithelial cell culture system: finding new anti-inflammatory immunobiotics. *FEMS Immunol Med Microbiol* **63**, 129–139.

- Garrett, W.S., Gordon, J.I. and Glimcher, L.H. (2010) Homeostasis and inflammation in the intestine. *Cell* **140**, 859–870.
- Hoarau, C., Lagaraine, C., Martin, L., Velge-Roussel, F. and Lebranchu, Y. (2006) Supernatant of *Bifidobacterium breve* induces dendritic cell maturation, activation, and survival through a Toll-like receptor 2 pathway. *J Allergy Clin Immunol* **117**, 696–702.
- Hou, C., Liu, H., Zhang, J., Zhang, S., Yang, F., Zeng, X., Thacker, P.A., Zhang, G. et al. (2015) Intestinal microbiota succession and immunomodulatory consequences after introduction of *Lactobacillus reuteri* I5007 in neonatal piglets. *PLoS ONE* **10**, e0119505.
- Imaoka, A., Shima, T., Kato, K., Mizuno, S., Uehara, T., Matsumoto, S., Setoyama, H., Hara, T. et al. (2008) Anti-inflammatory activity of probiotic *Bifidobacterium*: enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. *World J Gastroenterol* **14**, 2511.
- Jang, S., Uematsu, S., Akira, S. and Salgame, P. (2004) IL-6 and IL-10 induction from dendritic cells in response to *Mycobacterium tuberculosis* is predominantly dependent on TLR2-mediated recognition. *J Immunol* **173**, 3392–3397.
- Jeon, S.G., Kayama, H., Ueda, Y., Takahashi, T., Asahara, T., Tsuji, H., Tsuji, N.M., Kiyono, H. et al. (2012) Probiotic *Bifidobacterium breve* induces IL-10-producing Tr1 cells in the colon. *PLoS Pathog* **8**, e1002714.
- Johnson, C.M. and Tapping, R.I. (2007) Microbial products stimulate human Toll-like receptor 2 expression through histone modification surrounding a proximal NF- κ B-binding site. *J Biol Chem* **282**, 31197–31205.
- Jungersen, M., Wind, A., Johansen, E., Christensen, J.E., Stuer-Lauridsen, B. and Eskesen, D. (2014) The science behind the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12[®]. *Microorganisms* **2**, 92–110.
- Kaji, R., Kiyoshima-Shibata, J., Nagaoka, M., Nanno, M. and Shida, K. (2010) Bacterial teichoic acids reverse predominant IL-12 production induced by certain *Lactobacillus* strains into predominant IL-10 production via TLR2-dependent ERK activation in macrophages. *J Immunol* **184**, 3505–3513.
- Kandasamy, S., Chattha, K.S., Vlasova, A.N., Rajashekar, G. and Saif, L.J. (2014) Lactobacilli and Bifidobacteria enhance mucosal B cell responses and differentially modulate systemic antibody responses to an oral human rotavirus vaccine in a neonatal gnotobiotic pig disease model. *Gut Microbes* **5**, 639–651.
- Lacerte, P., Brunet, A., Egarnes, B., Duchêne, B., Brown, J.P. and Gosselin, J. (2016) Overexpression of TLR2 and TLR9 on monocyte subsets of active rheumatoid arthritis patients contributes to enhance responsiveness to TLR agonists. *Arthritis Res Ther* **18**, 10.
- Lähteinen, T., Lindholm, A., Rinttilä, T., Junnikkala, S., Kant, R., Pietilä, T.E., Levonen, K., von Ossowski, I. et al. (2014) Effect of *Lactobacillus brevis* ATCC 8287 as a feeding supplement on the performance and immune function of piglets. *Vet Immunol Immunopathol* **158**, 14–25.
- Latvala, S., Pietilä, T.E., Veckman, V., Kekkonen, R.A., Tynkkynen, S., Korpela, R. and Julkunen, I. (2008) Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World J Gastroenterol* **14**, 5570.
- López, P., Gueimonde, M., Margolles, A. and Suárez, A. (2010) Distinct *Bifidobacterium* strains drive different immune responses in vitro. *Int J Food Microbiol* **138**, 157–165.
- Mair, K., Sedlak, C., Käser, T., Pasternak, A., Levast, B., Gerner, W., Saalmüller, A., Summerfield, A. et al. (2014) The porcine innate immune system: an update. *Dev Comp Immunol* **45**, 321–343.
- Maynard, C.L., Hatton, R.D., Helms, W.S., Oliver, J.R., Stephensen, C.B. and Weaver, C.T. (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.
- Meng, D., Zhu, W., Ganguli, K., Shi, H.N. and Walker, W.A. (2016) Anti-inflammatory effects of *Bifidobacterium longum* subsp. *infantis* secretions on fetal human enterocytes are mediated by TLR-4 receptors. *Am J Physiol Gastrointest Liver Physiol* **311**, G744–G753.
- Mortaz, E., Adcock, I.M., Ricciardolo, F.L., Varahram, M., Jamaati, H., Velayati, A.A., Folkerts, G. and Garssen, J. (2015) Anti-inflammatory effects of *Lactobacillus rhamnosus* and *Bifidobacterium breve* on cigarette smoke activated human macrophages. *PLoS ONE* **10**, e0136455.
- Netea, M.G., Suttmüller, R., Hermann, C., Van der Graaf, C.A.A., Van der Meer, J.W.M., van Krieken, J.H., Hartung, T., Adema, G. et al. (2004) Toll-Like Receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol* **172**, 3712–3718.
- O'Mahony, D., Murphy, S., Boileau, T., Park, J., O'Brien, F., Groeger, D., Konieczna, P., Ziegler, M. et al. (2010) *Bifidobacterium animalis* AHC7 protects against pathogen-induced NF- κ B activation in vivo. *BMC Immunol* **11**, 63.
- Petrof, E.O., Kojima, K., Ropeleski, M.J., Musch, M.W., Tao, Y., De Simone, C. and Chang, E.B. (2004) Probiotics inhibit nuclear factor- κ B and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. *Gastroenterology* **127**, 1474–1487.
- Saraiva, M. and O'garra, A. (2010) The regulation of IL-10 production by immune cells. *Nat Rev Immunol* **10**, 170–181.
- Scotto, A., Djourie, S., Colavizza, M., Romond, P.-C. and Romond, M.-B. (2014) *Bifidobacterium breve* C50 secretes lipoprotein with CHAP domain recognized in aggregated form by TLR2. *Biochimie* **107**, 367–375.
- Solano-Aguilar, G., Dawson, H., Restrepo, M., Andrews, K., Vinyard, B. and Urban, J.F. (2008) Detection of

- Bifidobacterium animalis* subsp. *lactis* (Bb12) in the intestine after feeding of sows and their piglets. *Appl Environ Microbiol* **74**, 6338–6347.
- Tohno, M., Shimosato, T., Moue, M., Aso, H., Watanabe, K., Kawai, Y., Yamaguchi, T., Saito, T. *et al.* (2006) Toll-like receptor 2 and 9 are expressed and functional in gut-associated lymphoid tissues of presuckling newborn swine. *Vet Res* **37**, 791–812.
- Tomosada, Y., Villena, J., Murata, K., Chiba, E., Shimazu, T., Aso, H., Iwabuchi, N., Xiao, J.-Z. *et al.* (2013) Immunoregulatory effect of bifidobacteria strains in porcine intestinal epithelial cells through modulation of ubiquitin-editing enzyme A20 expression. *PLoS ONE* **8**, e59259.
- Trevisi, P., De Filippi, S., Minieri, L., Mazzoni, M., Modesto, M., Biavati, B. and Bosi, P. (2008) Effect of fructo-oligosaccharides and different doses of *Bifidobacterium animalis* in a weaning diet on bacterial translocation and Toll-like receptor gene expression in pigs. *Nutrition* **24**, 1023–1029.
- Vlasova, A.N., Chattha, K.S., Kandasamy, S., Liu, Z., Esseili, M., Shao, L., Rajashekara, G. and Saif, L.J. (2013) Lactobacilli and bifidobacteria promote immune homeostasis by modulating innate immune responses to human rotavirus in neonatal gnotobiotic pigs. *PLoS ONE* **8**, e76962.
- Weigmann, B., Tubbe, I., Seidel, D., Nicolaev, A., Becker, C. and Neurath, M.F. (2007) Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nat Protoc* **2**, 2307–2311.
- Zeuthen, L.H., Christensen, H.R. and Frøkiær, H. (2006) Lactic acid bacteria inducing a weak interleukin-12 and tumor necrosis factor alpha response in human dendritic cells inhibit strongly stimulating lactic acid bacteria but act synergistically with gram-negative bacteria. *Clin Vaccine Immunol* **13**, 365–375.
- Zeuthen, L.H., Fink, L.N. and 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.
- Zhu, Y.-H., Li, X.-Q., Zhang, W., Zhou, D., Liu, H.-Y. and Wang, J.-F. (2014) Dose-dependent effects of *Lactobacillus rhamnosus* on serum interleukin-17 production and intestinal T-cell responses in pigs challenged with *Escherichia coli*. *Appl Environ Microbiol* **80**, 1787–1798.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Figure S1 Swine monocytes subsets characterization by flow cytometry.

Figure S2 Swine MLN cells populations by flow cytometry.