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Cytokine 41 (2008) 223–231

Gut immune stimulation by non pathogenic Gram(+) and Gram(−) bacteria. Comparison with a probiotic strain

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Received 9 May 2007; received in revised form 30 October 2007; accepted 23 November 2007

Abstract

We analyzed the gut immune stimulation induced by Gram-positive bacteria: non probiotic *Lactobacillus acidophilus* CRL 1462 and *Lactobacillus acidophilus* A9; two potentially probiotic strains: *L. acidophilus* CRL 924 and *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 423; comparatively with a probiotic strain: *Lactobacillus casei* CRL 431. We also studied Gram-negative bacteria: *Escherichia coli* 129 and *E. coli* 13-7 in BALB/c mice. All the strains increased the number of IgA+ cells. We analyzed the cytokines IFNγ, TNFα, IL-17, IL-12, IL-6 and MIP-1α. The Gram(+) strains increased the number of IL-10+ cells. Gram(−) strains did not increase IL-10+ cells, but they increased the number of IL-12+ cells. The probiotic strain increased mainly IFNγ and TNFα. In the study of the receptors TLR-2, TLR-4 and CD-206, we demonstrated that only the probiotic strain increased the number of CD-206+ cells. All the Gram(+) strains increased the number of TLR-2+ cells and the Gram(−) strains of the TLR-4+ cells. The probiotic strain induced the release of IL-6 by a preparation enriched in intestinal epithelial cells (IEC). Gram(+) and Gram(−) bacteria activated different immune receptors and induced a different cytokine profile. The probiotic strain showed a great activity on the immune cells and the enriched population in IEC, activating mainly cells of the innate immune system.

Keywords: Gram-positive bacteria; Gram-negative bacteria; Intestinal immunity

1. Introduction

Mucosal immune responses to pathogenic gut bacteria have been studied intensely for decades. More recently, the influence of the resident non pathogenic or ''commensal'' microflora on mucosal immune function and gut health has emerged as an area of scientific and clinical importance. The relevance of the microflora and more specifically its composition in physiological processes in the human gastrointestinal tract is well documented [1,2]. The metabolism of nutrients, the development of the intestinal epithelium, the vasculature and the lymphoid tissue and the contribution to the phenomena of colonization resistance to pathogens, are only a few of the ways in which the host benefits from the resident microflora [3,4]. However, many clinically relevant diseases have been linked with dysfunctional immune responses directed against the commensal flora, since normal luminal bacteria and their products can induce and perpetuate chronic intestinal and systemic inflammation in genetically susceptible hosts [5–7].

The gastrointestinal tract harbors Gram-positive and Gram-negative bacteria that maintain an active dialogue with the host mucosal immune system. This cross-talk, mediated in part by toll like receptors (TLRs), affects immunological tolerance and homeostasis in the gut [8,9]. TLRs consist of a family of pattern recognition receptors that detect conserved molecular products of microorgan-

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doi:10.1016/j.cyto.2007.11.014

isms such as lipopolysaccharides (LPS) and lipoteichoic acid (LTA), recognized by TLR-4 and TLR-2, respectively [10].

Probiotics are known to affect the gastrointestinal tract and the associated immune system [11–13], but the mechanisms of probiotic activity are still poorly understood. In previous works, it was demonstrated that certain probiotic bacteria can modulate the cytokine release by cells of the Mucosal Immune System and increase the number of IgA-producing cells in the lamina propria of the gut [14–16]. In addition, as reported recently, one of the mechanisms of probiotic activity could work through the activation of the innate immune system [17].

The vast majority of probiotic bacteria are Gram-positive strains, mainly species of the *Lactobacillus* and *Bifidobacterium* genera; however, there are also some commercial probiotic preparations that contain Gram-negative bacteria such as *E. coli* Nissle strain [18,19]. Although lactic acid bacteria are known to have a wide range of effects on the immune system, evidence of *E. coli* strains from animal studies is contradictory [20,21]. This fact means that the immunomodulatory activity of probiotics is characteristic of a strain and that this property cannot be extrapolated to other genera or species.

At present, how the host distinguishes between commensal and non pathogenic bacteria is not well understood. In a previous work, we demonstrated that commensal and non commensal bacteria have a similar capacity of interaction with the gut. We observed fluorescent bacterial antigens in the inductor sites of the intestinal immune system (Peyer's patches), in the immune cells associated with the lamina propria of the small intestine and in nodules and crypts of the large intestine [22].

The aim of this work was to study comparatively the effect of Gram-positive lactobacilli, non probiotics, probiotic and potentially probiotics, and Gram-negative bacteria on the gut immune system through the cytokine profile, the number of IgA+ cells, the expression of the receptors TLRs and CD-206, in order to determine the differences or/and similarities between them in mucosal immune activation.

We also analyzed which are the immunological parameters that must be triggered, especially by probiotic bacteria, to induce mucosal immune activation in the gut ecosystem of the host harboring a complex microflora. The mucosal immune activation is an extremely important characteristic for the selection of probiotic bacteria.

2. Materials and methods

2.1. Animals

BALB/c adult mice (6–8 weeks old, weighing 25–30 g) were obtained from the close random bred colony kept in our department at CERELA. Each experimental and control group for each day and assay consisted of five mice. All the animals were fed *ad libitum* with a conventional balanced diet.

2.2. Bacteria and animal administration

Gram-positive and Gram-negative bacterial species were tested (Table 1). Gram-negative strains were cultured at 37 °C in brain heart infusion broth (BHI Britannia, Buenos Aires, Argentina) while Gram-positive strains were cultured at 37 °C in MRS broth (Britania, Buenos Aires, Argentina). All bacteria were harvested by centrifugation (5000*g*, 10 min), washed three times with phosphate-buffered saline (PBS), and diluted to a final concentration of 10^8 UFC/ml for Gram-positive strains and 10^6 UFC/ml for Gram-negative strains. Animals were given the cell suspension for 2, 5 or 7 consecutive days by gavage. The daily dose was previously selected by determination of absence of intestinal disturbance in the normal microflora, able to induce bacterial translocation to liver or spleen.

2.3. Histological samples

At the end of each administration period both test and control animals were sacrificed. The small intestine was removed and processed according to Sainte-Marie's technique for paraffin embedding [23].

2.4. Immunofluorescence assays for detection of IgA- and cytokine-positive cells

The number of IgA positive cells was determined on histological slices by a direct immunofluorescence assay. After deparaffinization with an immersion in xylene and rehydration in a graded ethanol series, paraffin sections (4 μm) were incubated with a 1:100 dilution of α-chain monospecific antibody conjugated with FITC (Sigma, St. Louis, MO, USA) for 30 min and observed with a fluorescent light microscope.

Cytokine producing cells were studied by an indirect immunofluorescence assay. After deparaffinization and rehydration, paraffin sections (4 μm) were incubated with

Table 1
Gram-positive and Gram-negative bacterial strains assayed

Bacterial species (strain)	Origin	Referred to as
Gram-positive		
Lactobacillus acidophilus CRL 1462	Mice small intestine	L. a CRL 1462
Lactobacillus acidophilus A9	Probiotic fresh cheese	L. a A9
Lactobacillus casei CRL 431	Human small intestine	L. c CRL 431
Lactobacillus acidophilus CRL 924	Fermented milk	L. a CRL 924
Lactobacillus delbrueckii subsp. b*ulgaricus* CRL 423	Yogurt	L. b CRL 423
Gram-negative		
Escherichia coli 129	Mice small intestine	E. c 129
Escherichia coli 13-7	Bovine intestinal tract	E. c 13-7

a 1% blocking solution of bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) for 30 min at room temperature. They were washed three times in PBS and incubated with normal goat serum (dilution 1:100) for 30 min. Rabbit anti-mouse IL-12, IL-10, IL-6, IL-17, MIP-1α, IFNγ and TNFα were applied to the sections for 60 min at room temperature. Then the slices were washed twice in PBS and incubated for 45 min with a 1:100 dilution of the goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research Labs Inc.) at room temperature and washed twice in PBS.

The number of fluorescent cells was counted in 30 fields at 1000× magnification and results were expressed as the number of positive fluorescent cells per ten fields.

2.5. Immunofluorescence assays for TLR-2, TLR-4 and CD-206 detection

The TLR-2 and TLR-4 positive cells were determined on histological sections from the small intestine of test and control mice. After deparaffinization and rehydration, paraffin sections (4 μm) were incubated with a 1% blocking solution of BSA for 30 min at room temperature and washed three times in PBS. Rat anti-mouse TLR-4 or TLR-2 (eBioscience, USA) was applied (dilution 1:300) to the sections for 60 min at room temperature. The slices were washed twice with PBS and incubated for 45 min with a 1:100 dilution of the goat anti rat antibody conjugated with FITC (Jackson Immuno Research Labs Inc.).

The number of CD-206 positive cells was also determined by an indirect immunofluorescence assay, using a 1:200 dilution of human anti-mouse CD-206 (BD Biosciences Pharmingen, USA) and a dilution 1:100 of goat anti rat antibody conjugated with FITC (Jackson Immuno Research Labs Inc) as second antibody.

The number of fluorescent cells was counted in 30 fields at 1000× magnification and results were expressed as the number of positive fluorescent cells per ten fields.

2.6. Intestinal epithelial cells isolation: ex vivo assays

To analyze the effect of the bacteria under study on the IL-6 released by the intestinal epithelial cells (IEC), they were isolated from the small intestine as previously described [24,25]. Animals were sacrificed after 7 days of administration for each bacterium assayed. The small intestine was removed and placed in cold Hank's balanced salt solution (HBSS) (Sigma–Aldrich, St. Louis, MO) containing glucose (2%; Sigma–Aldrich), penicillin (100 U/ml; Sigma–Aldrich) and streptomycin (0.1 mg/ml, Sigma–Aldrich). The intestines were opened longitudinally to expose the mucosal surface and washed five times with HBSS. Five-millimeter segments of tissue were then incubated in 20 ml of HBSS containing collagenase (300 U/ml, Sigma–Aldrich) and dispase (0.1 mg/ml; Gibco, Grand Island, NY) at 37 °C with constant gentle rotation for 45 min. Then 20 ml of Dulbecco's modified Eagle's medium (Gibco) supplemented with heat-inactivated fetal bovine serum (10%, ATCC, Manassas, VA), insulin–transferrin–selenium-A (2.50 μg/ml, 0.55 μg/ml and 1.68 pg/ml, respectively) from a 100× ready-to-use solution (Gibco) of penicillin (100 U/ml, Sigma–Aldrich) and streptomycin (0.1 mg/ml, Sigma–Aldrich) was added to the digest. Large fragments were allowed to settle on for 2 min, then the supernatant was recovered and transferred to centrifuge tubes and centrifuged at 100 g for 3 min. The pellet was washed twice with the culture medium and finally resuspended at a concentration of 2×10^5 organoids/ml. Trypan blue (0.4%) exclusion was used to assess cell viability. This cell suspension was transferred to 96-well cell culture plates and incubated for 8 h (37 °C, 5% CO_2). Supernatants were recovered for cytokine determination.

2.7. Flow cytometry

Contaminating hematopoietic ($CD45^+$) from the obtained cellular suspension as was describes above, was confirmed by antibody staining and flow cytometric analysis. This cell suspension was incubated with a dilution recommended by the manufacturer of Phycoerythrin-conjugated rat anti-mouse CD45 (BD Biosciences Pharmingen, USA). It was analyzed 20.000–30.000 cells with a FACS Partec Pas equipped with Flomax software and the percentage of positive cells was determined.

2.8. IL-6 quantification in supernatants

IL-6 was determined using commercially available BD OptEIA™ mouse cytokine ELISA kits (BD Biosciences San Diego, USA), according to manufacture's instructions.

2.9. Statistical analysis

Statistical analyses were performed using MINITAB 14 software. A factorial distribution (5×3, feeding procedure × days) was used; the comparisons were made using an ANOVA general linear model followed by a Tukey's post hoc test and $P < 0.05$ was considered significant.

Each experiment was repeated three times and all results (from the three trials) were analyzed together. No interactions between these three trials were observed when they were analyzed at different times (days) or when different lactobacilli were used.

3. Results

3.1. Determination of IgA+ cells

We observed that both Gram(+) and Gram(−) strains induced a significant increase in the IgA+ cells ($P \leqslant 0.01$) in the small intestine after 7 days of administration compared with the untreated control (Fig. 1). We did not observed differences among the bacteria assayed in the

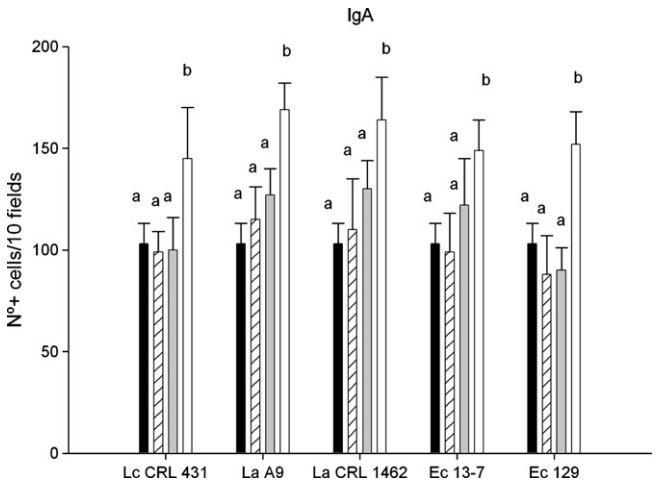

Fig. 1. Effect of bacterial administration on the number of IgA+ cells. Positive cells were counted in histological sections from small intestine of unfed control (black bar) 2 days group (diagonal lines), 5 days group (gray bar) and 7 days group (white bar). Values are means for $n = 5 \pm SD$. Means for each value without a common letter differ significantly ($P < 0.05$).

number of IgA+ cells, even for the probiotic strain *L. casei* CRL 431.

3.2. Effect of bacterial administration on the cytokine profile

The ability of the immune cells associated with the intestinal mucosa to respond to different strains was assessed by the cytokine release. We observed that both non probiotic strains, *L. acidophilus* CRL 1462 and *L. acidophilus* A9, induced a similar pattern of cytokine release (Fig. 2). In contrast, Gram(−) bacteria showed greater differences between them: *E. coli* 13-7 induced a significant increase in the number of all the assayed cytokines, except for the regulatory cytokine (IL-10), while *E. coli* 129 only increased the number of IFNγ and IL-12 positive cells. The probiotic strain, *L. casei* CRL 431, induced a great stimulation of the immune cells associated to the lamina propria of the small intestine, with a significant increase in the number of all the cytokines studied with the exception of IL-17. The most remarkable effect was found in the number of IFNγ and TNFα producing cells, when compared with the non probiotic strains. The above results obtained for the probiotic strain, led us to analyze these cytokines for the potentially probiotic strains *Lactobacillus acidophilus* CRL 924 and *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 423, because nowadays some strains of these last lactobacilli are considered probiotics [26]. We also determined the IgA+ cells for these strains.

We observed that both *L. acidophilus* CRL 924 and *L. delbrueckii subsp. bulgaricus* CRL 423 increased the number of IFNγ+ cells, while only *L. delbrueckii subsp. bulgaricus* CRL 423 was able to increase the number of TNFα+ cells after two days of administration. The values obtained for IgA+ cells showed similar variation in all the strains assayed (probiotic or non probiotic) (Table 2).

3.3. Effect of bacterial administration on TLR-2 and TLR-4 expression

When we determined the expression of TLRs involved in the intracellular signals, we observed that all the Gram(+) strains were able to increase the number of TLR-2+ cells. This effect was more relevant for *L. casei* CRL 431. The Gram(−) strains only induced a significant increase in the number of TLR-4+ cells, in both *E. coli* 13-7 and *E. coli* 129 (Fig. 3).

3.4. Determination of CD-206 positive cells

CD-206 is another pattern recognition receptor, besides toll like receptors, that has a high affinity for mannose residues. When we determined the expression of this receptor on the gut immune cells, we observed that only *L. casei* CRL 431 was able to induce an increase in the expression of this receptor (Fig. 4).

3.5. Determination of the percentage of CD45+ cells

This marker was determined by flow cytometry after isolation of IEC of the small intestine, in order to determine the contamination of this cell suspension with immune cells. We observed that the percentage of CD45+ cells was 17.34% (Fig. 5).

3.6. Effects of bacterial administration on IL-6 production by a preparation enriched in intestinal epithelial cells

Intestinal epithelial cells are source of various chemokines as IL-8, MCP, ENA and interleukins such as IL-6 or IL-1. We assessed the ability of a preparation enriched in IEC isolated from the small intestine to respond to different strains measuring IL-6 release in an ex vivo assay. This cytokine was selected because its release is induced by a diversity of stimulus, while IL-8 is induced preferentially by pathogen stimulation. The viability of this preparation enriched in IEC, determined by trypan blue exclusion, was higher than 90% and the percentage of CD45(−) cells was 82.66%, confirmed by flow cytometry.

Fig. 6 shows the IL-6 production by preparation enriched in IEC from mice that received the different strains for 7 days and from untreated (control) animals. We observed that only the probiotic strain (*L. casei* CRL 431) induced a significant production of this cytokine in the supernatant of this preparation with respect to the control animals.

4. Discussion

In the gut ecosystem of conventional mice harboring a complete microbiota, the network of signals between eukaryote and prokaryote cells is complex. We studied the

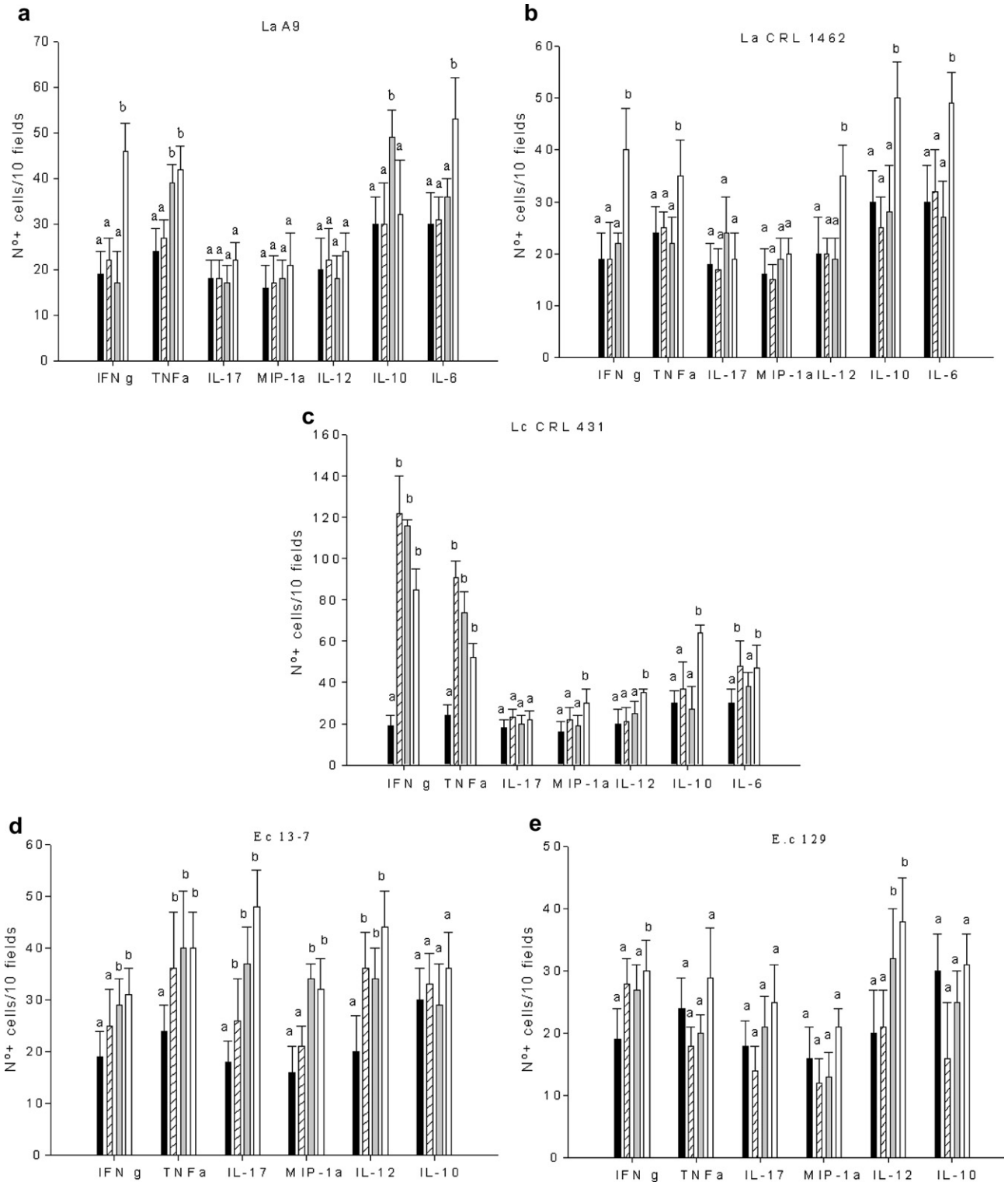

Fig. 2. Effect of the assayed strains on the cytokine profile on the small intestine. Positive cells for each cytokine were counted in histological sections from small intestine of unfed control (black bar) 2 days group (diagonal lines), 5 days group (gray bar) and 7 days group (white lines). Values are means for $n = 5 \pm$ SD. Means for each cytokine without a common letter differ significantly ($P < 0.05$).

contribution of Gram(+) and Gram(−) microorganisms in the stimulation of the gut immune system in conventional animals after oral administration of different strains, and how the stimuli induced after bacteria interaction enhance gut immunity.

In the gut, B cell population can be stimulated by antigens present in the food or by microbes. In conventional mice, B cells are specifically stimulated by microbial antigens and expand clonally. [27]. Cytokines such as IL-5, IL-6 and IL-10 are required to maintain the IgA+ B cell

Table 2
Influence of *Lactobacillus acidophilus* CRL 724 and *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 423 on number of IFNγ, TNFα and IgA positive cells

Experimental group	IFNγ	TNFα	IgA
L. a CRL 924			
2 days	68 ± 2^{a}	50 ± 7	173 ± 24^{a}
5 days	70 ± 2^{a}	40 ± 9	168 ± 25^{a}
7 days	67 ± 5^{a}	49 ± 11	135 ± 17^{a}
L.b CRL 423			
2 days	50 ± 19	80 ± 6^{a}	131 ± 26^{a}
5 days	73 ± 4^{a}	59 ± 11	91 ± 12
7 days	207 ± 6^{a}	44 ± 12	95 ± 18
Control	18 ± 1	24 ± 4	96 ± 17

Animals were given the strains assayed during 2, 5 or 7 days. Small intestine was removed after each period of administration. IgA, (direct immunofluorescence) IFNγ and TNFα (indirect immunofluorescence).
[a] Significant differences between test and control groups ($P < 0.05$).

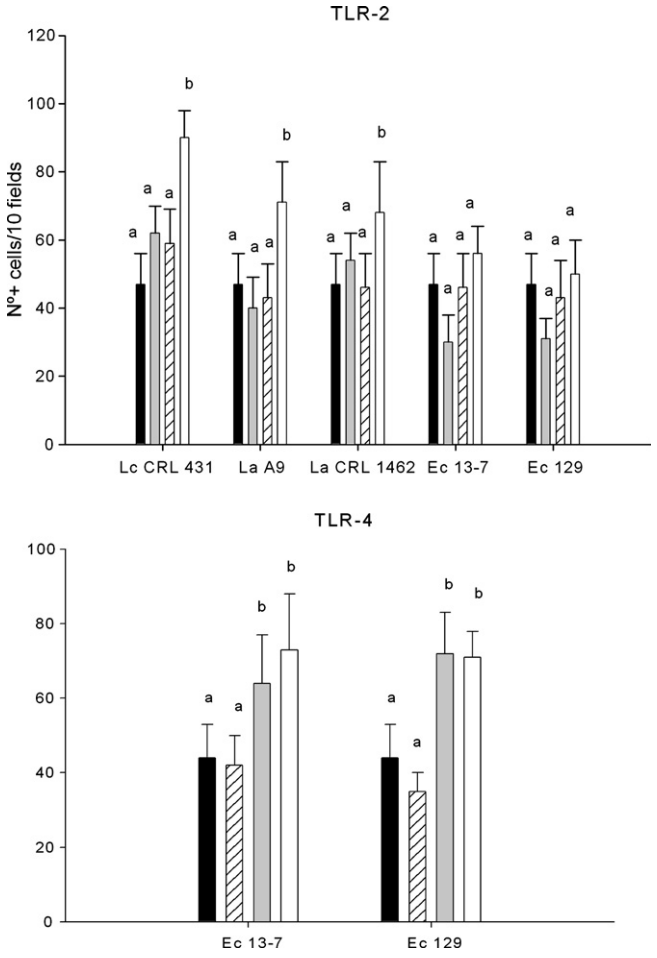

Fig. 3. Effect of the assayed strains on TLR expression. Positive cells for each TLR were counted in histological sections from small intestine of unfed control (black bar) 2 days group (diagonal lines), 5 days group (gray bar) and 7 days group (white lines). Values are means for $n = 5 \pm$ SD. Means without a common letter differ significantly ($P < 0.05$).

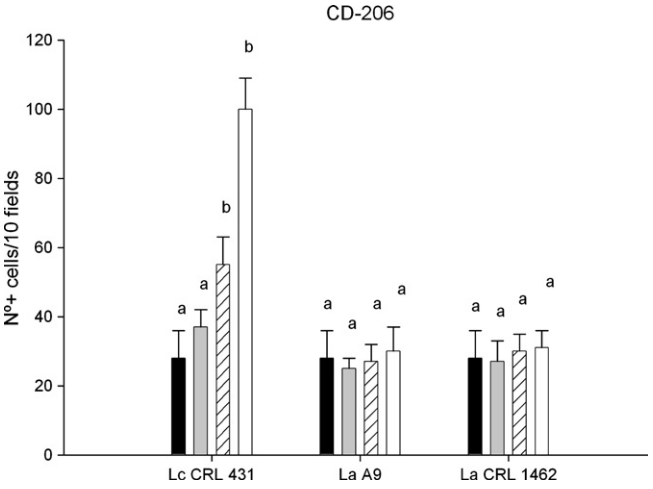

Fig. 4. Effect of the assayed strains on CD-206 expression. Positive cells were counted in histological sections from small intestine of unfed control (black bar), 2 days group (diagonal lines), 5 days group (gray bar) and 7 days group (white lines). Values are means for $n = 5 \pm$ SD. Means without a common letter differ significantly ($P < 0.05$).

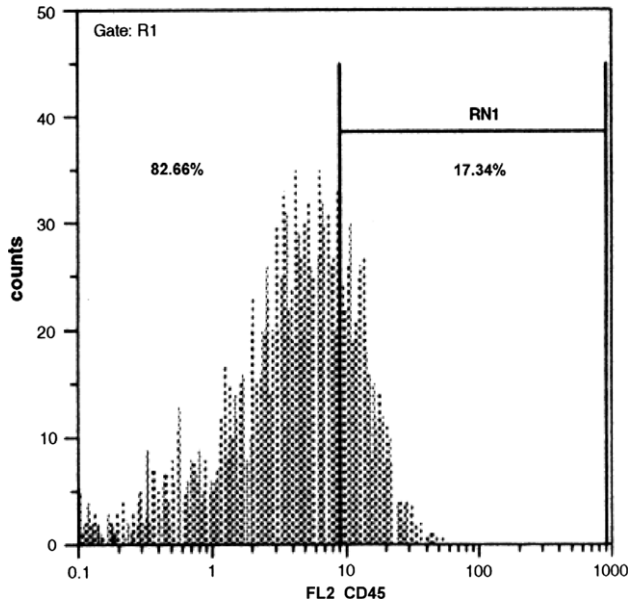

Fig. 5. Evaluation of purity of intestinal epithelial cells isolated from small intestine. R1 total cell population, RN1 CD45(+) cells. The results shown are the results of one representative experiment of three experiments performed.

population. B cell development and functioning can be independent of T lymphocyte, minimizing the lymphokines contribution mediated by T cells. [28]. When we compared the number of IgA producing cells after stimulation with the strains assayed, we determined significant differences with the untreated control, but we did not observe significant differences between either Gram(+) or Gram(−) bacteria or between the probiotic and potentially probiotic strains assayed (Fig. 1). This fact would mean that the increase in the IgA+ cells determined in the lamina propria of the small intestine would not be a good immunological marker for the selection of probiotic bacteria with an immunomodulatory capacity. Our results for Gram(+) and Gram(−) bacteria do not agree with those of Moreau

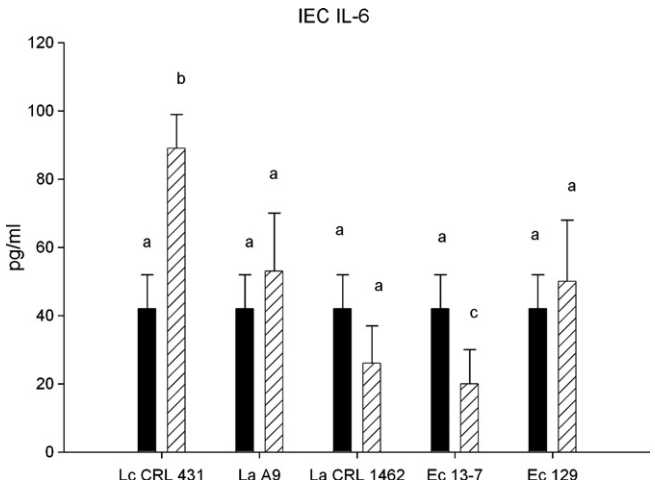

Fig. 6. Effect of the assayed strains on IL-6 secretion by a preparation of enriched IEC by ex vivo assay. Ex vivo effect after 7 days of bacterial administration (diagonal lines) compared to unfed control (black bar). Values are means for $n = 5 \pm$ SD. Means without a common letter differ significantly ($P < 0.05$).

et al. and Mac Ghee et al. [29,30] who demonstrated, in germ free animals, that Gram(+) bacteria had a slightly stimulating effect on the number of IgA+ cells and that Gram(−) microorganisms such as *E. coli* or *Bacteroides* induced an increase in the number of IgA+ cells, probably due to LPS present in the cell wall. We think that in conventional animals, the large numbers of different antigenic epitopes in the cell wall of both Gram(+) and Gram(−) bacteria, with adjuvant or inflammatory capacities [31] may have a modulatory effect on GALT functions, depending on bacterial equilibrium. We analyzed the cytokine producing cells in the lamina propria of the small intestine to determine the local effect induced in the gut after stimulation with the different strains assayed. We focused our study especially on those cytokines such as IFNγ and TNFα, whose main biological role is the activation of the innate immunity rather than proinflammatory activity [32–35]. In this respect, we measured IL-17, MIP-1α and IL−12, which promote chronic inflammation [36,37]. We also determined IL-6, which promotes both B cell maturation [38] and proinflammatory activity [39], and the regulatory cytokine IL-10. Our results for IFNγ and TNFα showed similar profiles for *L. acidophilus* A9 and for *L. acidophilus* CRL 1462. However, in the probiotic strain *L. casei* CRL 431 and in the potentially probiotics (as was previously demonstrated when analyzed the influence of the gut immune system (40)) *L. acidophilus* CRL 924 and *L. delbrueckii* subsp. *bulgaricus* CRL 423, a significant difference was found in the number of positive cells for these cytokines, specially for INFγ (Fig. 2 and Table 2). The results showed the importance of the increase in the number of IFNγ, TNFα and MIP-1α positive cells for the probiotic strain. Such increase would be due to a good activation of the immune cells, mainly macrophages and dendritic cells, involved in innate immunity. Several studies have suggested that macrophages and dendritic cells can also secrete IFNγ during the early immune response, when T cells are not activated, this cytokine playing a pivotal role in the control and development of effective host adaptative immune response against pathogens [32]. According to our results, IFNγ and TNFα, specially IFNγ, could be markers to characterize a probiotic strain with immunological activity, instead of IgA+ cells. Even though these cytokines could be considered proinflammatory markers, the activation of the immune cells by these non pathogenic bacteria maintains intestinal homeostasis, as demonstrated by previous works [21,40]. When we analyzed positive cells producing IFNγ and TNFα for Gram(−) bacteria (Fig. 2a–c), we found that *E. coli* 13-7 was able to increase the number of cells for these cytokines, but the values obtained were similar to those observed for the Gram(+) *L. acidophilus* A9 and *L. acidophilus* CRL 1462 strains. Nicaise et al. [41], using germ free animals, showed that Gram(−) bacteria could be responsible for the major source of production of IFNγ to maintain cellularity in the gut. We believe that the different results we found arise from the fact that our study was performed in conventional mice.

When we determined the proinflammatory cytokines IL-17, MIP-1α and IL-12 (Fig. 2), we observed there were no differences in IL-17 producing cells in the Gram(+) and the Gram(−) *E. coli* 129, bacteria assayed, with regard to the control. The only microorganism able to induce an increase in this cytokine was *E. coli* 13-7. IL-12 was similar for all Gram(+) and Gram(−) bacteria assayed. There is a pathophysiological function of IL-12 and IL-23 in the context of host defense and autoimmune inflammation [42]. IL-23 drives the development of auto reactive IL-17 producing cells and promotes inflammation whereas IL-12 drives the classical Th1 response [43]. IL-12 and IFNγ can suppress the IL-17 function [44]. We believe that intestinal homeostasis is maintained by the increased number of IFNγ and IL-12 positive cells induced by the Gram(+) bacteria, especially by the probiotic strain *L. casei* CRL 431. These results agree with the ones reported by Smits et al. [45]. With regard to the IL-6 producing cells determined in the immune cells associated to the lamina propria of the intestine, we observed an increased number of IL-6+ cells for all Gram(+) bacteria, with similar values. We believe that this cytokine was produced to favor the clonal expression of B cells after the non pathogenic stimulus. IL-10+ was induced only for all the Gram(+) bacteria, this effect being similar in all strains assayed. No differences with the probiotic bacteria were found (Fig. 2a–c). This result does not agree with those of other studies that indicate that Gram(−) bacteria are better producers of IL-10 than Gram(+) ones [46]. It should be noted that the induction of IL-10 producing cells after stimulation with Gram(+) bacteria is undeniable, by the immunomodulatory capacity of the peptidoglycan [31].

Intestinal epithelial cells (IEC), which are in continuous contact with vast microbial populations, have evolved an array of strategies for preventing bacterial invasion into deeper tissues and limiting the inflammatory response

toward the associated microbiota present in the lumen. These cells can express different toll like receptors (TLR-2; TLR-4; TLR-5) and are able to secrete different cytokines such as IL-6; IL-1 and IL-8 [47]. The oral administration of bacterial suspensions can imply some interaction with the mucosal surface; this interaction can be translated as an activation of IEC to induce cytokine release [24]. In our study, in a comparative assay using non pathogenic bacteria and a preparation enriched in intestinal epithelial cells (82.66%) in an ex vivo assay, we found that only the Gram(+) probiotic bacteria (*L. casei* CRL 431) was able to release IL-6 from this cell suspension after oral stimulation (Figs. 5 and 6). These results agree with those reported in a previous study [24]. We think that the physiological role for the IL-6 produced by a preparation enriched in IEC, after non pathogenic probiotic bacteria stimulation, would be responsible to initiate or maintain the cross-talk between the epithelial and immune cells associated with the gut. However, it is not possible to rule out non-epithelial cells as a possible source of this response, due to the percentage of CD45+ cells (17, 34%).

Toll like receptors are a family of pattern recognition receptors that link the innate and adaptative immunity and the TLR signaling influences CD4+ T helper cells during infection or vaccination [48,49]. TLR stimulation activates several intracellular signaling pathways that are crucial for the initiation and regulation of the innate immune response through the nuclear factor NF-KB. TLR signal in the host controls many cellular activities in the innate immunity and is important for the regulation of cytokine expression [50]. Thus, these receptors are the key sensors of the microbial product expressed in sentinel cells, most notably in macrophages and dendritic cells. We demonstrated an increase in the expression of TLR-2 for Gram(+) and TLR-4 for Gram(−) bacteria in the immune cells associated with the gut (Fig. 3) We think that this enhanced expression contributed to increase cellular activation and cytokine production and to reinforce the first line of host defense.

Mannose receptor (CD206) is another pattern of recognition of a broad spectrum of molecules of a wide variety of infectious agents. This receptor can bind altered self antigens and its role extends beyond the first line host defense; it can have a role as an inflammation modulator. The immune cells from the innate immunity can express CD206 [51]. We found that only the probiotic bacterium was able to increase the expression of CD206 receptor (Fig. 4). This finding would mean that probiotic bacteria can activate the immune cells through an enhancement in the expression of the CD206 receptor, thus favoring innate immunity.

We demonstrated that the oral administration of Gram(+) or Gram(−) bacteria to conventional mice helps to maintain a physiological state of immune cells activation through the innate immune system. This effect was more evident for the probiotic strain assayed, which indicates that the immune property of probiotics is characteristic only of some strains, perhaps due to the special chemical structure of the cell wall or to the antigenic molecules present in its cytoplasmatic content. The present study also showed that cytokines such as IFNγ and TNFα produced by macrophages or dendritic cells and IL-6 from a preparation enriched in IEC induced by the probiotic bacteria assayed, are more representative than IgA+ cells in the characterization of a probiotic strain.

Acknowledgments

This work was financially supported by PICT 00/10068, Agencia Nacional de Promoción Científica y Tecnológica (ANPyCT-FONCYT); PIP 5445, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) and CIUNT D351, Universidad Nacional de Tucumán, Argentina.

The authors are grateful to Analía Etcheverría from Universidad Nacional del Centro, Tandil, Argentina, who supplied the strain *Escherichia coli* 13-7.

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