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Probiotic bacteria cell walls stimulate the activity of the intestinal epithelial cells and macrophage functionality

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RESEARCH ARTICLE

Abstract

The effect of oral administration of probiotic bacteria cell walls (PBCWs) in the stimulation of the immune system in healthy BALB/c mice was evaluated. We focused our investigation mainly on intestinal epithelial cells (IECs) which are essential for coordinating an adequate mucosal immune response and on the functionality of macrophages. The probiotic bacteria and their cell walls were able to stimulate the IECs exhibiting an important activation and cytokine releases. Supplementation with PBCWs promoted macrophage activation from peritoneum and spleen, indicating that the PBCWs oral administration was able to improve the functionality of the macrophages. In addition, the PBCWs increased immunoglobulin A (IgA)-producing cells in the gut lamina propria in a similar way to probiotic bacteria, but this supplementation did not have an effect on the population of goblet cells in the small intestine epithelium. These results indicate that the probiotic bacteria and their cell walls have an important immunoregulatory effect on the IECs without altering the homeostatic environment but with an increase in IgA+ producing cells and in the innate immune cells, mainly those distant from the gut such as spleen and peritoneum. These findings about the capacity of the cell walls from probiotic bacteria to stimulate key cells, such as IECs and macrophages, and to improve the functioning of the immune system, suggest that those structures could be applied as a new oral adjuvant.

Keywords: lactic acid bacteria, mucosal immunity, innate immune cells, mucosal adjuvant

1. Introduction

The gastrointestinal mucosal immune system is constantly exposed to both self- and non-self-microorganisms (Westendorf *et al.*, 2010). This creates a requirement for a homeostatic balance between tolerance and immunity that represents a unique regulatory challenge to the mucosal immune system. There is a thin regulation mechanism between host mucosal defence and tolerance toward the resident microbiota, because an imbalance in this system might result in uncontrolled up-regulation of inflammatory responses (Otte and Podolsky, 2004). Intestinal epithelial cells (IECs) are thought to participate in the onset and regulation of the mucosal immune response by interacting with immune cells of Peyer's patches, lymphoid tissue in the lamina propria, and the intraepithelial lymphocytes. Under steady-state conditions, IECs generate a tolerogenic environment due to the continuous presence of specific bacterial components and metabolites that results in a status of hypo-responsiveness. They must discriminate between diverse elements of intestinal microbiota and respond to invading pathogens. In this sense, in the presence of pathogenic microorganisms, IECs are capable of secreting cytokines and chemokines that are crucial to immune cell recruitment and activation of the adaptive immune system.

The intestinal epithelium exhibits numerous adaptations to separate the host connective tissue from luminal

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environments (Artis, 2008). Tight junctions stop paracellular traffic, preventing microbial attachment and invasion. Immunoglobulin A (IgA) production limits bacterial internalisation and reduces the opportunity for commensal bacteria to activate innate immune cells in the lamina propria (Macpherson and Uhr, 2004). Goblet cells form a glycocalyx by the secretion and apical attachment of a heavily glycosylated mucin-rich layer. The epithelium layer also secretes a broad range of antimicrobial peptides via Paneth cells (Peterson and Artis, 2014).

Lactic acid bacteria (LAB) have been demonstrated to provide health-promoting effects upon administration (Dwivedi *et al.*, 2016; Kalliomäki and Salminen, 2007; Luoto *et al.*, 2014; Szajewska *et al.*, 2013). Our previous studies demonstrate that some LAB strains have an important immunomodulatory effect on mice in a dose-dependent manner, and they can interact with IECs as whole cells, but only LAB antigenic fragments are able to internalise into the cells for the subsequent activation (Galdeano and Perdigón, 2004; Maldonado Galdeano *et al.*, 2015; Perdigón *et al.*, 2001a, 2002).

Probiotic cell wall (CW) molecules are key ligands for interacting with the pattern-recognition receptors (PRRs) from the host and they could induce signalling pathways, resulting in a beneficial effect. Structural variations exist between different microorganisms, especially in the case of microorganism-associated molecular patterns (MAMPs) located on the CW. These variations lead to differences in the host-microbial interactions, and confer a strain-dependent effect. Probiotics are generally recognised as safe, and they are applied worldwide as food-grade bacteria; in this sense, molecules located on the probiotic CWs could interact with PRRs of the IECs and lamina propria immune cells, and act as potential mucosal adjuvants enhancing both local and systemic immune responses.

In order to study whether the CW of probiotic bacteria could be useful as oral adjuvant and exert a potential immunoregulatory effect – as was demonstrated for the whole bacteria (Maldonado Galdeano *et al.*, 2015) – the aim of the present work was to determine the effect of probiotic bacteria CWs administered by oral route, in activating intestinal epithelial barrier and macrophages from Peyer's patches, as well as macrophages at distant sites from the gastrointestinal tract. This study was carried out comparatively with whole probiotic bacteria.

2. Materials and methods

Bacterial strains and culture conditions

Lactobacillus casei CRL431 (Lc431) and *Lactobacillus acidophilus* CRL730 (La730) were obtained from the CERELA culture collection (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Tucumán, Argentina). *Lactobacillus paracasei* CNCM I-1518 (Lp1518) was provided by Danone Argentina. These strains were cultured for 16 h at 37 °C in a sterile De Man-Rogosa-Sharpe (MRS) broth (Britania, Buenos Aires, Argentina). *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) used in this study was obtained from Bacteriology Department of the 'Hospital del Niño Jesús' (San Miguel de Tucumán, Argentina) and was cultured for 16 h at 37 °C in a sterile Brain Heart Infusion broth (Britania).

Cell wall probiotic bacteria

For the isolation of CWs, the probiotic bacteria Lc431, Lp1518 and La730 were grown in MRS broth for 16 h at 37 °C, the biomass was harvested at 9,900×*g* for 10 min at 4 °C and washed three times with distilled sterile cold water. The cells were resuspended in one volume of distilled sterile cold water and were harvested at 33,000×*g* for 30 min. The pellet was lysed three times with a French press at 20,000 psi. The product obtained was harvested at 4,000×*g* for 15 min at 4 °C. The supernatant obtained was harvested at 30,000×*g* for 30 min at 4 °C. The pellet was delipidated by refluxing with methanol, methanol:chloroform and chloroform. The delipidated product was resuspended in phosphate buffered saline (PBS) 0.01 M pH 7.4 and treated with DNase I from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA) (50 µg/ml) and ribonuclease A (Sigma-Aldrich) (100 µg/ml) at 37 °C with stirring for 4 h. The insoluble product was washed with distilled sterile water and frozen at -80 °C; the products obtained were used as CWs from *L. casei* CRL431 (CW431), *L. paracasei* CNCM I-1518 (CW1518) and *L. acidophilus* CRL730 (CW730).

Animals and feed procedures

Male six-week-old BALB/c mice weighing 25 to 30 g were obtained from the inbred colony maintained at CERELA. The animals were fed on balanced rodent food and water *ad libitum*. The experimental protocol contained four experimental groups receiving by gavage 100 µl/day/mice. During a period of seven consecutive days, these animals received: (1) CW431; (2) CW1518; (3) CW730; and (4) PBS (control group), respectively (Supplementary Figure S1). To compare these results, other groups of mice received a viable *Lactobacillus* suspension in their drinking water for 7 consecutive days, either Lc431 or La730, and for 5 consecutive days Lp1518, respectively. These times were established according to previous results (Galdeano and Perdigón, 2004, 2006; Maldonado Galdeano *et al.*, 2011; Perdigón *et al.*, 2002). Overnight cultures (5 ml) of each strain were suspended in 5 ml sterile 10% non-fat milk and diluted in PBS to a final concentration of 10^8 cfu/ml. The amount of protein content between probiotic CW products and the viable *Lactobacillus* suspension was adjusted to ~1000 µg/ml. The animal protocol was pre-approved by

the Animal Protection Committee of CERELA (Protocol No. CRL-BIOT-LI-2011/1A) and all experiments complied with the current laws of Argentina.

Ex vivo phagocytic activity assay

At the end of each administration period both test and control animals were sacrificed. Peritoneal, spleen and Peyer's patches macrophages from BALB/c mice were obtained according to a previously described method in the laboratory (De Moreno de LeBlanc *et al.*, 2010). Briefly, macrophages were extracted from the peritoneal cavity with 5 ml of sterile PBS 0.01 M pH 7.4 containing 100 µg/ml of gentamicin (Gm). For the isolation of macrophages from Peyer's patches, the small intestine of each mouse was removed, washed and the Peyer's patches were excised in Hank's buffered salt solution (HBSS) (Sigma-Aldrich) containing 10% foetal bovine serum (FBS). The epithelium cells were separated with an HBSS/FBS solution containing EDTA. The sediments were incubated with collagenase/DNase solution and the mononuclear cells were recovered. These cells were collected from the supernatant and washed with RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA). For spleen macrophages isolation, the spleen was collected in 5 ml of HBSS solution containing 10% FBS and aseptically disrupted. The cells were harvested by centrifugation at 800-1000×*g* for 15 min at 4 °C. The resulting pellets were gently mixed with 2 ml of sterile red blood lysing buffer for 2 min. The haemolysis was stopped with PBS. The sample were again centrifuged and resuspended in RPMI-1640 medium containing 10% FBS. The macrophages were separated from the mononuclear population using their adherence property to glass slides.

Phagocytosis assay was performed using a *Saccharomyces cerevisiae* suspension at a concentration of 1×10^7 cells/ml. Opsonised yeast in mouse autologous serum (10%) was added to 200 µl of macrophage suspension. The mixture was incubated for 30 min at 37 °C. The percentage of phagocytosis was expressed as the percentage of phagocyting macrophages in 100 cells counted in an optical microscope.

Ex vivo microbicidal activity determination

Microbicidal activity was determined according to a previously described method (Maldonado Galdeano *et al.*, 2015). Briefly, peritoneal, spleen and Peyer's patches macrophages from BALB/c mice were isolated at the end of each administration period, cultured in RPMI-1640 medium containing 10% FBS and incubated in six wells sterile plates (JET Biofil, Guangzhou, China P.R.) (1 h; 37 °C; 5% CO_2), the final concentration was adjusted at 1×10^6 cells/ml/wells. Macrophages were infected with *S.* Typhimurium (1×10^7 cfu/ml). The bacterium/macrophage ratio was 10:1. After 30 min incubation at 37 °C, cells were gently washed and resuspended in RPMI-1640 medium plus Gm (200 µg/ml) to kill any remaining extracellular, but not intracellular bacteria, and incubated for 60 min at 37 °C. Finally, the cells were lysed with 1% Triton X-100/RPMI-1640 medium. Serial dilutions of the suspension of cells lysed were plated on SS agar (*Salmonella-Shigella* agar, Britania, Buenos Aires Argentina). After 24 h incubation at 37 °C, bacterial colonies were counted to determine the number of surviving intracellular bacteria.

Immunofluorescence assays for detection of IgA-positive cells in the gut lamina propria

The number of IgA+ cells was determined in the lamina propria of the small intestine on histological slices using a direct immunofluorescence assay. The small intestine was removed and processed according to Sainte-Marie's technique for paraffin embedding (Sainte-Marie, 1962). After deparaffinisation 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 fluorescein isothiocyanate (Sigma-Aldrich) for 30 min and observed with a fluorescent light microscope. 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 of vision.

Goblet cells counts in small intestine tissue

Slides from the small intestine of the different groups under study, were deparaffinised and rehydrated in a decreasing gradient of ethanol and incubated for 60 min in 1% Alcian Blue 8Gx solution (Merck, Darmstadt, Germany) in 3% acetic acid. Histological slides were then incubated for 3 min in eosin solution and then 40 min in 0.5% safranin solution in 0.1 N HCl. They were then dehydrated and finally mounted using synthetic Canada Balsam (Ciccarelli Lab., San Lorenzo, Argentina). Goblet cells were stained blue with this methodology. The results are expressed as the number of goblet cells per ten intestinal villi.

Intestinal epithelial cells isolation and cytokines determination

To analyse the effect of the probiotic bacteria CWs under study in the IECs, interleukin (IL)-6, IL-10 and monocyte chemoattractant protein 1 (MCP-1) were measured in the supernatant culture. The IECs were isolated from the small intestine according to Vinderola *et al.* (2005). Briefly, at the end of each administration period, animals were sacrificed and the small intestine was removed and placed in cold HBSS, containing 100 µg/ml Gm. The intestines were opened longitudinally to expose the mucosal surface and washed five times with the same cold buffer. 5 mm segments of tissue were collected in HBSS with 1 mM

dithiothreitol (Sigma-Aldrich), 10 mM EDTA, 8% FBS and Gm (100 µg/ml) for 15 min at room temperature to remove the mucus. Then, segments of tissue were incubated in HBSS containing 8% FBS, Gm (100 µg/ml) and 30 mM EDTA at room temperature and agitated for 15 min. Supernatant was collected and centrifuged for 5 min at 300×*g*. The pellet was washed twice with the culture medium and finally resuspended in the same culture medium at a concentration of 1×10^6 to 5×10^6 IEC cluster/ml. IEC suspensions were then transferred to six-well sterile plates (JET Biofil) (1 ml/well) and incubated for 8 h (37 °C; 5% CO_2). Supernatants were recovered for cytokine determination. IL-6, IL-10 and MCP-1 were determined using the corresponding enzyme-linked immunosorbent assay set according to the manufacturer's instructions (BD OptEIA; BD Biosciences, San Diego, CA, USA).

Transmission electron microscopy

BABL/c mice received by gavage 100 µl of each probiotic bacteria (10^8 cfu/ml) or their CWs assayed. They were sacrificed 30 min after administration. This time was established according to a previous report, where antigens probiotics were found 10 min after administration on IECs (Galdeano and Perdigón, 2004). The small intestine of each mouse was removed. The intestinal contents were eliminated by washing with 3 ml of PBS and 0.5 mm segments of tissue were fixed in (Karnovsky, 1965) solution (2.66% formaldehyde, 1.66% glutaraldehyde), and sodium phosphate buffer 0.1 M pH 7.4 and incubated overnight at 4 °C. The fixed samples were washed three times with the sodium phosphate buffer for 10 min every time, and then fixed with a 1:1 solution of sodium phosphate buffer/2% osmium tetroxide (OsO_4), overnight. The samples were washed three times with distilled water for 1 min each time, and a 1:1 solution of phosphate buffer/uranyl acetate was added. After 30 min incubation in the dark, the solution was discarded and the samples rapidly washed with ethanol 70, 90 and 100% for 1 min, and then dehydrated three times with ethanol 100%, 20 min each time, and three times in acetone for 10 min each time. The samples were incubated for 30 min in a 1:1 solution of acetone/Spurr resin. The specimens were included in Spurr resin, and heated at 60 °C for 24 h. Ultra-thin sections were made with an ultramicrotome, mounted on copper grids and contrasted with uranyl acetate and lead citrate (Venable and Coggeshall, 1965). The samples were observed with a Zeiss EM109 (Carl Zeiss NTS GmbH, Oberkochen, Germany) transmission electron microscope. The processing of the samples, pictures and analysis of them has been made by experts of the 'Centro Integral de Microscopía Electrónica' (CIME-CONICET), Tucumán, Argentina.

Statistical analysis

For all the studies, each group assayed was from three mice. Each experiment was performed three times. All statistical analyses and plotting were carried out using GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA, USA). The significance of the difference was determined using one-way-ANOVA when more than 2 groups were conducted, whereas T test was conducted to compare two experimental groups. A $P<0.05$ was considered as statistically significant.

3. Results

Evaluation of phagocytosis and microbicidal activity of macrophages

Macrophages are key immune effector cells. They can link the innate and adaptive immune response. It was determined if the probiotic bacteria CWs possess the capacity to activate the macrophages. Comparatively, whole probiotic bacteria were administered via the drinking water to other groups of mice; the activity of peritoneal, spleen and Peyer's patches macrophages was analysed. The administration of the three lactobacilli CWs significantly increased the phagocytic activity of peritoneal macrophages in comparison with the control (Figure 1A). There was also a significant increase in phagocytic activity of peritoneal macrophages from mice treated with CWs compared to the probiotic bacteria (Figure 1A). CW431 and CW1518 showed a significant increase in the phagocytosis in spleen macrophages compared to the control; however, CW730 did not induce any increase in the phagocytic activity compared to the control (Figure 2B). CW1518 and CW730 showed a significant increase in comparison to their whole bacteria (Lp1518 and La730, respectively) (Figure 1B). Lc431 administration increased the phagocytosis of Peyer's patches macrophages compared to the control, and CW1518 administration showed a significant increase in phagocytosis of Peyer's patches macrophages compared to Lp1518 (Figure 1C).

An important feature of macrophages is the ability to kill microorganisms after the phagocytic process. In this sense, based on the phagocytic activity data, the microbicidal activity from peritoneal, spleen and Peyer's patches macrophages against *S.* Typhimurium was measured by *ex vivo* assay after administration period. A significant increase was observed in the microbicidal activity of peritoneal macrophages in mice treated with CW431, CW1518 and CW730, as evidenced by lower cfu/ml of *S.* Typhimurium compared to the control (Figure 2A). An increase in the microbicidal activity of peritoneal macrophages of Lc431- and La730-treated mice was observed, as well as a significantly increased microbicidal activity of spleen macrophages by Lc431 and Lp1518 administration,

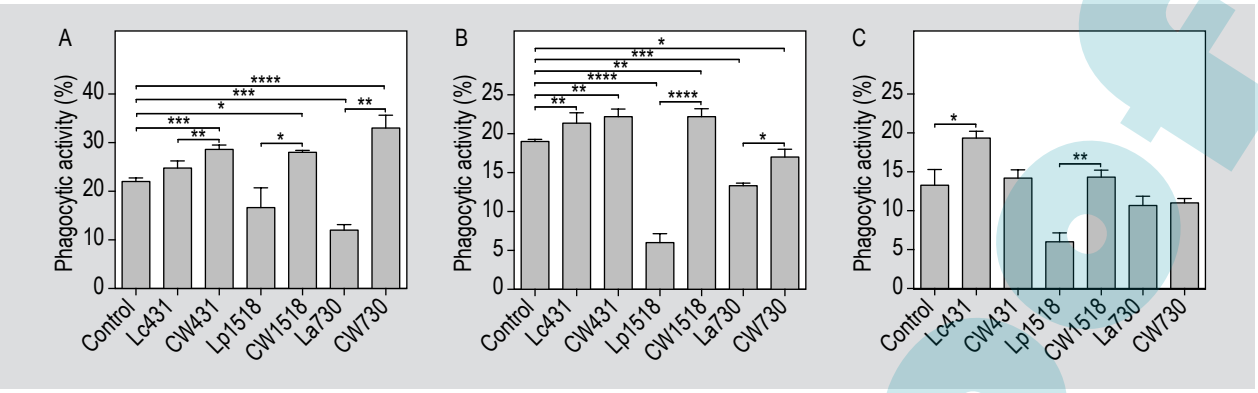

Figure 1. Macrophages activity induced after oral delivery of probiotic bacteria and their cell walls (CW). BALB/c mice were administered with probiotic bacteria Lc431, Lp1518, La730 or purified water as control, and other groups were administered with probiotic bacteria CWs CW431, CW1518, CW730 or PBS as control by gavage. At the end of the administration period, phagocytic activity of (A) peritoneal, (B) spleen, and (C) Peyer's patches macrophages were determined. Values are means (n=9) ± standard error of the mean from each group of three mice; data are representative of three independent experiments. * *P*<0.05; ** *P*<0.01; * *P*<0.001; **** *P*<0.0001.**

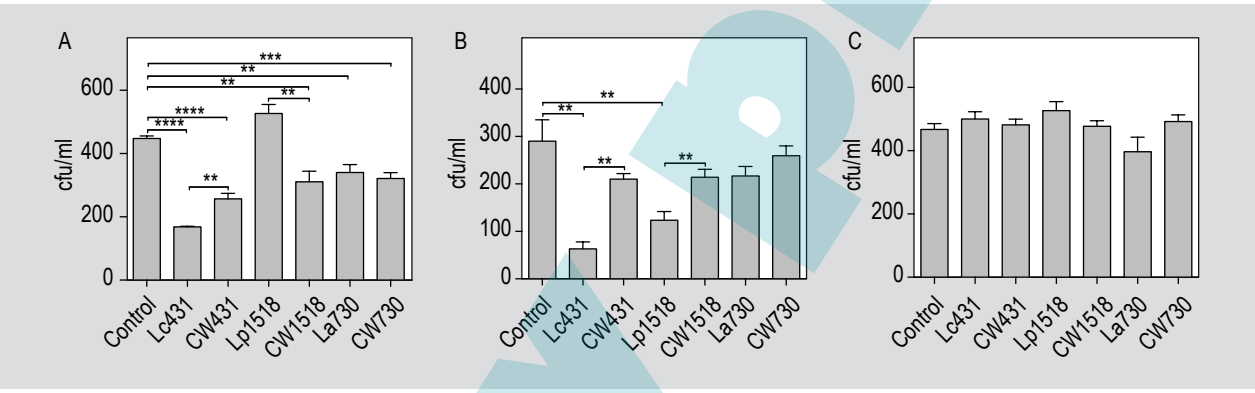

Figure 2. Microbicidal activity of (A) peritoneal, (B) spleen and (C) Peyer's patches macrophages. *Ex vivo* assays of macrophages of mice that had received probiotic bacteria Lc431, Lp1518, La730 or purified water as control, and other groups were administered with probiotic bacteria cell walls CW431, CW1518, CW730 or PBS as control by gavage. Values are means (n=9) ± standard error of the mean from each group of three mice; data are representative of three independent experiments. * *P*<0.05; ** *P*<0.01; * *P*<0.001; **** *P*<0.0001.**

compared to the control group. Administration of CW431, CW1518, La730 and CW730 did not induce any change compared to the control group (Figure 2B). The microbicidal activity of Peyer's patches macrophages from all treated groups was similar to the untreated control (Figure 2C).

Determination of IgA+ cells in lamina propria of the small intestine

The IgA helps to protect the mucosal tissue against internalisation of commensal and non-commensal microorganisms. It was determined if CWs and the whole probiotic bacteria assayed were able to increase the number of IgA+ cells in the lamina propria of the small intestine at the end of the administration period. It was observed that the administration of probiotic bacteria assayed significantly increased the number of IgA+ cells in lamina propria of the small intestine in comparison to the normal control. A rise in IgA+ cells with the CWs (CW431- and CW1518-treated mice) when compared with the normal control was also observed. Lc431 administration significantly increased IgA+ cells compared to its CW (CW431) (Figure 3).

Effect on the gut barrier: goblet cell determination

The intestinal epithelium is covered by a mucus gel that is synthesised and secreted by goblet cells that form a mucosal barrier. To investigate if CWs and probiotic bacteria could influence the population of goblet cells, the number of goblet cells in the small intestine at the end of administration period was measured. It was observed that none of the different treatments induced any change in the number of goblet cells in the small intestine epithelium compared to the control group (Supplementary Figure S2).

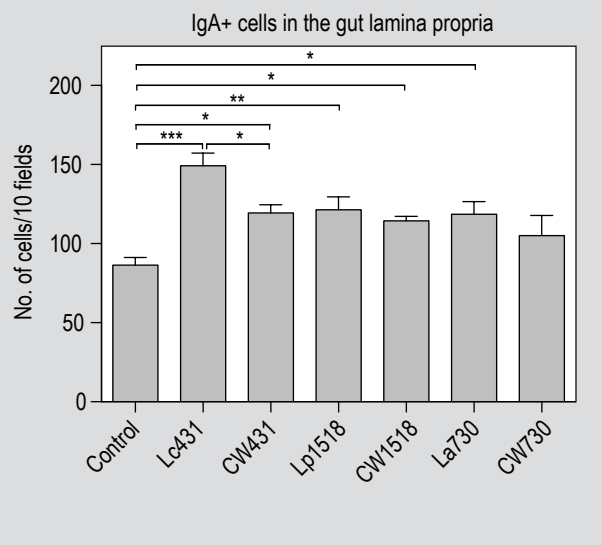

Figure 3. IgA+ cells in the lamina propria of the small intestine of mice. IgA+ cells were determined by direct immunofluorescence on slides from small intestine of BALB/c mice at the end of the administration period with probiotic bacteria Lc431, Lp1518, La730, and other groups of mice were administered with CW431, CW1518, CW730 or PBS as control group by gavage. Results are expressed as number of positive cells per 10 fields of vision at 1000x magnification. The data are shown as the mean values (n=9) ± standard error of the mean for three independent experiments. *P<0.05; ***P<0.001.

Effects of probiotic bacteria and their cell wall administration on cytokine production by IECs

IECs are a source of various cytokines and chemokines. The ability of a preparation enriched with IECs isolated from the small intestine to respond to probiotic bacteria or their CWs, measuring IL-6, IL-10 and MCP-1 release by an *ex vivo* assay, was assessed. All treatments increased the production of IL-6 in the IECs compared to the control group; a difference was observed between Lp1518- and CW1518-treatments (Figure 4A). Administration of Lc431, CW431, Lp1518, La730 and CW730 increased the production of IL-10 in the IECs; increased levels in the concentration of this cytokine with Lp1518 was observed compared to its CW (CW1518) (Figure 4B). CW1518 administration showed an increase level of MCP-1 compared to the control; significant differences between respectively Lp1518 and CW1518 administration, and La730 and CW730 administration were also observed, whereas Lc431 and its CW (CW431) did not induce changes in the concentration of MCP-1 (Figure 4C).

Study of intestinal epithelial cell activation by transmission electron microscopy

BALB/c mice were challenged with probiotic bacteria CWs and the whole probiotic strains to observe the activation of the IECs by transmission electron microscopy. Administration of three lactobacilli CWs or their whole bacteria induced greater activation in the IECs as evidenced by the abundance of mitochondria, Golgi, vacuoles, and lysosomes present in the cytoplasm (Figure 5B-G) compared to the control group (Figure 5A); this activation was more significant in both Lc431- and CW431-treated mice.

4. Discussion

It is well known that adjuvants are extensively used in order to increase the immune response to vaccines, triggering the signals necessary to recruit and activate innate immune cells enhancing immunogenicity (Pasetti *et al.*, 2011). In this sense, adjuvants may act as an immunomodulatory compound or association of compounds that potentiate or modulate the immune system against an antigen. The oral adjuvant capacity of some probiotic bacteria has been well

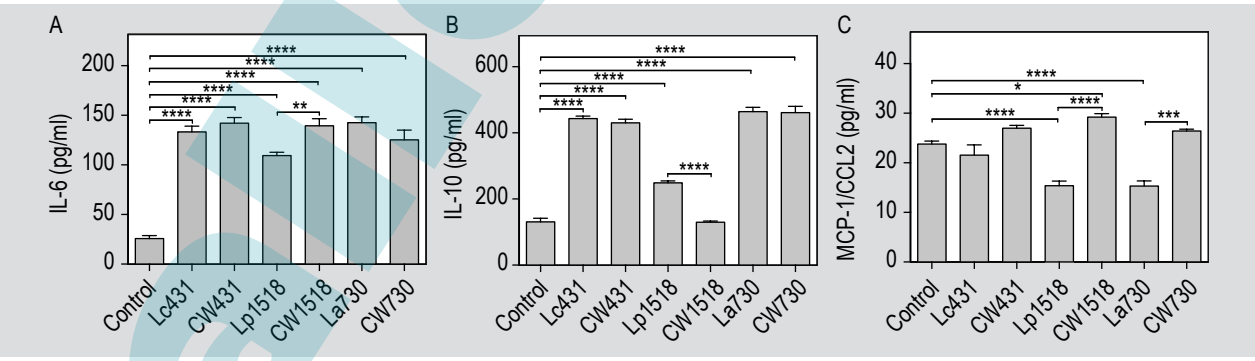

Figure 4. Cytokine production by a preparation of enriched intestinal epithelial cells (IECs) by *ex vivo* assay. BALB/c animals were administered with probiotic bacteria Lc431, Lp1518, La730 or purified water as control, and other groups were administered with probiotic bacteria CWs CW431, CW1518, CW730 or PBS as control by gavage. At the end of the administration period the levels of (A) interleukin (IL)-6, (B) IL-10 and (C) monocyte chemoattractant protein 1 (MCP-1) in the supernatant of IECs were determined by ELISA. Data represent the means (n=9) ± standard error of the mean from each group of three mice; data are representative of three independent experiments. * P<0.05; **** P<0.0001.

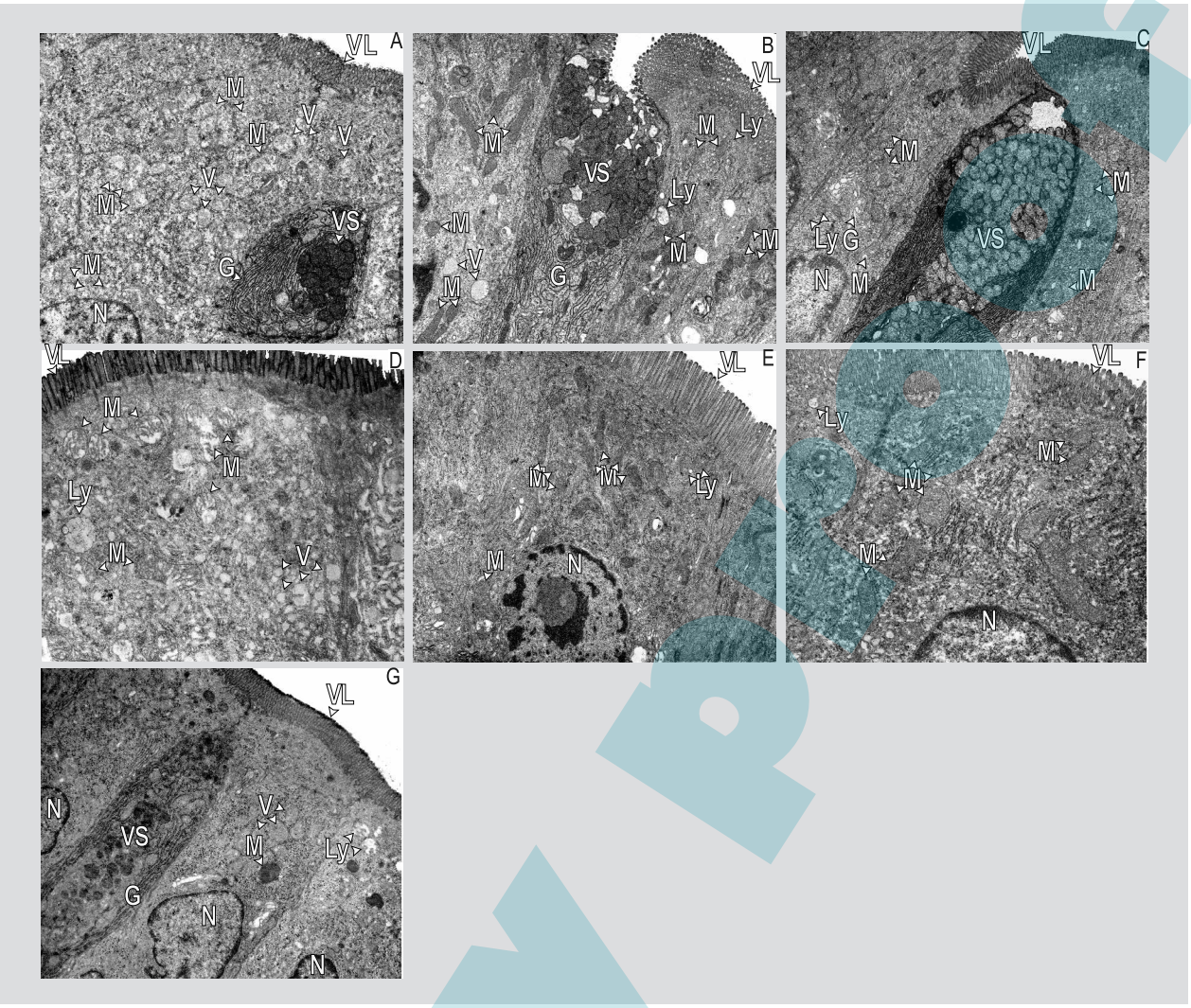

Figure 5. Transmission electron micrographs of intestinal epithelium challenged with probiotic bacteria cell walls (CW) and whole probiotic strains. BALB/c mice were administered by gavage with probiotic bacteria Lc431, Lp1518 and La730, and their CWs CW431, CW1518, CW730 or PBS as control. After 30 min, mice were sacrificed and the small intestine of each mouse was removed and fixed in the appropriate medium. PBS (A, magnification 4800×), Lc431 (B, magnification 4,800×), CW431 (C, magnification 3,400×), Lp1518 (D, magnification 7,500×), CW1518 (E, magnification 7,500×), La730 (F, magnification 7,500×), CW730 (G, magnification 3,400×). N = nucleus; M = mitochondria; V = vacuole; G = Golgi; VS = vesicle; Ly = lysosome; VL = villi. Pictures are representative of three mice.

demonstrated in our laboratory (Vitiñi *et al.*, 2000). Thus, the development of a mucosal delivery vehicle based on bacteria or their compounds generally considered as safe, such as *Lactobacillus* spp., becomes a promising strategy for modulating the intestinal immune system necessary for regulating a vaccine-induced immune response. It was reported that non-living bacteria-like particles derived from the food-grade bacterium *Lactococcus lactis* improved the systemic and mucosal immune response, potentiating immunogenicity of co-administered antigens (Saluja *et al.*, 2010a,b). Thus, the identification of safe and effective simple adjuvant components, such as the cell wall from probiotics, able to induce appropriate mucosal immune responses, would be a powerful tool for enhancing immunogenicity for orally delivered antigens, especially in immunocompromised hosts such as malnourished patients, where the administration of beneficial bacteria is still controversial.

Considering that the gut-associated lymphoid tissue (GALT) is a mucosal inductive site, the immune response induced in the small intestine can spread through the systemic immune system and reach mucosal and non-mucosal sites by lymphatic and blood circulation (Czerkinsky and Holmgren, 2012). In a previous work, it was demonstrated that oral administration with Lc431 increased phagocytosis and microbicidal activity of macrophages at distant sites from the gastrointestinal tract (Maldonado Galdeano *et*

al., 2015). In addition, the treatment of a malnutrition model mouse with a probiotic fermented milk increased the phagocytosis activity of peritoneal and spleen macrophages (Maldonado Galdeano *et al.*, 2011). The present work studied the effect of oral administration of CWs, isolated from lactobacilli strains, on the macrophages activity from the inductor sites of the intestinal immune response (Peyer's patches), as well as on macrophages from distant sites from the gastrointestinal tract, such as peritoneum and spleen. Macrophages act as innate effector cells that play a role in inflammation and in the host defence. Functions including production of cytokines and mediators, engulf and clear bacteria, and antigen presentation (Epelman *et al.*, 2014). These properties are controlled by numerous stimuli from the tissue microenvironment. It has been reported that CW of LAB can stimulate macrophages to produce IL-6 and TNF-α (Tejada-Simon and Pestka, 1999). Our *ex vivo* studies showed that all functions of peritoneal macrophages are increased by lactobacilli CW. It was found that CW431 and CW1518 increased the microbicidal and phagocytic activity of spleen macrophages; our results indicate that the activation of macrophages at distant sites from the gastrointestinal tract is dependent on the strain of CW employed. On the other hand, in the Peyer's patches CW administration did not enhance the microbicidal and phagocytic activity of macrophages. These finding may be due to the fact that the Peyer's patches macrophages are constantly exposed to the microbiota, causing the cells to acquire a tolerisation state against microorganisms, different from pathogenic bacteria (De Moreno de LeBlanc *et al.*, 2010). In addition, intestinal macrophages normally express lower levels of TLRs and are hyporesponsive to TLR signalling to dampen inflammatory responses to the commensal microbiota (Smythies *et al.*, 2005).

The gut mucosa releases anti-microbial proteins and IgA, an antibody isotype specialised in mucosal protection that plays an important role as the first line of defence and aids the regulation of bacteria-host interaction. B cells in the gut lamina propria can be activated by MAMPs from the microbiota (Gutzeit *et al.*, 2014) and differentiate into IgA-secreting plasma cells where secretory IgA has multiple roles in the mucosal defence. We showed that the administration of CW431 and CW1518 increased the number of IgA+ cells. Other LAB fractions, such as exopolysaccharides have been shown to increase the IgA+ cells in the small and large intestine lamina propria (Vinderola *et al.*, 2006). Moreover, the three *Lactobacillus* strains (Lc431, Lp1518 and La730) significantly increased the IgA+ cells in the lamina propria of the small intestine. This effect has been also observed in previous work, where Lc431 administration increased the IgA+ cells in the lamina propria after 7-day administration (Galdeano and Perdigon, 2006). We believe that the LAB antigen uptake can be carried out by M cells in the Peyer's patches or villi or may be sampled by dendritic cells as whole cells or their antigenic fragments. Furthermore, the microenvironment cytokines would favour the clonal expansion of IgA B lymphocytes, increasing the number of IgA-producing cells and the passage of them to plasmatic cells in the lamina propria of the gut.

As a mucosal surface, intestinal epithelium is covered by a mucus layer that is a first line of defence against microbes and they are a constituent part of the intestinal barrier. Mucus is produced by goblet cells, and products of microbiota help induce mucin production. Studies have demonstrated that mice deficient in Muc2, the most abundantly secreted gastrointestinal mucin by goblet cells, have increased translocation of commensal and pathogenic bacteria, and spontaneously develop colitis (Wenzel *et al.*, 2015). It was shown that CW and probiotic bacteria administration did not induce variation in the number of goblet cells in the villi of healthy mice. These results are in line with previous studies showing that the consumption of a probiotic fermented milk after weaning did not induce any modification in the goblet cell population reaching values similar to the control (De Moreno de LeBlanc *et al.*, 2008). In contrast, it was recently reported in a murine model of irinotecan-induced mucositis that *S. cerevisiae* UFMG A-905 treatment prevented a decrease in goblet cells and stimulated the replication of cells in the intestinal crypts of mice with experimental mucositis (Bastos *et al.*, 2016). Our results indicate that in an immunocompetent host, probiotics or their fragments are not an important stimulus in increasing the number of goblet cells in the small intestine, and they would act in similar way to the commensal bacteria.

IECs are considered to participate in the initiation and regulation of the mucosal immune response to bacteria by interacting with immune cells of the GALT, lamina propria cells and intraepithelial lymphocytes (Kagnoff and Eckmann, 1997). The crosstalk between IECs and the immune cells present in the lamina propria play a crucial role in keeping the intestinal homeostasis and coordinating an appropriate immune response to pathogenic microorganisms. In addition, IECs are presumed to be the first and most important target cells of probiotic action. Thus, lactobacilli and their molecules can interact and beneficially influence the optimal functioning of the gut epithelium, related to its barrier function and as a source of cytokine, chemokines and growth factors able to enhance the mucosal response. IECs are able to secrete several pro-inflammatory cytokines such as MCP-1, IL-6 and TNF-α on stimulation by pathogens (Haller *et al.*, 2000). *Ex vivo* studies showed that the administration of the three CW (CW431, CW1518 or CW730) and the probiotic bacteria (Lc431, Lp1518 or La730) significantly increased the levels of IL-6 and only CW1518 showed increased levels of MCP-1, which may be explained as both a strain-specific and/or a host-specific phenomenon. These facts correspond to a previous report about other probiotic strains, *L. rhamnosus*

CRL1505 and *L. rhamnosus* CRL1506, which induce IL-6 mRNA expression in porcine intestinal epithelial cells but not MCP-1 (Villena *et al.*, 2014). IL-10 is one of the major regulatory cytokines required to maintain intestinal homeostasis mediated through host-bacterial interactions. Previous work demonstrated that stimulation with probiotic bacteria induced signals in the epithelial and immune cells that evoked an increase in the regulatory cytokine IL-10 for the probiotic strains assayed (Maldonado Galdeano *et al.*, 2007, 2015). In this work, it was demonstrated that CW431 and CW730 increased the production of IL-10 in the IECs. Moreover, the administration of the three strains Lc431, Lp1518 or La730 stimulated production of IL-10 in the IECs. This fact contrasts with a previous report in which a *Lactobacillus* strain used at different bacterial concentrations did not by itself up-regulate mRNA for IL-10 in the human colon epithelial cell line T84, HT-29, or Caco-2 (Ma *et al.*, 2004). Moreover, the electron microscopy studies confirm the findings that after 30 min of CW or probiotic bacteria administration there is a high activation of the IECs. This fact is in line with previous reports where the probiotic strain Lc431 interacted with epithelial cells of the small intestine and their fragments could internalise and activate the IECs (Galdeano and Perdigón, 2004; Perdigón *et al.*, 2005). Furthermore, it was demonstrated when Lc431 labelled with fluorescein isothiocyanate was given to mice, that fluorescent cells at different levels of the intestine in Peyer's patches, lamina propria of the villi, and nodules of the large intestine were found after 5 minutes of probiotic administration (Galdeano and Perdigón, 2004; Perdigón *et al.*, 2001b). Our finding suggests that CW or probiotic bacteria interact with the IECs, improve their functionality and this activation highlights the possible mechanism by which IECs could activate immune cells distant from the gut. We believe that this crosstalk of IECs and bacteria occurs through the PRRs, which are selectively expressed on the cell surface, internal compartments or cytoplasm of the IECs, thus this interaction would activate signalling pathways that lead to secrete immune mediators which drive the activation of the immune cells present in the lamina propria. In this way, the increases in IL-6 levels allow us to suggest that it could be related to the enhancement of the IgA B-cell population, and the IL-10 production acts as an important mechanism of immunoregulation.

In summary, we have demonstrated that the stimulation effect of CW probiotic bacteria is achieved by the activation of the IECs with the simultaneous production of cytokines. This creates the intestinal microenvironment that influences the clonal expansion of the IgA B-cell population, and acts as a biological mediator to activate immune cells distant from the gastrointestinal tract. These findings suggest that the CWs from probiotic bacteria could be used as mucosal adjuvants. Studies are required to evaluate the mucosal adjuvant effect against a pathogen challenge and gain more insight into the mechanism by which CWs exert their action from the gut.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2016.0220.

Figure S1. Experimental protocol used in this study.

Figure S2. Goblet cells in the small intestine of BALB/c mice.

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