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In vitro immunomodulatory activity of *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713: two probiotic strains isolated from human breast milk

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ABSTRACT

Commensal bacteria, including some species of lactobacilli commonly present in human breast milk, appear to colonize the neonatal gut and contribute to protection against infant infections, suggesting that lactobacilli could potentially modulate immunity. In this study, we evaluated the potential of two Lactobacillus strains isolated from human milk to modulate the activation and cytokine profile of peripheral blood mononuclear cell (PBMC) subsets *in vitro*. Moreover, these effects were compared to the same probiotic species of non-milk origin. *Lactobacillus salivarius* CECT5713 and *Lactobacillus fermentum* CECT5716 at 10^5, 10^6 and 10^7 bacteria/mL were co-cultured with PBMC (10^6/mL) from 8 healthy donors for 24 h. Activation status (CD69 and CD25 expressions) of natural killer (NK) cells ($CD56^+$), total T cells ($CD3^+$), cytotoxic T cells ($CD8^+$) and $CD4^+$ T cells was determined by flow cytometry. Regulatory T cells (Treg) were also quantified by intracellular Foxp3 evaluation. Regarding innate immunity, NK cells were activated by addition of both Lactobacillus strains, and in particular, the $CD8^+$ NK subset was preferentially induced to highly express CD69 ($\sim$90%, $p < 0.05$). With respect to acquired immunity, approximately 9% of $CD8^+$ T cells became activated after co-cultivation with *L. fermentum* or *L salivarius*. Although $CD4^+$ T cells demonstrated a weaker response, there was a preferential activation of Treg cells (CD4+CD25+Foxp3+) after exposure to both milk probiotic bacteria ($p < 0.05$). Both strains significantly induced the production of a number of cytokines and chemokines, including TNFα, IL-1β, IL-8, MIP-1α, MIP-1β, and GM-CSF, but some strain-specific effects were apparent. This work demonstrates that *L salivarius* CECT5713 and *L. fermentum* CECT5716 enhanced both natural and acquired immune responses, as evidenced by the activation of NK and T cell subsets and the expansion of Treg cells, as well as the induction of a broad array of cytokines.

Introduction

The neonatal immune system is highly influenced by maternal factors, both before and after birth. Before birth, the mother's immunoglobulin (Ig) G reaches the foetus by crossing the placenta, while postnatally, breast milk becomes the route of communication between the maternal and infant immune system (Chirico et al., 2008). Components of breast milk not only educate the infant immune system, but also confer multiple means of protection from pathogens by their effects on the infant gut microbiota (Field, 2005; Kelly and Coutts, 2000). These effects may be due to the synergistic action of breast milk Ig and many other bioactive molecules, such as growth factors, cytokines, nucleotides, cellular components, oligosaccharides and lipids (Gil and Rueda, 2002; Hanson and Silfverdal, 2009; Lonnerdal, 2003). While it is well known that breast milk is rich in oligosaccharides, which have prebiotic activity and may help to protect against infections (Arslanoglu et al., 2007; Dai and Walker, 1999), it has only recently become accepted that breast milk constitutes a source of micro-organisms that may colonize the infant gut and modulate its function (Martin et al., 2003, 2004; Lara-Villoslada et al., 2007).

Human breast milk includes several predominant bacterial species, such as staphylococci, streptococci, micrococci, lactobacilli, enterococci, lactococci and bifidobacteria (Gueimonde et al., 2007; Heikkila and Saris, 2003; Martin et al., 2003, 2004, 2009; Beasley and Saris, 2004). Thus, breastfeeding could be a significant source of lactobacilli, and other probiotic species, for the infant gut. The *Lactobacillus* genus contains more than 25 species of gram-positive, catalase-negative, non-sporulating and rod-shaped organisms, which constitutes less than 1% of total intestinal bacteria in adults but is found in a higher proportion in neonate

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and infant intestines (between 10^5 and 10^8) (Heilig et al., 2002; Sghir et al., 2000). The lactobacilli species isolated in breast milk to date are *L. gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. fermentum* and *L. salivarius* (Heikkila and Saris, 2003; Martin et al., 2003). Several of these strains (derived from sources other than breast milk) have been demonstrated to have immunomodulatory properties (Lomax and Calder, 2009). *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713 were isolated from breast milk of two different healthy women. Both strains have probiotic potential (Martin et al., 2005, 2006), both strains are protective in an animal model of colitis (Peran et al., 2005, 2007), and both strains reduced the lipopolysaccharide-induced inflammatory response of murine bone marrow-derived macrophages, although *L.* CECT5713 was more effective, probably because of its ability to induce interleukin (IL)-10 (Díaz-Ropero et al., 2007). This strain has also been shown to be effective in the treatment of infectious mastitis during lactation (Jimenez et al., 2008). *L. fermentum* CECT5716 has been shown to be protective in an experimental murine model of septic shock (Arribas et al., 2009), and to enhance the immune response to influenza vaccination after supplementation to human volunteers (Olivares et al., 2007).

Despite growing evidence for immunomodulatory effects of breast milk-derived Lactobacillus species, there is still relatively little information regarding their mechanisms of action. The aim of this study was to investigate the effects of *L. fermentum* CECT5716 and *L. salivarius* CECT5713 on specific components of innate and acquired immunity in an *in vitro* human cell model.

Materials and methods

Preparation of bacteria

L. fermentum CECT5716 and *L. salivarius* CECT5713 were kindly provided by Puleva Biotech SA (Granada, Spain). Both strains were grown in Man-Rogosa-Sharpe (MRS) agar and broth medium (Oxoid, Basingstoke, UK) at 37 °C in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK) under 10% H_2; 10% CO_2; 80% N_2 conditions. Specific bacterial growth curves, i.e. correspondence between the optical density at 620 nm (OD_{620}) and colony-forming units (CFU), were developed for each strain. Bacteria were subsequently collected when their OD_{620} during the log phase corresponded to 1×10^8 cell/mL. Harvested cells were washed twice with sterile phosphate buffered saline (PBS) and centrifuged at low speed (800 *g*, 10 min). The final working concentration in medium (Roswell Park Memorial Institute; RPMI 1640) was adjusted to 1×10^8, 1×10^7 and 1×10^6 cells/mL. Two Lactobacillus strains of non-milk origin were included in some experiments; *L. fermentum* NCIMB701751 and *L. salivarius* NCIMB11795 which were originally derived from saliva (NCIMB, Scotland, UK).

Subjects and cell preparation

Blood samples were taken from 8 healthy volunteers aged 25–34 years (3 males and 5 females) in sodium heparin vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by layering blood over an equal volume of Fycoll density gradient (Lympholyte®-H, Cedarlane, Ziereikzee, The Netherlands) and centrifuging at 1000 *g* for 20 min. Cells collected at the interface were washed with PBS and the Lympholyte separation repeated to achieve a lower degree of contamination with erythrocytes. After washing the cells with RPMI 1640 (Roswell Park Memorial Institute, Autogen Bioclear Ltd., Wiltshire UK) containing 2 mM L-glutamine (Sigma), cells were counted using a cell counter (Coulter, Fullerton, CA, USA) and resuspended at a working concentration of 2×10^6 cells/mL.

Bacterial co-culture and stimulation of PBMCs

Cell cultures were set up in 24-well plates (Cellstar, Greiner Bio-one Ltd., Gloucestershire, UK) containing a final concentration of 1×10^6 PBMC/mL. Cells were stimulated with either the polyclonal T cell mitogen, concanavalin A (ConA) at 1 μg/mL, or with 1 ng/mL of *Esherichia coli* lipopolysacharide (LPS) 0127:B8, both from Sigma (St. Louis, MO, USA), or with live probiotic bacteria in a cell:bacteria ratio of 10:1, 1:1 or 1:10. Negative control cultures contained unstimulated PBMCs. The plates were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Culture supernatants were collected and stored at −80 °C until cytokine analysis and cells were used immediately for staining.

Immunofluorescence staining and flow cytometric analysis

Cultured cells were harvested, distributed in tubes (3×10^5 cells/tube) and triple stained with mouse anti-human mAb conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll-a protein (PercP). These included FITC conjugated anti-CD69 (FN50) and anti-CD25 (M-A251), PE conjugated anti-CD8α (RPA-T8), anti-CD3 (UCHT1), anti-CD4 (RPA-T4) and PercP anti-CD56 (B159) mAb, all obtained from BD Biosciences (Oxford, UK). For intracellular staining of Foxp3, PCH101 clone mAb from eBioscience Ltd. (Haltfield, UK) was used. Briefly, cell surface staining was performed as follows. Cells were incubated with a mixture of saturating concentrations of FITC-, PE- and PercP-conjugated mAb at 4 °C in the dark for 20 min. Stained cells were washed with PBS and fixed with 0.5% p-formaldehyde (BD Biosciences) and stored at 4 °C in the dark until analysis by flow cytometry. For Foxp3, after extracellular staining, cells were fixed, permeabilized (60 min at 4 °C), and washed before incubating with anti-Foxp3-PercP for 30 min at 4 °C in the dark. Cells were washed again and finally fixed. Analyses were performed with a FACS Calibur flow cytometer (Becton Dickinson Corp., Hialeah, USA). Phenotypical results were expressed as the percentage of positive cells with respect to the total number of gated lymphocytes or with respect to a particular cell subset (i.e NK cells or T cells).

Cytokine screening by proteome profiler array

For preliminary screening of PBMC cytokines induced by bacteria we used a semi-quantitative method to simultaneously profile the relative levels of 32 selected cytokines and chemokines (C5a, CD40L, G-CSF, GM-CSF, GXCL1, 8 and 10–12, CCL1–CCL5, sICAM-1, IFNγ, IL-1α, Il-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, MIF, Serpin E-1, TNFα). Briefly, a representative sample for each stimulatory condition (control cultured PBMC, LPS-stimulated PBMC, and bacteria stimulated PBMC at a 1:1 ratio) were incubated overnight at 2–8 °C on a rocking platform in a nitrocellulose membrane with the specific capture and detection antibodies (Proteome Profiler™ Array with human cytokine array panel A, R&D Systems Europe Ltd., Abingdon, UK). After washing, streptavidin-HRP was added and after 30 min incubation, the nitrocellulose was exposed to ECL reagent (GE Healthcare UK Limited, Buckinghamshire, UK) for 2 min. In a dark room, membranes were exposed to hyperfilm for 2 min in an auto-radiographic cassette and developed for 5 min. Protein spots were quantified using Quantity One v4 software (Bio Rad, Hertfordshire, UK).