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Modulation of the cytokine profile in Caco-2 cells by faecal lactobacilli and bifidobacteria from individuals with distinct dietary habits

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ABSTRACT

Enterocytes are actively involved in the defense against pathogens and they limit penetration of commensal microbes into tissues. They also have an important role in gut immunity as enterocytes confer mucosal dendritic cell specialisation. On the other hand, the microbiota is directly involved in the development and modulation of the intestinal immune system. Particularly, lactobacilli and bifidobacteria play a primary role in shaping the immune response. We further explored this issue by evaluating whether functional differences in Caco-2 cells could characterise faecal populations of lactobacilli (155 samples) and bifidobacteria (110 samples) isolated from three dietary cohorts (omnivores, ovo-lacto-vegetarians and vegans) recruited at four Italian centres (Turin, Parma, Bologna and Bari). According to our findings, tested bacteria were unable to modulate expression of IL-8, IL-10, TGF-β or thymic stromal lymphopoietin (TSLP) cytokines in unstimulated Caco-2 cells. Conversely, in phorbol 12-myristate 13-acetate and ionomycin (PMA/Io) stimulated Caco-2 cells, lactobacilli from the omnivorous group and all bifidobacteria significantly down-regulated IL-8. Notably, both genera also lowered the TSLP expression in stimulated Caco-2 cells, regardless of the diet regimen. By further examining these data on the basis of geographical origin, we found that lactobacilli from the vegetarian group recruited in Bari, significantly up-regulated this cytokine. In conclusion, we highlighted a peculiar immune-modulatory activity profile for lactobacilli on enterocytes undergoing a stimulatory signal, which was associated with a specific dietary habit. Furthermore, the geographical area had a significant impact on the inflammatory potential of members of the *Lactobacillus* genus.

1. Introduction

The intestinal epithelium lies at the interface between the microbiota and the gut-associated lymphoid tissue (GALT). In addition to the barrier function of enterocytes, they actively defend against pathogens and limit penetration of commensal microbes into underlying tissues. Specifically, enterocytes play an important role in the intestinal immune system as they regulate mucosal dendritic cell (DC) specialisation. Among other unidentified mediators, enterocytes release thymic stromal lymphopoietin (TSLP) which blocks interleukin (IL)-12 production by DCs in response to bacteria and drives Th2-polarising cells, inhibiting the inflammatory potential of DCs [1]. Interestingly, the majority of enterocytes isolated from patients with Crohn's disease (CD) were shown not to express TSLP failing to control the DC pro-inflammatory response [1]. On the other hand, the gut microbiota is directly involved in the development and modulation of the intestinal immune system. In particular, lactobacilli and bifidobacteria are considered key players because they constitute essential members of the normal intestinal microbiota in animals and humans, particularly bifidobacteria in infants [2,3]. Changes in diet,

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; GSR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; LAB, lactic acid bacteria; Io, ionomycin; PMA, phorbol 12-myristate 13-acetate; TSLP, thymic stromal lymphopoietin.

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use of antibiotics and intestinal colonisation by helminths can modify intestinal microbial communities [4,5] Furthermore, alterations in intestinal microbiota have been reportedly documented in a growing list of diseases, such as inflammatory bowel disease [6] and celiac disease [7]. The role of diet, in particular the impact of dietary macronutrients (carbohydrates, protein and fats) in microbial ecology, is significant. Very recently, the gut microbiota and metabolome in 153 Italian individuals recruited from different regions in Italy, who followed omnivore, ovo-lacto-vegetarian or vegan diets, were analysed. Results showed that a high-level of consumption of plant foodstuffs was associated with beneficial microbiome-related metabolomic profiles in subjects consuming a Western diet [8]. Interestingly, the subsequent analysis of their faecal microbiota indicated that the samples clustered differently, according to the recruitment site, highlighting a greater impact of geographical location than type of diet [9]. In the present work, we further analysed the same populations by addressing the immune mechanisms by which lactobacilli and bifidobacteria from these individuals may influence the enterocyte response. Accordingly, we evaluated, for the first time, a large microbiological screening of lactobacilli and bifidobacteria isolated from individuals undergoing omnivorous, vegan and ovo-lacto-vegetarian diets to determine the role of diet in modulating *in vitro* immune markers of Caco-2 cells. Our data revealed peculiar modulatory activities of selected bacteria on inducible cytokines produced by enterocytes undergoing a stimulatory signal, which were found to be dependent on dietary habit for lactobacilli. Furthermore, the geographical area also influenced the pro-inflammatory activity of lactobacilli in Caco-2 cells.

2. Materials and methods

2.1. Participant recruitment and faecal sample collection

Healthy adult volunteers (n = 155) who followed an omnivorous (n = 55), ovo-lacto-vegetarian (n = 53) or vegan (n = 47) diet were recruited from 4 four Italian centres (Bari, Bologna, Parma and Turin) [8,9] (https://clinicaltrials.gov; ClinicalTrials.gov Identifier: NCT02118857; MRMOVVD), as indicated in Table 1. The exclusion criteria were dietary regimen followed for less than 1 year, age under 18 or over 60 years, regular consumption of drugs, regular supplementation with prebiotics or probiotics, consumption of antibiotics in the previous 3 months, evidence of intestinal pathologies (Crohn's disease, chronic ulcerative colitis, bacterial overgrowth syndrome, constipation, celiac disease, irritable bowel syndrome), and other pathologies (type I or type II diabetes, cardiovascular or cerebrovascular diseases, cancer, neurodegenerative disease, rheumatoid arthritis, and allergies), pregnancy and lactation. Three faecal samples/volunteer (ca. 15 g) were collected for three consecutive weeks (once per week) at home, transferred to sterile tubes containing 10 ml of liquid Amies transport medium (Oxoid, Milan, Italy) and stored at 4 °C. The specimens were then transported to the laboratory within 12 h and immediately processed. The research was conducted according to the Declaration of Helsinki. Informed consent was obtained from all subjects.

2.2. Isolation and growth of lactobacilli and bifidobacteria

Ten grams of faeces from each volunteer was homogenised with 90 ml of Ringer's solution (Oxoid) for 2 min in a stomacher (LAB Blender 400, PBI, Italy) at room temperature. Serial dilutions were prepared in Ringer's solution, and 100 μl aliquots of each dilution were placed into Rogosa Agar (Oxoid) with 21 μM acetic acid or spread onto Bifidobacterium Agar (Becton Dickinson, Milan, Italy) [9]. The first agar is an effective, selective medium for lactobacilli because the high acetate concentration and low pH suppress many strains of other lactic acid bacteria. The latter is a slight modification of the original medium developed by Beerens [10]; it is supplemented with lactulose, a sugar used as a prebiotic that is preferentially fermented by bifidobacteria. The low pH of Bifidobacterium Agar and the presence of propionic acid have been shown to inhibit fungi and many bacteria other than bifidobacteria. Growth conditions were aerobic at 30 °C for 48 h and anaerobic at 37 °C for 48–72 h for selection of mesophilic lactobacilli and bifidobacteria, respectively [9]. For each faecal sample, 10 random colonies were picked from appropriate plate dilutions for analysis *in vitro* [11,12]. Cell morphology and cell motility of selected colonies were evaluated for genus confirmation: all bifid-shaped rods were considered bifidobacteria, whereas all non-spore forming straight rods were considered lactobacilli [13]. Bifidobacteria were cultured in trypticase-phytone-yeast extract (TPY; Oxoid) at 37 °C under anaerobic conditions and lactobacilli in de Man, Rogosa and Sharpe medium (MRS; Oxoid) at 30 °C under aerobic conditions; cells were collected during the exponential growth phase. Cell concentration was evaluated by measuring optical density at 600 nm and converting this value to the corresponding CFU/ml value by plate counting. Bacteria were irradiated with 2800 Gy of γ-irradiation by the Gammacell 1000 (MDS Nordion, Canada) to prevent their proliferation before being used as a stimuli for Caco-2 cells.

2.3. PCR amplification and DGGE analysis

DNA was isolated from bacterial cultures by using the ZR Fungal/Bacterial DNA MicroPrep™ Kit (Zymo Research Corp, Irvine, USA) according to the manufacturer's instructions. 100 ng of DNA, was used as a template in the PCR reaction. The V3 region of the 16S rRNA gene was amplified and the PCR products were analysed by DGGE as recently described [9]. Selected DGGE bands, specific of each media, were excised from the gel with sterile pipette tips and purified in water. One microlitre of the eluted DNA was used for the re-amplification [9] and the PCR products were checked by means of DGGE. The original PCR product was run on the gel as the control. Products that migrated as a single band and at the same position as the control were sent for sequencing to GATC-Biotech (Cologne, Germany). Searches were performed in public data libraries (GenBank) with the Blast search program (http://www.ncbi.nlm.nih.gov/blast/) in order to determine the closest known relatives of the obtained partial 16S rRNA gene sequences.

Table 1
List of lactobacilli and bifidobacteria isolates from human faecal samples.

Diet	Recruitment centre			
	Bari (l; b)[a]	Bologna (l; b)[a]	Parma (l; b)[a]	Turin (l; b)[a]
Omnivorous	15; 15	15; 2	10; 8	15; 15
Vegetarian	12; 11	14; 1	15; 11	12; 13
Vegan	10; 12	12; 2	12; 9	13; 11

[a] number of analysed lactobacilli (l) and bifidobacteria (b).