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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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Abbreviations used in this paper: 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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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-b 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.

Keywords: diet, enterocyte, immunity, microbiota.

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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, 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, 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 hours 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 ml aliquots of each dilution were placed into Rogosa Agar (Oxoid) with 21 mM 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
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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 microliter 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.

2.4. In vitro culture of Caco-2 cells

Caco-2 cells were obtained from American Type Culture Collection and cultured at 37 °C in an atmosphere of 5% CO_2. Cells were maintained in DMEM with 4.5 g/l glucose, 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM HEPES, 1% essential and nonessential amino acids,
and 10% FCS (complete medium). Confluent monolayers were then used for the bacterial challenge experiments on day 14. To study the effect of phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), differentiated Caco-2 cells were incubated for different times with 20 ng/ml PMA plus 1 µg/ml Io (PMA/Io).

2.5. Microbial challenge

Differentiated Caco-2 cells were incubated for 0-72 h with irradiated bacteria resuspended in complete DMEM medium at a 30:1 bacteria (CFU): Caco-2 cell (n) ratio. In some experiments following bacteria incubation, cells were stimulated with PMA/Io for 24-72 h in complete medium.
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Cells were collected for RNA, NF-κB and RedOx status analyses. Spent media were centrifuged at 10,000 x g for 10 min to eliminate any residual cells and cell debris, and supernatants were stored at -80 °C. No pH change occurred in the medium after 72 h of bacterial incubation.

2.6. Analysis of cytokine production

Total RNA was extracted from Caco-2 cells using the TRIzol Reagent (Life Technologies Italia, Monza MB, Italy) according to the manufacturer's instructions and quantified by fluorimetry using

the RiboGreen RNA Reagent (Invitrogen Corp., Carlsbad, CA, USA), and RNA quality was verified by denaturing gel electrophoresis. Complementary DNA was prepared from 1 mg of total RNA by reverse transcription with M-MLV Reverse Transcriptase (Invitrogen) and Oligo-(dT)12-18 Primer at 42 °C for 60 min. Real-time PCR was performed using the iCycler iQTM Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Amplification was conducted in a total volume of 25 µl, containing iQTM SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and cDNA. The reaction conditions for 44 cycles were 95 °C for 30 s, 54.6 °C (IL-10), 60 °C (GAPDH, IL-8, TGF-b), or 62.0 °C (TSLP) for 30 s, and 72 °C for 40 s. The relative gene expression levels of TGF-b, IL-8, TSLP and IL-10 were calculated using the DDCT method and presented as the fold change in gene expression after normalisation to the GAPDH housekeeping gene. The following primer sequences were designed and used in this study: GAPDH, forward 5′- GAA GGT GAA GGT CGG AGT C -3′, reverse 5′- GAA GAT GGT GAT GGG ATT TC -3′; IL-8, forward 5'- CTG
CAC CCA GTT TTC -3', reverse 5'- ACT GAG AGT GAT TGA GAG TGG AG -3'; TSLP, forward 5'- GTT CTG TCA GTT TCT TTC AGG -3', reverse 5'- CTC GGT ACT TTT GGT CCC AC -3'; IL-10, forward 5'- GCT GGA GGA CTT TAA GGG TTA CCT -3', reverse 5'- CTT GAT GTC TGG GTC TTG GTT CT -3'; TGF-b forward 5'- GCG CAT CCT AGA CCC TTT CTC -3', reverse 5' - CAG AAG GTG GGT GGT CTT GAA -3'. Supernatants from Caco-2 cell cultures were analysed for IL-8, TSLP and IL-10 protein levels by sandwich-type ELISA. First, 100 µl of capture antibody solution (BioLegend, San Diego, CA) was dispensed into each well of a 96-well plate (Nunc Maxisorb; eBioscience Inc., San Diego, CA) and incubated overnight at 4 °C. After removal of the capture antibody solution, 100 µl of PBS supplemented with 1% BSA (blocking buffer) was added to each well and incubated at room temperature for 2 h. Next, cytokine standards and samples diluted in blocking buffer supplemented with 0.05% Tween-20 were added to the respective wells and incubated at room temperature for 2 h. After incubation, three washing steps with PBS supplemented with 0.05% Tween-20 were performed, and 100 µl of biotinylated antibody
solution was added to the wells and incubated for 1.5 h at room temperature. After three washes, streptavidin–horseradish peroxidase conjugate (1:1500 dilution; BioLegend) was added to the wells
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and incubated for 1 h at room temperature. Finally, after washing, 100 µl of 63 mM Na_2HPO_4,
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mM citric acid (pH 6.0) containing 0.66 mg/ml o-phenylenediamine/HCl and 0.05% hydrogen peroxide were dispensed into each well, and the wells were allowed to develop. The absorbance was read at 415 nm and the cytokine concentrations were calculated using standard curves and expressed as pg/ml.

2.7. Analysis of NF-κB and redox status

Cytoplasmic and nuclear protein fractions were prepared from Caco-2 cells and used for phospho-NF-κB and NF-κB immunodetection, respectively [14]; phospho-NF-κB p65 (Ser536) (93H1) rabbit monoclonal antibody (#3033) and NF-κB p65 (D14E12) XP® rabbit monoclonal antibody (#8242; Cell Signaling Technology, Danvers, MA, USA) were used as probes. Cytoplasmic extracts were also used for the evaluation of RedOx status (GSH and GSSG content) according with previously published protocols [15]. Glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GSR) activities were spectrophotometrically evaluated in cytoplasmic extracts and their activities, upon normalization to protein content, were expressed as IU or as nmoles NADPH mg^{-1} min^{-1} [16, 17]

2.8. Statistical analysis

Statistical significance for cytokine assessment was determined by the Kruskal-Wallis test and Dunn's post-hoc test analysis using GraphPad PRISM 4.0 software (GraphPad Software, Inc., La Jolla, CA). A P-value of 0.05 or less was considered to be significant. For all the other parameters, ANOVA analyses were performed with the SPSS 22.0 statistical software package (SPSS Inc.,

Cary, NC, USA). The Duncan HSD test was applied when ANOVA revealed significant differences ($P < 0.05$).
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3. Results
3.1. Plate counts and DGGE analysis
We used cell morphology and cell motility of colonies grown on selective media as general principle for genus confirmation [13]. The average numbers of lactobacilli and bifidobacteria found in the different dietary groups was then calculated and reported in Table 2. In agreement with previous observations [9], counts were found lower in the vegan group than in the other two groups for both examined genera ($P < 0.05$). To further characterize the cultivated microbial populations, randomly selected cultures were analysed by PCR-DGGE and sequencing of amplimers.
PCRDGGE
fingerprints obtained from cells isolated from MRS cultures showed the presence of several lactic acid bacteria belonging to Lactobacillus fermentum, L. rhamnosus, Pediococcus lollii and P. pentosaceus, as well as few bands belonging to Enterococcus sp. On the contrary, PCR-DGGE analysis from TPY cultures showed the presence of bands identified as Bifidobacterium longum as well as a few bands belonging to Lactobacillus crispatus (data not shown). These data indicated that both selected lactobacilli and bifidobacteria populations were not homogeneous but still had some degree of contamination by other LAB.
3.2.Modulation of cytokine expression in unstimulated Caco-2 cells
In order to establish test conditions to be adopted for in vitro assessment of a large number of bacterial samples, we preliminarily determined the time course of IL-8, TGF-b, IL-10 and TSLP mRNA expression induced by PMA/Io stimulation in a monolayer of differentiated Caco-2 cells, an in vitro model of enterocytes. We found a significant increase in IL-8 transcript levels starting at 3 h and until 6h, which then rapidly decreased (Fig. 1). All the other examined cytokines did not show any significant regulation in response to the pro-inflammatory stimulus. We then examined the effect of bacterial co-incubation with Caco-2 cells by testing two randomly selected lactobacilli populations from individuals undergoing different diet regimens (an omnivorous and a vegetarian sample from Bari and Turin, respectively, Table 1). Transcriptional analysis indicated that bacterial challenge induced a significant although late up-regulation of IL-10 (Fig. 2a). Similarly, TSLP mRNA significantly peaked at 18 h following incubation with both examined lactobacilli populations. On the contrary, IL-8 and TGF-b mRNA levels were not changed (Fig. 2a). The time course of cytokine protein expression, performed for transcriptionally regulated cytokines, showed that only IL-8 was significantly increased from 24 h until 72 h, whereas IL-10 and TSLP levels remained low in the same time frame (Fig. 2b).
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3.2. Modulation of cytokine expression in PMA/Io stimulated Caco-2 cells
To further highlight the modulatory ability of selected bacteria on Caco-2 monolayers, we tested the putative immunomodulatory effects by bacteria pre-incubation on PMA/Io-stimulated cells. Differentiated Caco-2 cells were treated with irradiated lactobacilli and then pulsed for 24 h with PMA/Io. We found that both tested lactobacilli populations significantly reduced IL-8 protein levels (Fig. 3). Interestingly, one population also down-regulated TSLP expression following PMA/Io challenge; on the contrary, a partial recovery of IL-10 secretion was reported for lactobacilli from the vegetarian individual, but not for those from the omnivorous subject (Fig. 3).
3.3. Modulation of the cytokine profile by diet-selected lactobacilli and bifidobacteria
Based on the initial analysis, the protocol of bacteria pre-incubation was adopted to study the entire faecal lactobacilli and bifidobacteria collections from healthy adult volunteers who followed omnivorous, ovo-lacto-vegetarian or vegan diets, recruited from four Italian centres. Samples were screened by determining cytokine protein levels; data were expressed as percentages of the positive control (PMA/Io pulsed Caco-2 cells). Results from each independent experiment were pooled and reported in Figure 4. It was confirmed that PMA/Io challenge of differentiated Caco-2 cells caused

a significant increase in the expression of IL-8 (Fig. 4a, b). Furthermore, analysis of regulatory TSLP in a larger sample size confirmed that this cytokine was not modulated by PMA/Io (Fig. 4c, d). Pre-incubation with both lactobacilli and bifidobacteria collections generated a wide range of values in the cytokine response. Nevertheless, analysis of medians indicated that only lactobacilli from the omnivorous group induced a significant down-regulation of IL-8 expression in stimulated monolayers (Fig. 4a). On the contrary, a less heterogeneous response was found for bifidobacteria that induced a diet-independent down-regulation of IL-8 (Fig. 4b). Pre-incubation with both lactobacilli and bifidobacteria isolates significantly down-regulated the TSLP expression in stimulated Caco-2 cells, independent of the diet regimen (Fig. 4c, d). Furthermore, no significant induction was reported for IL-10 by all examined bacteria (data not shown). By evaluating these data on the basis of geographical origin, the statistical relevance of the response was lost in different experimental groups, as a consequence of the wide heterogeneity of both IL-8 (Fig. 5a,b) and TSLP responses (Fig.5c,d). Nevertheless, lactobacilli collected from the vegetarian group recruited in Bari experienced a significant increase in IL-8 expression (Fig. 5a). Furthermore, the Dunn's post-hoc test analysis revealed significant differences between isolates from this group and those from the other two groups in Bari.

3.4. Mechanisms of IL-8 down-regulation

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It is known that the activation of NF-κB is required for the transcription of IL-8 [18]. Furthermore, the redox status is known to modulate cytokine expression. So, we analysed the ability of tested bacteria to modulate both these parameters. Three lactobacilli populations, randomly selected from Turin and Bari centres, previously found to be down-regulatory, were analysed. The proinflammatory
effect associated to PMA/Io exposure of Caco-2 cells was indicated by a slight
increase of nuclear phospho-NF-κB levels (Fig.6A). Interestingly, the examined lactobacilli were found unable to decrease these levels. The cell RedOx status (GSH/GSSG content) and the activities of phase 2 enzymes (G6PD and GSR) were then evaluated. G6PD is the rate-limiting enzyme in the pentose phosphate pathway and a major source of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which regulates numerous enzymatic activities including GSR; the latter is involved in the reduction of intracellular pool of oxidized glutathione (GSSG). As shown in Fig. 6B, the significantly higher GSH and GSSG yields, measured in Caco2 cells pretreated
with lactobacilli, suggested changes of cellular RedOx status (upper panel). This finding was associated with an increased activity of both antioxidant enzymes (Fig.6, middle and lower panels).

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4. Discussion

In this study, we compared faecal populations of lactobacilli (155 samples) and bifidobacteria (110 samples) isolated from three dietary cohorts (omnivores, ovo-lacto-vegetarians and vegans). We found that both genera down-regulated cytokine expression in Caco-2 cells undergoing a proinflammatory
stimulus; however, only lactobacilli activity was influenced both by the dietary habit
and by the geographical origin. A major challenge for understanding the microbiota-host interactions is the heterogeneity of microbial communities that can colonise the intestine and other body sites. Different components of microbiota can have very different effects on the host; the composition of microbial communities can be influenced by a variety of exogenous factors, including diet. By considering this complex scenario, we specifically focused on culturable lactobacilli and bifidobacteria isolated from faecal samples; these genera play a crucial role in the immune homeostasis of the gut [2,3]. To analyse the immune effects of bacterial-enterocyte interactions, we used monolayers of fully differentiated Caco-2 cells as a model of intestinal epithelium. Ten phenotypically identified colonies [13] were picked from adequate plate dilutions for each faecal sample, corresponding to the numerically most important bacteria that represent the

sampled community [11,12]. Furthermore, a preliminary study was designed to select immunological parameters to screen this large number of bacteria on enterocytes. Accordingly, we initially focused on the transcription of four cytokines: the pro-inflammatory IL-8 and the regulatory TGF-b, IL-10 and TSLP. Caco-2 cells were stimulated with a combination of phorbol myristate acetate (PMA) and ionomycin (Io). Ionomycin causes disruption of Ca^{2+} homeostasis in Caco-2 cells [19]. Phorbol myristate acetate is a known protein kinase C activator, and stimulation with both of these compounds bypasses the cell membrane receptor complex and leads to activation of several intracellular signalling pathways, resulting in cell activation and production of a variety of cytokines. We confirmed that PMA/Io incubation induced mRNA levels only of IL-8 among the tested cytokines, indicating activation of the nuclear factor B (NF- B) pathway [18, 20]. On the contrary, incubation of Caco-2 cells with two different lactobacilli populations, randomly selected from our collection, up-regulated IL-10 and TSLP mRNA levels. Interleukin 10 is one of the major anti-inflammatory cytokines required to control the host immune response to intestinal bacteria [21]. Epithelial-cell-derived TSLP might represent one of the factors initiating the allergic response, as TSLP-activated human DCs produce Th2-attracting chemokines and induce T cell differentiation into effector cells with a pro-allergic phenotype [22]. In addition, during homeostasis, enterocytederived
TSLP promotes the development of tolerogenic DCs [23], whereas this control is absent in Crohn's disease [6] and celiac disease [24]. Previous studies showed that bifidobacteria and

lactobacilli isolated from healthy intestinal tracts significantly reduced IL-10 transcripts in Caco-2 cells [25]. On the contrary, IL-10 mRNA was induced in Caco-2 cells by lipopolysaccharide [26], in line with our data. However, both IL-10 and TSLP protein levels were essentially unchanged after bacterial challenge in our assays, indicating that post-transcriptional regulatory mechanisms are still operative. To gain more insight about the modulatory role of the examined bacteria, we analysed their potential in preventing a pro-inflammatory stimulus in Caco-2 cells. Our findings indicated that bacterial challenge effectively reduced the production of IL-8, whereas a modulation of TSLP and IL-10 secretions was not consistently found. These results are in agreement with different studies suggesting that lactobacilli elicit an anti-inflammatory response [27] essentially by down-regulating IL-8 [28]. Accordingly, this protocol was adopted to screen the large cohorts of isolated faecal lactobacilli and bifidobacteria populations. Immunological outcomes widely varied within each diet group. Nevertheless, due to the large number of individuals included in the examined groups, most data were found to be significant. Specifically, we showed that bacteria belonging to the Lactobacillus genus isolated from the omnivorous group significantly decreased the production of IL-8. A similar activity was consistently registered for all examined bifidobacteria. Another remarkable finding was the bacterial-driven reduced expression of TSLP in PMA/Io-stimulated cells. A possible outcome of this apparently contradictory activity could be its interference with the enterocyte:DC crosstalk in the presence of a stimulatory signal (i.e. PMA/Io); consequently, the number of activated DCs involved in the T cell differentiation of effector cells could be reduced [22]. In line with this speculation, lactobacilli treatment was found to suppress TSLP responses in a mouse model of allergy [29]. In the examined populations, the long-term omnivorous regimen did generate a significant higher anti-inflammatory potential in faecal lactobacilli. This result was in agreement with the lower culturable lactic acid bacteria (LAB) loads in the vegan and vegetarian groups, previously found [9]. Taken together, these data suggested that the presence of food containing LAB (i.e. dairy and fermented meat products) in the omnivorous diet, can enrich gut lactobacilli populations providing beneficial effects on intestinal immunity. Furthermore, we detected an inflammatory potential in the lactobacilli isolates collected from the vegetarian group in Bari compared to those from other centres. More specific studies concerning the influence of genetic or unselected environmental factors are thus required to further address this issue. Results from PCR-DGGE suggested that, even if Lactobacilli and Bifidobacteria genera in the community structure are dominant populations, there was occurrence of other contaminating

LAB. On the other hand, due to the high number of screened samples, the presence of contaminating LAB in both examined populations did not overshadow the statistical relevance of our findings. Finally, the significantly higher GSH and GSSG yields, measured in Caco-2 cells pre13
treated with lactobacilli were associated with an increased activity of antioxidant enzymes, indicating a possible protective mechanism triggered in Caco-2 cells by lactobacilli exposure. Furthermore, our data provided evidence that NF-κB was not involved in the herein reported downregulation
of IL-8.
In conclusion, by examining the faecal lactobacilli and bifidobacteria isolates in groups with large sample sizes, we highlighted the peculiar immune-modulatory activity of both genera on enterocytes undergoing a stimulatory signal. We also confirmed the association between the immune features of lactobacilli and a specific dietary habit. Finally, the geographical area did have a significant impact on the inflammatory potential of members of the Lactobacillus genus.
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Conflict of interest

The authors have no financial or personal conflicts of interest.
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Figure Legends

Figure 1. Setup of cytokine transcription by Caco-2 cells. Kinetic analysis of IL-8, IL-10, TGF-b and TSLP transcription in Caco-2 cells in response to PMA/Io stimulation; values were calculated as fold change in gene expression (absolute units, AU). Cells were collected at 0, 3, 6, 17 and 24 h after stimulation and analysed for cytokine mRNAs by using real-time PCR. Points represent the mean ± SD and are representative of three independent experiments. **, $P < 0.01$.

Figure 2. Comparison between mRNA (a) and cognate protein levels (b) for IL-8, IL-10, TGF-b and TSLP of Caco-2 cells pre-treated with PMA/Io or lactobacilli from two randomly selected lactobacilli populations from a vegetarian (veget) and an omnivorous subject (omniv) collected in Turin and Bari, respectively. Cells and culture supernatants were recovered and analysed for cytokine mRNAs and secreted protein expression by using real-time PCR and sandwich-type ELISA, respectively. Values represent the mean ± SD and are representative of three independent experiments. **, $P < 0.01$.

Figure 3. Protein levels for IL-8, IL-10, TGF-b and TSLP secreted by Caco-2 cells pre-treated with lactobacilli from test samples for 72 h and then pulsed for 24 h with PMA/Io. Culture supernatants were collected and analysed by using sandwich-type ELISA. Columns represent the mean ± SD and are representative of three independent experiments. *, $P < 0.05$.

Figure 4. Protein expression of IL-8 and TSLP by stimulated Caco-2 cells. Caco-2 cells were pretreated
with irradiated lactobacilli (a, c) or bifidobacteria (b, d) isolates from adults who followed an omnivorous, ovo-lacto-vegetarian or vegan diet (Table 1) before PMA/Io stimulation. Culture supernatants were collected after 24 h and analysed for IL-8 (a, b) and TSLP (c, d) expression by sandwich-type ELISA. Data were expressed as a percentage of the positive control (unchallenged PMA/Io treated Caco-2 cells). Bars represent medians. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 5. Immunomodulatory potential of lactobacilli and bifidobacteria analysed across diet and geography. Cytokine values were reported for lactobacilli and bifidobacteria populations from adults recruited at four Italian centres who followed an omnivorous, ovo-lacto-vegetarian or vegan diet (Table 1). Bars represent medians. *, $P < 0.05$.

Figure 6. Mechanisms underlying the immunomodulatory activity of lactobacilli. A. Western blot analysis of cytoplasmic (upper panel) and nuclear extracts (lower panel) for NFkB and phospho-NFkB, respectively. B. Intracellular GSH and GSSG concentrations to evaluate cellular RedOx status (upper panel); cytoprotective enzymes G6PD (middle panel) and GSR activities (lower panel) evaluated in cytoplasmic extracts and expressed as IU or as nmoles NADPH mg^{-1} min^{-1}. Each bar represents the mean values ± SD from triplicate analysis on three different cultures. ctr, control (untreated Caco-2 cells); *, $P < 0.05$.

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

diet	recruitment center							
	Bari (l; b)*		Bologna (l; b)*		Parma (l; b)*		Turin (l; b)*	
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	

*: number of analysed lactobacilli (l) and bifidobacteria (b).

Table 2. Average numbers of lactobacilli and bifidobacteria (Log CFU/g) as a result of the different diets.

media	vegetarian

(mean + SD)
vegan
(mean + SD)
omnivorous
(mean + SD)
Rogosa agar 30°C 4.62 + 1.41$_b$ 4.10 +1.73$_a$ 4.72 + 1.69$_b$
Bifidobacterium agar modified 8.76 +1.07$_b$ 8.38 + 1.33$_a$ 8.73 + 1.13$_b$
Differing superscript letters indicate statistically significant differences ($P < 0.05$).
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