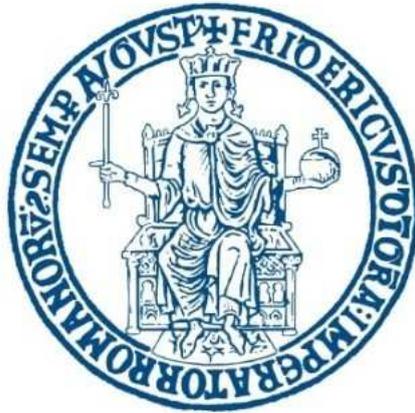


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Use of GRAS bacteria for the modulation of immune functions in cellular and mouse models.

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Abstract

Intestinal bacteria play a pivotal role in shaping gut immunity. Epidemiologic evidence also suggests that *Lactobacilli* and *Bifidobacteria* are key players in this context. Furthermore, literature data reported that the immune modulatory activity of generally recognized as safe (GRAS) bacteria could be associated with the secretion of specific molecules, like enzymes or other unidentified substances. Based on these findings, the general objective of this Doctoral Thesis was to evaluate the modulation of the immune response by using different bacterial populations, isolated from the human intestine, or already available as GRAS strains, according with the American FDA designation. Both *in vitro* (Caco-2 cell line and mouse dendritic cells) and *in vivo* (HLA-DQ8 transgenic mice) models were adopted to address this issue. In the first study, the immunomodulatory effects of various probiotic strains of *L. paracasei* dendritic cells were determined. We found that bacterial metabolites from only some of tested strains showed an anti-inflammatory activity.

In a second study, another GRAS bacterial strain, *Streptomyces mobaraensis*, was assessed. In particular, new culture conditions were developed to optimize the secretion of microbial transglutaminase (mTG), previously found to be able to block the inflammatory response induced by gluten in Coeliac disease (CD) patients. Importantly, we found that wheat flour treated with sterile-filtered supernatant of *S. mobaraensis* culture, resulted also effective in modulating the immune response to gluten. Furthermore, bread manufactured with treated flour had only minor changes in the baking parameters.

In other two studies, we specifically focused on the relationship between the dietary habit and immunomodulatory abilities exerted *in vitro* by intestinal *Bifidobacteria* and *Lactobacilli*, isolated from individuals following omnivorous, vegetarian and vegan diets. Interestingly, both *Lactobacilli* and *Bifidobacteria* showed a genus-specific ability of modulating *in vitro* innate immunity associated with a specific dietary habit.

In conclusion, these data highlighted different applications of GRAS bacteria and their metabolites with possible implications for the management of inflammatory diseases like CD.

Abstract

I batteri che popolano il nostro intestino giocano un ruolo fondamentale nel plasmare l'immunità intestinale. In particolare l'evidenza epidemiologica suggerisce che sia i *Lattobacilli* che i *Bifidobatteri* sono attori chiave in questo contesto. I dati riportati in letteratura mostrano che l'attività immunomodulatoria dei batteri GRAS (Generalmente riconosciuti come sicuri) potrebbe essere associata con la secrezione di molecole specifiche, come enzimi o altre sostanze non ancora identificate. Sulla base di questi dati, l'obiettivo generale di questa tesi di dottorato è stato quello di valutare la modulazione della risposta immunitaria utilizzando diverse popolazioni batteriche, isolate dall'intestino umano, o batteri già riconosciuti come ceppi GRAS, secondo la designazione dell'americana FDA. Per lo studio sono stati adottati sia modelli *in vitro* (linea cellulare Caco-2 e cellule dendritiche di topo) che modelli *in vivo* (topi transgenici HLA-DQ8).

Nel primo studio sono stati determinati gli effetti immunomodulatori di tre ceppi probiotici di *L. paracasei* sulle cellule dendritiche. I risultati hanno evidenziato che i metaboliti batterici di uno solo dei ceppi testati presentano un'attività anti-infiammatoria.

In un secondo studio è stato valutato un altro ceppo batterico GRAS, *Streptomyces mobaraensis*, produttore naturale di transglutaminasi microbica (mTG). In particolare, sono state sviluppate metodiche per ottimizzare la secrezione di mTG precedentemente individuata come enzima in grado di bloccare la risposta infiammatoria indotta dal glutine nei pazienti affetti da malattia celiaca. È importante sottolineare che i risultati ottenuti hanno rivelato che la farina di frumento trattata con surnatante sterile filtrato della cultura di *S. mobaraensis*, è risultata efficace nel modulare la risposta immunitaria al glutine. Inoltre, il pane prodotto con farina trattata ha presentato solo lievi modifiche nei parametri di cottura.

Altri due studi sono stati incentrati sulla relazione tra le abitudini alimentari e la capacità immunomodulatoria esercitata *in vitro* da *Bifidobatteri* e *Lattobacilli* intestinali, isolati da individui con diete onnivore, vegetariane e vegane. È interessante notare, che dai risultati emersi, sia *Lattobacilli* che *Bifidobatteri* hanno mostrato una capacità specifica di genere nella modulazione dell'immunità innata, associabile ad una specifica abitudine alimentare.

In conclusione, i dati raccolti hanno evidenziato nel loro insieme diverse possibilità applicative dei batteri GRAS e dei loro metaboliti, con potenziali opportunità per la gestione del decorso di malattie infiammatorie come la stessa malattia celiaca.

Introduction

1. Research aims and objectives

The global aim of this Doctoral Thesis was to evaluate the modulation of the immune response driven by different bacterial populations, isolated from the human intestine, or already available as generally recognized as safe (GRAS) strains, according with the American FDA designation. Both *in vitro* (Caco-2 cell line and mouse dendritic cells) and *in vivo* (HLA-DQ8 transgenic mice) models were adopted. Main research objectives were then addressed to investigate the following issues:

- To examine the influence of culture supernatant filtrates of previously characterized strains of *Lactobacillus paracasei* on the maturation of immunocompetent cells (dendritic cells).
- To examine the ability of mTG-active supernatants from *Streptomyces mobaraensis* to modulate the immune response to wheat gluten, the triggering agent of Coeliac disease.
- To examine the influence of different dietary habits on the immunomodulatory ability of intestinal *Lactobacilli* and *Bifidobacteria*.

2. Relevant Nomenclature

GRAS bacteria

"GRAS" is an acronym for **Generally Recognized As Safe**. Microbes have been used in food for millennia and fermented foods were traditionally prepared as a means of preservation. Over the years, the FDA has published a number of regulations in the Code of Federal Regulations title 21 that detail the allowed uses of microorganisms, often as sources of enzymes used to produce food. Food ingredients may be "food additives" that are approved by FDA for specific uses or GRAS (generally recognized as safe) substances. A substance may be GRAS only if its general recognition of safety is based on the views of experts qualified to evaluate the safety of the substance. Among GRAS bacteria, Probiotics are live microorganisms which upon ingestion in sufficient concentrations can exert health benefits to the host. This definition of probiotics was derived in 2001 by the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO), and has been the term of reference for science and regulation thereafter (FAO/WHO 2002).

In April 2003, responsibility for the safety assessments of food/feed undertaken by the Scientific Committees of the Commission formally passed to the European Food Safety Authority (EFSA). In 2007, EFSA's Scientific Committee recommended that a Qualified Presumption of Safety (QPS) approach should be implemented across EFSA. This should apply equally to all safety considerations of biological agents that EFSA assesses. The Scientific Committee also set out the overall approach to follow and established the first list of proposed biological agents for QPS status. The QPS list is reviewed by EFSA's Panel on Biological Hazards (BIOHAZ). New biological agents recommended for QPS status are regularly added to the 2013 QPS list through a Panel statement.

Demand for food containing probiotics are expanding globally due to the continuous generation of research evidence indicating their potential health benefits to consumers. Hundreds of different

bacteria species are the natural and predominant constituents of intestinal microbiota. Among the numerous intestinal microbes, those anticipated to exhibit potential health benefits to the host through modulation of the intestinal microbiota are commonly selected as probiotics. Species belonging to the genera *Lactobacillus* and *Bifidobacterium* have been reported to be the beneficial probiotic bacterial strains. The representative species include *L. acidophilus*, *L. casei*, *L. plantarum*, *B. animalis lactis*, *B. longum*, and *B. bifidum* (Kailasapathy & Chin 2000; Ishibashi & Yamazaki 2001). Some of the major health benefits attributed to probiotics include improvement of gastrointestinal microflora, enhancement of immune system, reduction of serum cholesterol, cancer prevention, treatment of irritable bowel-associated diarrhoea, antihypertensive effects as well as improvement of lactose metabolism (Saarela *et al.* 2000; Nagpal *et al.* 2012). There are a number of criteria that must be met during the selection of a probiotic bacterial strain with utmost importance placed on safety issues. Strains of the *Lactobacillus* and *Bifidobacterium* genera are usually regarded as safe for long-term human use. Members of other genera such as *Bacillus licheniformis* have also been investigated to be used as probiotics. However, it should not be concluded that all members belonging to the *Bacillus* genus can be used as probiotics. This is because there are some strains from the *Bacillus* genus that are associated with diseases such as *Bacillus cereus*, which can cause food-borne illnesses. It is critical to perform safety assessment when the probiotics are not from the genera of *Lactobacillus* or *Bifidobacterium* (Leuschner *et al.* 2010). The viable cell numbers of probiotics in a product should be at least 10^6 CFU/mL at the expiry date for health and functional claiming as the recommended minimum effective dose per day is 10^8 – 10^9 cells. Many factors such as pH, titrable acidity, molecular oxygen, redox potential, hydrogen peroxide, flavouring agents, packaging materials, and packaging conditions are associated with viable cell count of a microorganism in a product throughout the manufacturing and shelf-life periods (Mortazavian *et al.* 2012). Another important selection criterion for a probiotic is the ability to adhere to host tissues especially to the intestinal mucus and epithelial cells to promote efficient host-microbial interactions. This interaction is particularly important to prolong the retention period of the specific strain in the gut. However, continuous intake of orally administered probiotics is necessary because permanent colonisation of probiotics is uncommon. Many factors are involved in the adhesion of probiotic microorganisms to the host tissues. Microbial cell density, buffer components, fermentation duration, and growth medium are associated to the *in vitro* culture parameters while intestinal microflora, digestion, and the food matrix are referred to *in vivo* conditions (Ouweland & Salminen 2003). There are ongoing studies on the identification of new strains for potential exploitation as probiotics concurrently with existing strains being explored for novel applications. These new strains need to be evaluated and assessed based on established selection criteria which include safety and, functional and technological characteristics prior to the selection of a particular strain for probiotic application.

Intestinal Microbiota

The human large intestine is a very complex ecosystem that is still not fully understood. The intestinal microbiota is considered to play numerous functions in the human body, such as metabolic, protective and structural functions. The composition of the microflora in the human large intestine, as estimated using culturing techniques, is usually dominated by the genera *Bacteroides*, *Eubacterium*, and *Bifidobacterium*, with several other predominant genera,

such as *Clostridium*, *Peptostreptococcus*, *Enterococcus*, *Lactobacillus*, and members of the family *Enterobacteriaceae* (Ju-Hoon Lee et al 2010). The number of species estimated by culturing techniques is approximately 400. Non culturing analysis of the gut microflora was greatly facilitated by the direct isolation of DNA from feces and by amplification of the 16S rRNA genes representing the entire microflora. Cloning and sequencing of individual rRNA genes enabled the numerically dominant genera of bacteria to be identified.

This molecular analysis of the intestinal microflora in fecal and colonic samples initially suggested that the human large intestine contains more than 500 different bacterial species and that about 75% of them are non culturable (Duncan et al 2007). However, a recent extensive metagenomic analysis revealed that this is an over estimation, with most individuals harboring approximately 160 different bacterial species, the majority of which are “known”. While the total number of species present in the human gut is not known, recent molecular studies indicate that it is in excess of 1,000, with an upper estimate of 1,150 suggested by an extensive metagenomic analysis of 124 individuals (Ju-Hoon Lee et al 2010).

Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are a functional group of microorganisms comprising Gram-positive, catalase negative bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation. Among LAB, *Lactobacillus* is the genus including a high number of GRAS species (Generally Recognized As Safe) and many strains are among the most important bacteria in food microbiology and human nutrition, due to their contribution to fermented food production or their use as probiotics. From a taxonomic point of view, the genus *Lactobacillus*, until October 2012, consist of 152 validly described species, and it belongs to the family *Lactobacillaceae* together with genus *Pediococcus*, with whom it is phylogenetically intermixed. (Salveti E. et al , 2012). Instead, the phylogenetic analysis based on 16S rRNA gene sequence revealed that the family is divided into 15 groups of three or more species, 4 couples and 10 single lines of descents. Lactobacilli are rod-shaped, Gram+, no spore forming, catalase-negative and anaerobic but oxygen tolerant. Normally they form straight rods but under certain conditions spiral or coccobacillary forms have been observed. In most cases they form chains of varying length. The majority of lactobacilli's species are very demanding from a nutritional point of view, requiring the substrates for growth complexes containing in addition to carbon sources and nitrogen, compounds phosphorus and sulfur. As their name implies, they produce lactic acid and derive energy from the fermentation of lactose, glucose and other sugars to lactate via homofermentative metabolism. About 85-90% of the sugar utilized in the fermentative process is converted to lactic acid. However, there are some heterofermentative lactobacilli that produce alcohol in addition to lactic acid from sugars. This acid-producing mechanism inhibits growth of other organisms and favours the growth of lactobacilli that thrive in low pH environments. They are widely distributed in nature, finding himself in different habitats: the oral cavity, the human and animal intestinal tract. They can grow in a temperature range between 5 ° C and 53 ° C with optimal values of 30-40 ° C. They are acidurici, with an optimum growth pH of 5.5-5.8 being able grow even at pH <5. Probiotic bacteria reduce inflammation associated with allergy both in murine models and human diseases . Probiotics affect the induction phase of immune responses and decrease IgE responses by altering antigen presentation , by reversing the Th1/Th2 polarization, and/or by inducing Tregs (L Cassard, et al 2016). In literature was reported

that *Lactobacillus paracasei* CNCM I-1518 affects the effector phase of immune responses by inhibiting IgE-dependent human basophil and mouse mast cell activation. *Lactobacillus paracasei* has been demonstrated, also, to inhibit the growth of many pathogenic microbes such as *Streptococcus mutans*, in vitro (L. Cassard, et al 2016). *Lactobacillus paracasei* are commonly used in dairy product fermentation and probiotics. It is commonly found as common inhabitants of the human intestinal tract and mouths as well as sewages, silages, and previously mentioned dairy products. This species is generally recognized as safe (GRAS status) because of a long history of safe human consumption and now was used as ingredients of functional foods.

Bifidobacteria

Bifidobacteria were first isolated from the feces of breast-fed infants in 1899, by Henri Tissier, and were designated *Bacillus bifidus*. Even though Orla-Jensen proposed the genus *Bifidobacterium* in 1924 (Ju-Hoon Lee et al, 2010) *bifidobacteria* were classified into other taxonomic groups, such as *Bacillus bifidus*, *Bacteroides bifidus* and *Lactobacillus bifidus*, for several decades. In 1973, Poupard (Poupard, et al 1973), and subsequently *Bergey* (Bergey, et al 1974), reclassified them as a separate taxon and designated the genus *Bifidobacterium*, consisting of 11 species. Scardovi (Scardovi V. et al 1969.) updated this to 24 species in 1986, and currently there are 31 proposed species that have been isolated from the intestines of humans, animals, and insects, and also from human dental caries and raw milk.

Bifidobacteria are nonmotile, non-spore-forming, non-gas-producing, Gram-positive, anaerobic, catalase-negative bacteria with a high G+C content (55 to 67%). Their morphology is generally referred to as bifid or irregular V- or Y-shaped rods resembling branches. They have been recommended as dietary supplements as freeze-dried bifidobacterial preparations, sometimes with *Lactobacillus acidophilus*, have been used for the treatment of gastrointestinal (GI) disorders (Prevot, A. R. et al, 1971). Currently, *Bifidobacteria* are added to numerous foods, specifically for their perceived probiotic activities. Numerous studies have suggested that the presence of *Bifidobacteria* in the human large intestine is associated with many human health benefits.

Celiac Disease

Celiac disease is a lifelong autoimmune condition where the body's immune system reacts to gluten, an alcohol-soluble protein fraction found in Cereals, wheat, barley and rye. The body's reaction to gluten causes damage to the lining of the intestine, the place where food and nutrients are absorbed. This can lead to malnutrition. Celiac disease (CD) is characterized by very variable clinical picture, ranging from profuse diarrhea with marked weight loss, in extra-intestinal symptoms, the association with other autoimmune diseases. Unlike allergies to wheat, Celiac Disease and Dermatitis herpetiformis are not induced by skin contact with gluten, but only from its ingestion.

Celiac disease can be identified with the serological test and biopsy of the duodenal mucosa during endoscopy. The gluten-free diet is the only treatment available for celiac disease, it should be carried out rigorously for life. CD is a complex genetic disorder that involves multiple chromosomal regions. The strong influence of genetic factors is shown in studies carried out on monozygotic twins showed a concordance rate of 86%, while in dizygotic twins it only reaches 20%. Several are the genes involved

in the development of celiac disease, certainly as long known genes of the major histocompatibility complex (HLA / MHC) located on chromosome 6 have a significant influence: in fact about 95% of patients are carriers of the genes encoding the molecules HLA-DQ2, while those who do not have this, in most cases, have the haplotype HLA-DQ8 (A. K Taylor, et al, 2008). Numerous studies have shown the existence in celiac patients, but not in control groups, of CD4 T cells specific HLA-DQ based on the deaminated peptides by transglutaminase (J. May, et al 2010).

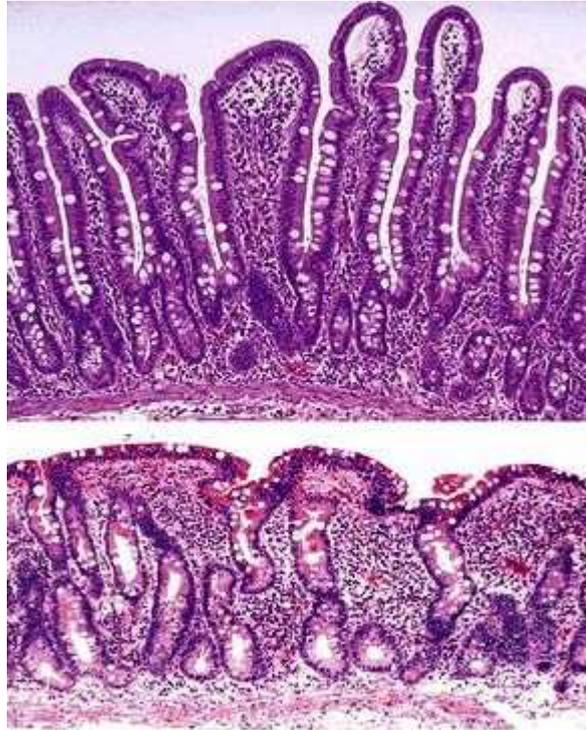


Fig.1: Intestinal villi from the epithelial lining in a non-Celiac individual (top) as compared to an individual with Celiac disease (bottom). Image from the Celiac Disease Foundation.

Some deaminated peptides have a high affinity for the DQ2 and DQ8 molecules and have a greater stimulatory capacity (A. Camarca, et al, 2009). In sensitive individuals, the adaptive response, mediated by the activation of antigen-specific T cells, drives a proinflammatory response, particularly the production of IFN-gamma, culminating in an immune-mediated response, when villous atrophy, crypt hyperplasia, and increased infiltration of intraepithelial lymphocytes are the typical result.

Actually there is an alternative therapeutic strategy for celiac patient characterized by a biochemical modification of wheat flour by pretreatment with the transglutaminase. The transglutaminase is not only the key enzyme of the mechanism to development of celiac disease, but it can also be used in the laboratory to change the gluten structure to not activate the immune response. It has been demonstrated that the transamidation in the laboratory of wheat flour by the *Streptovercillium* bacterial transglutaminase reduces the immunological toxicity of gluten in an animal model of celiac disease (DQ8 mice), and biopsies of celiac patients. (Lombardi et al., 2013).

Streptomyces mobaraesis

Streptomyces a genus of filamentous bacteria of the family Streptomycetaceae (order Actinomycetales) that includes more than 500 species occurring in soil and water. Many species are important in the decomposition of organic matter in soil, contributing in part to the earthy odour of soil and decaying leaves and to the fertility of soil. Certain species are noted for the production of broad-spectrum antibiotics, chemicals that the bacteria naturally produce to kill or inhibit the growth of other microorganisms. *Streptomyces* are characterized as gram-positive aerobic bacteria of complex form. They form a thread like net called mycelium that bears chains of spores at maturity. Their branching strands are 0.5 to 1.0 micrometers in diameter. Streptomycetes comprise very important industrial bacteria, producing two-thirds of all clinically relevant secondary metabolites. They are mycelial microorganisms with complex developmental cycles that include programmed cell death (PCD) and sporulation. Industrial fermentations are usually performed in liquid cultures (large bioreactors), conditions in which *Streptomyces* strains generally do not sporulate, and it was traditionally assumed that there was no differentiation.

The traditional *Streptomyces* developmental cycle mainly focused on the sporulation phases occurring in solid cultures. After spore germination, a completely viable vegetative mycelium (substrate) grows on the surface and inside agar until it differentiates to a reproductive (aerial) mycelium that grows into the air, producing spores at the end of the cycle (Flårdh & Buttner, 2009). A young, compartmentalized mycelium (MI) was reported to die early on, following a highly ordered sequence (Manteca et al., 2005, 2006a). Subsequently, the viable segments of this mycelium differentiate into a multinucleated second mycelium (MII). MII grows inside the culture medium (substrate mycelium) until it starts to express hydrophobic covers and grows into the air (aerial mycelium) and ends by forming spores (Manteca et al., 2007). Prior to sporulation, there is a second round of PCD affecting substrate and aerial mycelium (Wildermuth, 1970; Mendez et al., 1985; Miguelez et al., 1999).

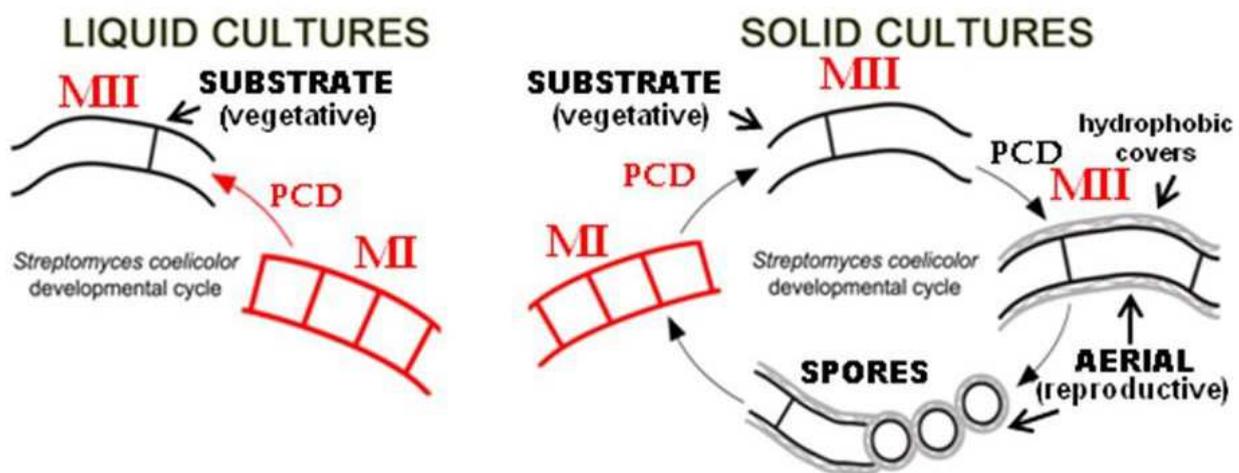


Fig. 2: (Sourceyague et al, 2013) *Streptomyces* developmental cycle and mycelium differentiation. (a) *Streptomyces* developmental cycle in liquid (left) and solid (right) cultures. Newly described structures and the proposed nomenclature (Manteca et al., 2005) are indicated in red: MI, first compartmentalized mycelium; MII, second multinucleated mycelium.

Streptomyces mobaraensis is a spore forming bacterium species from the genus of Streptomyces. *Streptomyces mobaraensis* produces bleomycin, detoxin, piericidin A, piericidin B, reticulol and a Ca²⁺-independent transglutaminase (TGase) that is activated by removing an N-terminal peptide from a precursor protein. *Streptomyces mobaraensis* is used in the food industry to produce transglutaminase to texture meat and fish products.

Chapter 1

Effect of *Lactobacillus paracasei* culture filtrates and artichoke polyphenols on cytokine production by dendritic cells.

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Abstract

The most recent trend in research on probiotic bacteria aims at the exploitation of bioactive bacterial compounds that are responsible for health-promoting effects and suitable for medical applications. Therefore, the main purpose of this study was to ascertain if the immunomodulatory effects of *L. paracasei* strains on dendritic cells (DCs) were caused by bacterial metabolites released in the culture medium. For that reason, bacterial strains were grown in two media generally used for the culture of DCs, and the effects of culture filtrates on the maturation of DCs and cytokine production were evaluated. Moreover, to reveal potential synergistic effects on the immunomodulation of DCs, an artichoke phenolic extract (APE) was added to the media before bacterial growth. The experiments pointed out an interesting anti-inflammatory activity of a culture filtrate obtained after growing a probiotic *L. paracasei* strain in one of the media supplemented with APE. Therefore, this culture filtrate which combines the anti-inflammatory activity and the other well-known health-promoting properties of artichoke phenolic compounds could represent the basis for future particular exploitations.

Keywords: probiotic bacteria, immunomodulatory bacterial metabolites, dendritic cells, artichoke phenolic compounds

Introduction

Bacterial strains of the species *Lactobacillus paracasei* can be considered as common inhabitants of the human intestinal tract, and they are usually also isolated from foods such as milk, dairy products, and fermented vegetables. This species is generally recognized as safe (GRAS and “Qualified Presumption of Safety”-QPS status) because of a long history of safe human consumption [1,2]. Accordingly, a number of *L. paracasei* strains have been studied to define their strain-specific health-promoting properties and selected to be used as probiotics. In particular, strain *L. paracasei* IMPC 2.1 has been studied and used for the development of an innovative patented functional food [3,4,5,6,7] whose commercialization has been authorized by the Italian Ministry of Health, based on the association of that probiotic strain with vegetables. The probiotic/vegetable joining has the aim of broadening the variety of probiotic food types using a suitable vegetable carrier rich in functional components. In this regard, artichokes contain polyphenols in particular flavonoids and hydroxycinnamic acid derivatives which are known for their antioxidant properties. The main phenolic compounds contained in artichoke head are caffeoylquinic acids, above all chlorogenic acid (5-*O*-caffeoylquinic acid) and two dicaffeoylquinic acids (1,5-*O*- and 3,5-*O*-dicaffeoylquinic acid) [8,9]. Therefore, artichoke was selected as a possible vegetable carrier of strain *L. paracasei* IMPC 2.1, whose probiotic aptitude and efficacy were also studied in association with that food matrix. In particular, the probiotic activities of strain IMPC 2.1 (recognized by the European Food Safety Authority, EFSA, as sufficiently characterized and identified at the strain level [10]) include inhibition of the growth of the food-borne human pathogen *Yersinia enterocolitica* [11] and anti-proliferative activity on both gastric and colon cancer cell lines [12]; more significantly, in vivo human trials demonstrated the effect of *L. paracasei* IMPC 2.1 associated with artichoke on the modulation of fecal biochemical and microbiological parameters [13] and its efficacy in reducing symptoms of functional constipation [14], as also evaluated in a meta-analysis of randomized controlled trials on the effect of probiotics on gastrointestinal dysfunction [15]. The interaction with the immune system and its modulation are probably the most important mechanisms underlying the health-promoting effects of probiotic bacteria [16]. The immunomodulatory properties of strain IMPC 2.1 were evaluated in comparison to other strains of the same species, studying the effects of their interaction with dendritic cells (DCs), which are important in the earliest bacterial recognition and in determination of the subsequent T-cell responses, playing a pivotal role in both innate and adaptive immunity [16]. The results of the study [17] indicated that strain IMPC 2.1, as well as the other strains, stimulated phenotypic maturation of DCs. Moreover, the study confirmed that different strains even of the same species may show very diverse immunomodulatory properties. In fact, strain IMPC 2.1 induced a low pro-inflammatory response (enough to induce a state of alertness of the immune system), while strain IMPC 4.1 (genetically similar to IMPC 2.1 [10]) was characterized by very interesting and peculiar anti-inflammatory properties. Although a number of studies indicate that the immunomodulatory properties of probiotic bacterial strains are due to molecules associated to the bacterial cell surface [18,19], recent increasing evidence indicates that they can also be due to the production of specific compounds which are secreted in the culture medium. For example, Thomas et al. [20] demonstrated that the probiotic strain *Lactobacillus reuteri* 6475 secretes histamine, which inhibits the production of the pro-inflammatory cytokine tumor necrosis factor (TNF). In another study, *L. plantarum* 10hk2 was shown to produce a protein fraction estimated to have a molecular weight of 8.7 kDa with anti-inflammatory activity [21]. A proteinaceous compound with a molecular weight of 50 kDa is secreted

in the culture supernatant of *Bifidobacterium animalis* subsp. *lactis* strain BB12, and it was found to be involved in the anti-inflammatory effects caused in stimulated Caco-2 cells by that bacterial strain [22]. Concerning *L. paracasei*, an anti-inflammatory activity was also detected in a culture supernatant of strain B21060, as it was able to inhibit the production of inflammatory cytokines by DCs in response to *Salmonella typhimurium* [23]. Likewise, a more recent work [24] highlighted that yet-unknown substances produced by a different *L. paracasei* strain were responsible for its anti-inflammatory properties, also indicating the possibility of exploiting this strain's feature directly using a milk fermented with the strain. In those cases, *L. paracasei* compounds responsible for the anti-inflammatory activity remained unidentified, while von Schillde et al. [25] identified a lactocepine with a molecular weight higher than 100 kDa as a protease secreted by a strain of *L. paracasei*, exerting an anti-inflammatory activity by degrading pro-inflammatory chemokines. The above-mentioned studies indicated that the specific immunomodulatory activity of different probiotic strains can be due to diverse compounds whose production also depends on the composition of the culture medium. In this regard, it is important to note that the culture medium itself can also exert an immunomodulatory activity, thus hiding the results of in vitro experiments carried out to evaluate the immunomodulatory activity of bacterial culture filtrates [24]. In this context, the aim of this study is to further characterize the *L. paracasei* strains used in our previous work [17] in particular strain IMPC 2.1 to ascertain if their immunomodulatory properties are related to metabolites released in the culture medium. Taking into account that the immunomodulatory activity of each strain is strictly strain-specific, and that this activity could be caused by diverse immunomodulatory compounds, the work carried out in this study represents an opportunity to highlight different immunomodulatory activities caused by novel molecules. Moreover, considering that several probiotic features of the strain *L. paracasei* IMPC 2.1 are exerted in the presence of the artichoke matrix, in order to reveal a potential symbiotic activity, we also evaluated the effect of an artichoke phenolic extract fermented by those *L. paracasei* strains on the immunomodulation of DCs. A further element of the novelty of this study is the development of an experimental system in which the bacterial strains were grown directly in media originally designed for the culture of DCs, thus avoiding the interfering effect of bacterial culture medium on DCs [24].

Materials and methods

Artichoke Phenolic Extract Preparation and Assay

The edible part of fresh artichoke (cv. Violetto di Provenza) buds was used to prepare an artichoke phenolic extract (APE) in the form of a lyophilized powder, according to Di Venere et al. [9], with some modifications. Briefly, about 100 g of fresh tissue were extracted after homogenization (1:5 w/v) by refluxing in boiling methanol (twice for 1 h). The obtained extract was filtered through a Whatman 1 filter paper and then concentrated under reduced pressure by a rotary evaporator. The obtained dry residue was finally dissolved in 500 mL of distilled water. The resulting solution was filtered through a Whatman 1 filter paper, immediately frozen, and then lyophilized to obtain about 4.3 g of dry powder (APE), which was stored under vacuum at $-25\text{ }^{\circ}\text{C}$ until use. The phenolic content of the APE was assayed as follows. A weighed aliquot of APE was dissolved in a known volume of distilled water to realize a solution at concentration of about 4 mg/mL (w/v). The total phenolic concentration of the solution was quantified by high performance liquid chromatography (HPLC), as described below, and the total phenol (TP) content of APE was calculated as the sum of the contents of single peaks identified by HPLC analysis and expressed as mg of TP/g of dry powder.

Phenolic Composition and Concentration Assay

The analysis of phenolic compounds in artichoke extract, in the medium (used as a control), and in bacterial culture filtrates was performed by HPLC according to Gatto et al. [26]. Chromatographic peaks were identified according to Mileo et al. [27]. Calibration curves built for each compound using commercial standards were used to quantify the identified phenolics: 5-*O*-caffeoylquinic acid (5-*O*-CQA, also known as chlorogenic acid, CHLGA), 1-*O*-CQA, 3-*O*-CQA, 4-*O*-CQA, 1,3-dicaffeoylquinic acid (1,3-DCQA, also known as cynarin, CYN), 1,4-DCQA, 4,5-DCQA, 3,5-DCQA, 1,5-DCQA, 3,4-DCQA, and an apigenin glycoside (APG-GLYC), this latter being quantified as apigenin-7-glucoside.

Chemicals

HPLC grade water was obtained by a Milli-Q system (Millipore, Bedford, MA, USA). Methanol (Chromasolv[®] gradient grade) was purchased from Sigma-Aldrich (Milan, Italy). All HPLC standards (chromatographic purity > 95%) were purchased from Phytolab GmbH and Co. KG (Vestenbergsgreuth, Germany).

Bacterial strains and culture conditions

L. paracasei LMG 23554 (=strain YS8866441) was used as a certain non-probiotic strain [28], it was obtained from the Belgian Coordinated Collections of Microorganisms, Ghent, Belgium. *L. paracasei* IMPC 2.1 and IMPC 4.1 (both isolated from human intestine) were obtained from the Culture Collection at the Istituto di Microbiologia, Università Cattolica, Piacenza, Italy and deposited as strain LMG P-22043 and LMG S-27068 in the Belgian Coordinated Collections of Microorganisms, respectively. For long-term storage, 1 mL aliquots of fresh cultures with 20% Bacto glycerol (Difco, Detroit, MI, USA) were frozen at $-80\text{ }^{\circ}\text{C}$ in 2 mL sterile cryovials (Nalgene,

Rochester, NY, USA). For preparation of working cultures, strains were 2% (v/v) inoculated in de Man Rogosa Sharpe (MRS) broth (Difco, Detroit, MI, USA), grown for 24 h at 37 °C under anaerobic conditions, and subcultured in the same medium twice before use in experiments. To obtain culture filtrates to be tested on DCs, strains were grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 10% fetal calf serum (Sigma, St. Louis, MO, USA), 1% L-glutamine (Sigma, St. Louis, MO, USA), and 1% non-essential amino acid (EuroClone, Milan, Italy) (complete RPMI 1640) or X-Vivo 15 medium (without Phenol red and Gentamicin, Lonza, Verviers, Belgium) with or without the addition of APE. A suitable amount of APE was dissolved in the culture media to realize a final TP concentration of about 500 mg/L (about 3.3 mg of APE/mL of medium), then the media were sterilized by filtration (0.22 µm) before strain inoculation. To this purpose, working cultures were harvested by centrifugation (9000× g for 10 min at 4 °C), washed twice with sterile saline solution (NaCl 0.85%, w/v) and re-suspended in the same volume of supplemented RPMI 1640 medium or X-Vivo 15 medium (with or without APE). These cultures were 2% (v/v) inoculated in 30 mL of the same media and incubated at 37 °C under anaerobic conditions. The experiment was carried out in triplicate. The growth of each strain was monitored by measuring the plate counts and the optical density (OD) value at 600 nm. When OD₆₀₀ reached the value 0.6 (corresponding to a cell density of ca. 3×10^8 cfu/mL), cell-free supernatants were obtained by centrifugation (9000× g, 4 °C, 10 min) and filtration through 0.22 µm filters (International PBI, Milan, Italy).

Mice

BALB-c mice were maintained under pathogen-free conditions at the animal facility of the Institute of Food Sciences (accreditation no. DM.161/99). Mice were used at the age of 6–12 weeks and were euthanized by inhalation of anaesthesia with isoflurane. All experiments with mice were performed in accordance with European Union Laws and guidelines. All animal studies were approved by the review committee of the Health Ministry, General Division of Animal Health and of Veterinary Medicine, and performed according to European regulations (EU Directive 2010/63/EU).

Isolation and Growth of Bone Marrow-Derived Dendritic Cells

Murine DCs were generated according to a previously published method [29]. In brief, bone marrow cells from the femurs and tibiae of mice were flushed and bone marrow cell aliquots (1×10^6) were diluted in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with antibiotics (penicillin 100 IU/mL; streptomycin 100 IU/mL) and 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (culture medium) before being seeded in 12-well plates (Falcon, Heidelberg, Germany). On days 3, 5, and 7, 1 mL/well of cell supernatant was centrifuged and pellet re-suspended in freshly prepared culture medium. On day 9, cells were pre-incubated with bacterial filtrates. Cell viability was microscopically evaluated by dye-exclusion test using Nigrosin (1% solution), and >90% live cells were found in all experiments. Fluorescence-activated cell sorting (FACS) analysis revealed that cells resulted >75% CD11b⁻ CD11c⁺.

Microbial Filtrate Challenge

Immature DCs (iDCs) were incubated for 24 h in the presence of undiluted microbial filtrates in RPMI 1640 or X-Vivo 15 media with or without the addition of APE. Following incubation, cells were

treated with 1 µg/mL lipopolysaccharide (LPS) for 6 h (LPS pulse) to induce the maturation of DCs and were cultured for an additional 24 h in RPMI 1640 or X-Vivo 15 media. Cells were collected for FACS analysis. Spent media were centrifuged at 10,000× *g* for 10 min to eliminate any residual cells and cell debris, and supernatants were stored at −80 °C.

Fluorescence-Activated Cell Sorting (FACS) Analysis

DCs were stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies (Abs) (BioLegend, San Diego, CA, USA) against CD80 and CD86. Cell staining was analysed using a CyFlow Space flow cytometer (Partec, Munster, Germany) and FlowJo software (Tree Star Inc., Ashland, OR, USA). For each Ab, an isotype control of the appropriate isotype subclass was used.

Analysis of Cytokine Production

Supernatants from DCs cultures were analysed for IL-12, TNF- α , and IL-10 protein levels using an in-house sandwich ELISA. First, 100 µL aliquots of capture antibody solution (BioLegend, San Diego, CA, USA) were plated into ELISA wells (Nunc Maxisorb; eBioscience Inc., San Diego, CA, USA) and incubated overnight at 4 °C. After the removal of the capture antibody solution, 100 µL of blocking buffer (phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA)) were 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 respective wells and incubated for 2 h at room temperature. At the end of the incubation, three washing steps with PBS supplemented with 0.05% Tween-20 were performed, and 100 µL aliquots of biotinylated antibody solution were added to the wells and incubated for 1.5 h at room temperature. After three washes, streptavidin–horseradish peroxidase conjugate solution (1:1500 dilution; BioLegend, San Diego, CA, USA) was then added to the wells and incubated for 1 h at room temperature. Finally, after washing, 100 µL of 63 mM Na₂HPO₄, 29 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.

Statistical Analysis

Statistical significance was determined by *t*-test or ANOVA using GraphPad PRISM 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value of 0.05 or less was considered to be significant.

Results

Phenolic Content of Artichoke Extract and of Bacterial Culture Filtrates

The APE was found to contain CQAs, DCQAs, and small amounts of an APG-GLYC. The main components were CHLGA, 4-*O*-CQA, and two DCQAs (3,5-DCQA and 1,5-DCQA) (Figure 1). The total phenolic content of APE was found to be 151 mg TP/g dry powder. The concentration of the different phenolic compounds in complete RPMI 1640 supplemented with APE at the beginning (control $t = 0$) and at the end (control) of incubation, as well as in the bacterial culture filtrates after bacterial growth was determined (Figure 1). When APE was incubated in the presence of growing bacterial strains, a lower concentration of 3-*O*-CQA and CYN (about 50%) and a higher concentration of CHLGA, 3,5- and 1,5-DCQA (about 200%) were observed in comparison to the control (Figure 1). Such high differences in concentration were probably produced by phenolic structural changes occurring in the control during the incubation time, due to the slight alkaline pH of the culture broth (pH = 7.8). Actually, a slow isomerisation and breakdown of phenolic compounds at pH ≥ 7 have already been reported [30]. Therefore, the minimal changes in concentration of the different phenolic compounds observed in the three bacterial treatments might be explained by the progressive lowering of pH (down to pH = 6) produced by the bacterial growth in such samples during incubation, which probably contributed to an inhibition of the above-mentioned phenomena. No significant differences in phenolic composition was recorded among the three bacterial culture filtrates (Figure 1).

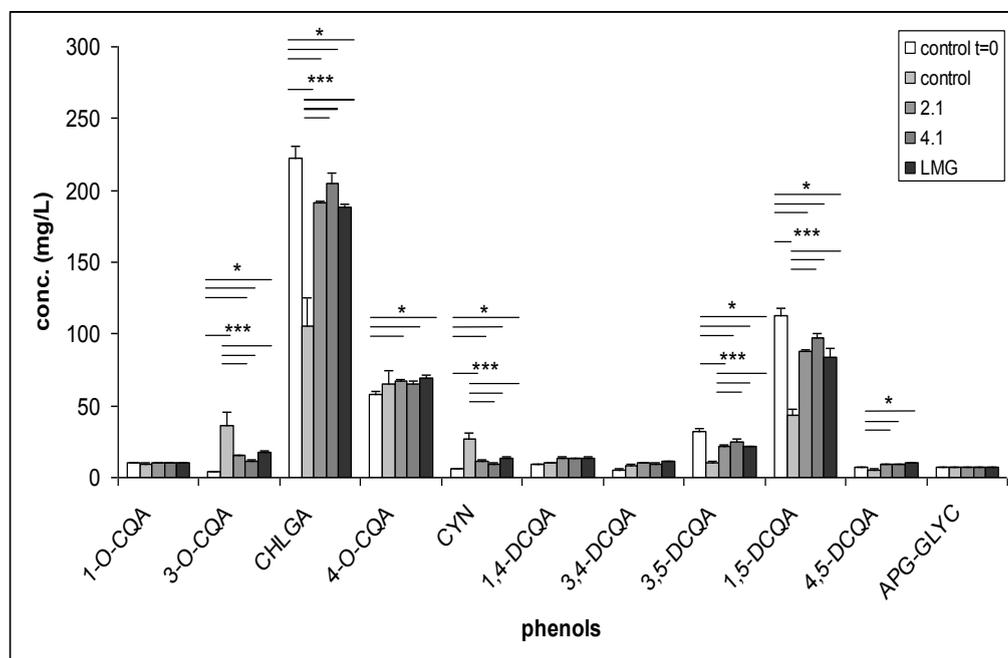


Figure 1: Concentration of phenolic compounds in the control (RPMI 1640 medium supplemented with artichoke phenolic extract) at the beginning (control $t = 0$) and at the end of incubation (control), as well as in bacterial culture filtrates after bacterial growth. Values represent the mean \pm SD of three replications and statistical significance from ANOVA (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) is reported.

2.1: *Lactobacillus paracasei* IMPC 2.1; 4.1: *L. paracasei* IMPC 4.1; LMG: *L. paracasei* LMG 23554; CQA: Caffeoylquinic acid; CHLGA: Chlorogenic acid; CYN: Cynarin; DCQA: Dicafeoylquinic acid; APG-GLYC: Apigenin glycoside.

Growth of *L. paracasei* Strains in RPMI 1640 and X-Vivo 15 Media

Bacterial culture filtrates tested on DCs were obtained by growing bacterial strains in complete RPMI 1640 medium and X-Vivo 15. All three strains were similarly able to grow in complete RPMI 1640 medium, the presence of fetal calf serum being essential for growth, but notably also in X-Vivo 15, which it is a specific serum-free medium. The addition of APE to both media markedly improved the growth of the strains; in fact, an $OD_{600} = 0.6$ (corresponding to a cell density of ca. 3×10^8 cfu/mL) was reached after about 24 h of incubation without APE and after 9 h with APE addition.

Effect of Culture Media on the Maturation of Dendritic Cells and Cytokine Production

Intestinal DCs are able to directly sample luminal antigens by extruding dendrites between epithelial cells. To reproduce this interaction *in vitro*, we pulsed bone marrow-derived immature DCs (iDCs) with LPS to obtain mature DCs (mDCs). Moreover, the effects of the two culture media were compared on both iDCs and mDCs (Figure 2). Following LPS-pulse, mDCs expressed significantly high levels of CD80 and CD86 co-stimulatory markers. This increase was comparable in both media (Figure 2A). Next, the effect of medium on the cytokine profile of DCs was analyzed by evaluating the production of interleukin IL-12 and IL-10, a pro- and an anti-inflammatory cytokine, respectively. As reported in Figure 2B, IL-12 significantly increased in mDCs, independently of the medium. On the contrary, RPMI 1640 favoured the expression of IL-10 in mDCs, even if levels were not found statistically relevant.

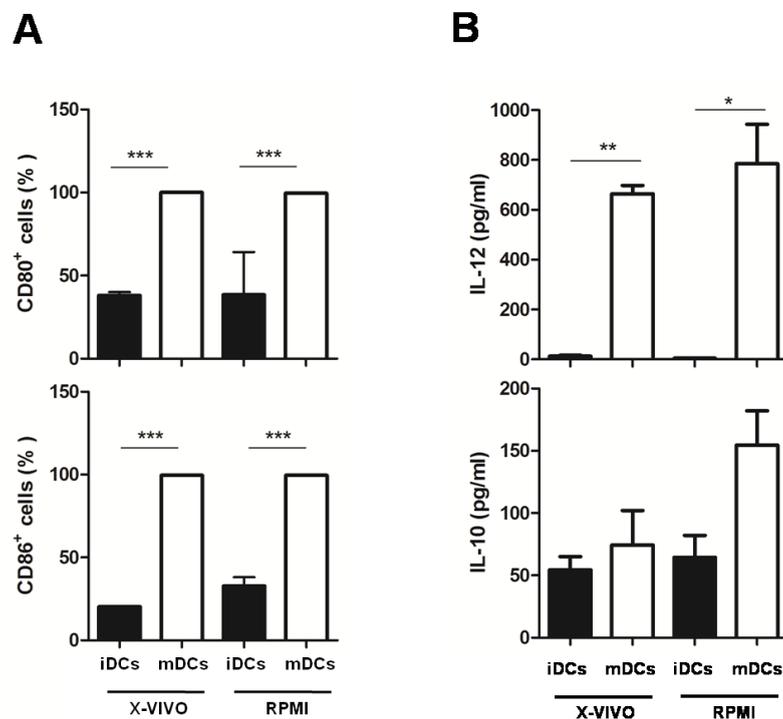


Figure 2: Effect of the culture medium RPMI 1640 (RPMI) or X-Vivo 15 (X-VIVO) on maturation of dendritic cells (DCs) and cytokine production. (A) Effect on maturation (induced by lipopolysaccharide (LPS) stimulation) revealed by the expression of CD86 and CD80 molecules; representative dot plots are also reported; (B) Effect on cytokine production. mDCs: Mature DCs; iDCs: Immature DCs; IL: Interleukin. The statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) is reported.

Effect of Bacterial Culture Filtrates with or without Artichoke Phenolic Extract on the Maturation of Dendritic Cells

To evaluate the immunomodulatory potential of metabolites released in the culture media in the presence or absence of APE, iDCs were incubated for 24 h with filtrates derived from bacterial growth in complete RPMI 1640 and X-Vivo 15 media supplemented or not with APE. Following this treatment, iDCs were LPS-pulsed and cultured for an additional 24 h in the same respective medium. As reported above, LPS induced increased expression of both CD80 and CD86 surface markers in both examined media (Figure 3A,B). Pre-treatment of iDCs with the culture filtrate obtained after the growth of strain LMG 23554 in RPMI 1640 medium (Figure 3A) did not further stimulate their maturation, whereas the levels of CD80 and CD86 induced by IMPC 2.1 culture filtrate were significantly lower. On the contrary, IMPC 4.1 induced CD86 expression. In presence of APE-supplemented complete RPMI 1640 medium, LPS-induced CD80 and CD86 expressions were significantly reduced in mDCs. This inhibition was partially limited for CD86 by the bacterial fermentation of RPMI 1640 medium supplemented with APE (Figure 3A). Different effects on marker expressions were observed when DCs were pre-incubated with culture filtrates obtained after bacterial growth in X-Vivo 15 medium (Figure 3B). In fact, an increase of both DC markers was reported for all the three strains, although it was statistically significant only for CD80 expression observed after pre-incubation with IMPC 4.1 and LMG 23554. Additionally, the addition of APE to X-Vivo 15 medium caused a marked increase of both co-stimulatory markers. Pre-treatment of DCs with IMPC 2.1 and LMG 23554 filtrates obtained after growth in the presence of APE reduced this effect, even if this was not found to be statistically significant.

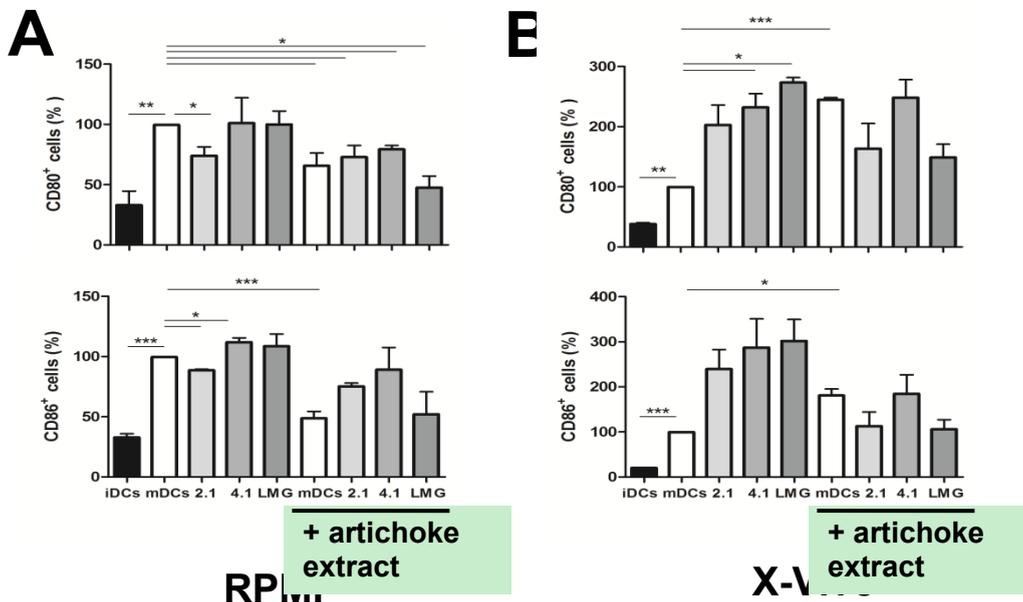


Figure 3: Effect of bacterial culture filtrates on the maturation of dendritic cells (DCs) as revealed by the expression of CD86 and CD80 molecules. (A) Effect caused by culture filtrates obtained after bacterial growth in RPMI 1640 medium without or with artichoke phenolic extract; (B) Effect caused by culture filtrates obtained after bacterial growth in X-Vivo 15 medium without or with artichoke phenolic extract. mDCs: Mature DCs (obtained by LPS treatment); iDCs: Immature DCs; 2.1: Bacterial strain *Lactobacillus paracasei* IMPC 2.1; 4.1: Bacterial strain *L. paracasei* IMPC 4.1; LMG: Bacterial strain *L. paracasei* LMG 23554. The statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) is reported.

Effect of Bacterial Culture Filtrates with or without Artichoke Phenolic Extract on Cytokine Production by Dendritic Cells

Next, the production of IL-12 and IL-10 by challenged DCs was determined. Treatment with culture filtrates obtained after the growth in RPMI 1640 medium did not modify IL-12 secretion of mDCs, but determined differential (albeit non-significant) effects on IL-10 production (Figure 4A). In particular, culture filtrates of strains IMPC 2.1 and LMG 23554 reduced the production of IL-10, whereas strain IMPC 4.1 increased the production of that cytokine. Notably, the addition of APE to the culture medium significantly reduced the production of both IL-12 and IL-10 by mDCs, with the exception of strain IMPC 2.1 for IL-10. In parallel, treatment of DCs with culture filtrates obtained after the growth of bacterial strains in X-Vivo 15 medium determined a decrease of IL-12 production; this effect was statistically significant for IMPC 2.1 and IMPC 4.1 strains (Figure 4B). On the contrary, culture filtrates tended to increase IL-10 production in mDCs. Notably, the addition of APE to X-Vivo 15 medium completely suppressed LPS-induced production of IL-12 and blocked IL-10 induction by filtrates. In this regard, it is interesting to note that the treatment of DCs with bacterial culture filtrates obtained after growth in X-Vivo 15 medium was cytokine-specific. In fact, a filtrate-dependent trend toward an increased production of TNF- α was observed in the presence of APE, even if it was not found to be statistically significant.

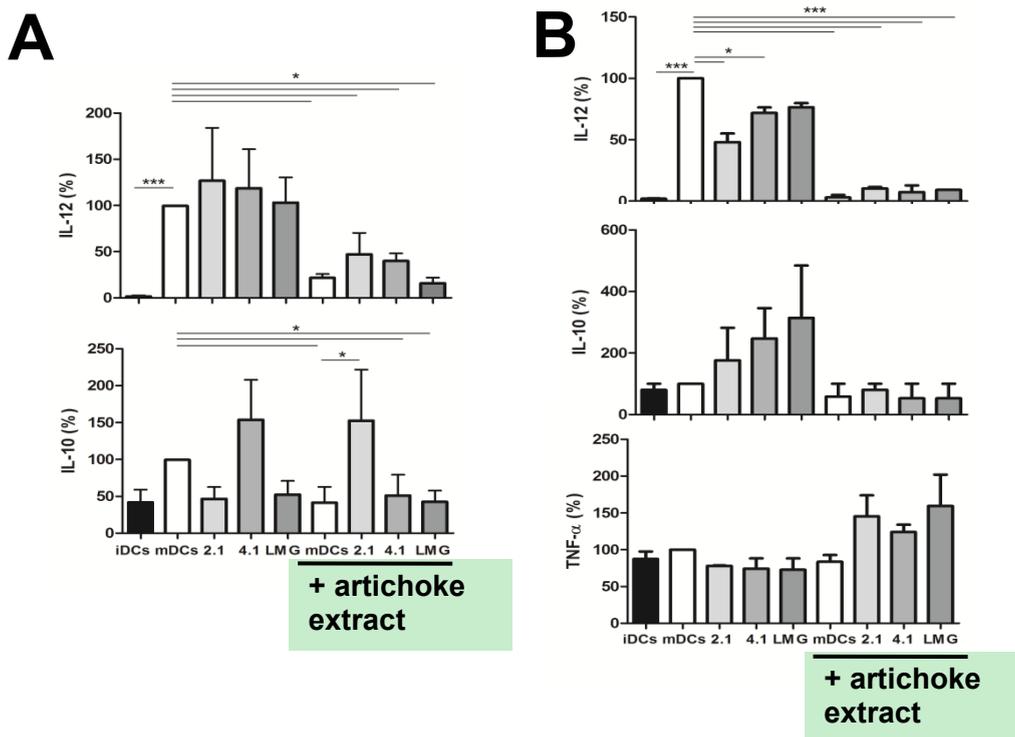


Figure 4 Effect of bacterial culture filtrates obtained after the growth in (A) RPMI 1640 medium or (B) X-Vivo 15 medium with or without artichoke phenolic extract on cytokine production by dendritic cells (DCs). mDCs: Mature DCs (obtained by LPS treatment); iDCs: Immature DCs; 2.1: Bacterial strain *Lactobacillus paracasei* IMPC 2.1; 4.1: Bacterial strain *L. paracasei* IMPC 4.1; LMG: Bacterial strain *L. paracasei* LMG 23554; TNF: Tumor necrosis factor; IL: Interleukin. The statistical significance (* $p < 0.05$; *** $p < 0.001$) is reported.

Discussion

In this study, *L. paracasei* strains have been further characterized to reveal whether their strain-specific immunomodulatory properties [17] could be related to the production of bioactive molecules in a culture medium. The most advanced trend in research on probiotic bacteria aims at the exploitation of these molecules [24] for medical applications. These compounds could allow more specific therapeutic effects to be obtained, even if their use would require complex studies to get the desired clinical results. Nevertheless, it should be considered that the identification of bioactive compounds responsible for a probiotic effect is very challenging, because the peculiar immunomodulatory properties of each bacterial strain could be the overall result of still-undefined factors, including a very complex mixture of bacterial metabolites [18,31]. Therefore, the use of a fermented milk possibly containing a mixture of bioactive compounds has been proposed to obtain an anti-inflammatory effect [24]. Moreover, the choice of the experimental conditions plays a fundamental role. Previous studies [24] demonstrated that a common medium (i.e., MRS) for the growth of lactobacilli itself has an effect on cytokine production by DCs, thus possibly hiding and interfering with the effect of bacterial metabolites. A result of our study is that both complete RPMI 1640 and the serum-free X-Vivo 15 were found to be suitable for bacterial growth, thus avoiding the above-mentioned problem and making the experimental conditions more appropriate for in vitro assessment of DC functionality. Notably, treatment of DCs with bacterial culture filtrates obtained after growth in X-Vivo 15 (but not in RPMI 1640) partially replicated the effects caused by the direct interaction with bacterial cells [17]. Specifically, increased expression of CD86 and of the anti-inflammatory cytokine IL-10 in mDCs were confirmed mainly for filtrates from X-Vivo 15. On the contrary, these filtrates decreased IL-12 levels in mDCs, rather than inducing, as reported following bacterial cell interaction [17]. Importantly, these data strongly suggest the existence of soluble metabolites that were functionally active (immunomodulatory) only in a serum-free medium. Further studies are then requested to assess if interaction with protein components or hydrolases can dampen these activities in complete RPMI 1640 medium. Nevertheless, interesting results were obtained in both media when an artichoke phenolic extract (APE) was added. It is interesting to note that APE addition did not inhibit bacterial growth, but improved it in both media. As the analysis of artichoke phenols did not reveal significant variations, this effect could be due to unidentified compound/s. Concerning the immunomodulatory effect, the addition of APE significantly reduced the expression of both IL-12 and IL-10 in mDCs. Among the examined filtrates, only that derived from the growth of strain IMPC 2.1 in RPMI 1640 was able to recover IL-10 production. It is well known that the association of a probiotic bacterium with the appropriate food matrix is very important for its efficacy and in determining the kind of health-promoting effect [32]. In this case, the addition of APE to the RPMI 1640 medium resulted in a culture filtrate obtained after the growth of strain IMPC 2.1 with interesting anti-inflammatory properties, as it increased the production of the anti-inflammatory cytokine IL-10 while decreasing IL-12 production. It is noteworthy that strain IMPC 2.1 is the *L. paracasei* strain used for the development of the innovative “probiotic artichoke” [4], and that the anti-inflammatory effect is strictly strain-specific, differentiating strain IMPC 2.1 from the other tested strains. Therefore, the anti-inflammatory effect is not due to the APE itself but to the interaction between APE and the strain metabolism. As the analysis of artichoke phenols in bacterial culture filtrates did not reveal significant differences between strains, it could be hypothesized that APE modified IMPC 2.1 metabolism, inducing the production of one or more still-unknown anti-

inflammatory metabolites. Another plausible hypothesis is that APE did not modify strain metabolism, but that the anti-inflammatory effect is the result of a complex interaction between bacterial metabolites and APE compounds with DCs. Evaluation of these hypotheses could be the subject of future interesting studies. In conclusion, the results of this study indicate that the immunomodulatory properties of a probiotic strain may also depend on the particular medium used for its growth. Therefore, the choice of the right culture medium plays a fundamental role in research activities aimed at the production of immunomodulatory compounds. In this case, an interesting anti-inflammatory activity was detected in the culture filtrate obtained by growing a probiotic *L. paracasei* strain in a medium for DCs supplemented with APE. Therefore, the anti-inflammatory activity of a probiotic strain is synergistically joined with the antioxidant and the other health-promoting properties of artichoke phenolic compounds in that culture filtrate [27,33,34], paving the way to future interesting practical applications.

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Authors Contribution

A.S., P.L., and M.R. conceived and designed the study; D.L., L.T., P.D.B. and D.D.V., performed the experiments; P.D.B., D.D.V., D.L. and M.R. analyzed the data; A.S., D.D.V., P.L. and M.R. wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results

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Chapter 2

“Development of gluten with immunomodulatory properties using mTGactive food grade supernatants from *Streptomyces mobaraensis* optimized Fermentations”

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Abstract

Transamidation by microbial transglutaminase (mTG) blocks gliadin immunotoxicity in Coeliac disease (CD), however, mTG is expensive and limits cost effectiveness. Herein, we set up an inexpensive treatment by employing culture supernatant of *Streptomyces mobaraensis*. A food grade medium and fermentation workflow were developed to obtain inexpensive mTG (2.6 U/ml at 120 h). Incubation of wheat flour with culture supernatant and lysine ethyl ester solubilised both gliadin and glutenin, similarly to the process using commercial mTG. The water-soluble protein fraction (spfc) cross-reacted against gliadin, as it stimulated gliadin-specific spleen cells from HLA-DQ8 transgenic mice, a model of gluten sensitivity. Notably, splenocytes from spfc immunised mice induced high levels of anti-inflammatory IL-10 when challenged with gliadin. Bread manufactured with treated flour had only minor changes in the baking parameters. In conclusion, our data highlighted the immunomodulatory properties of a bread produced by applying this protocol, with implications for the management of CD patients.

Keywords: Coeliac disease; immunomodulation; wheat gliadin; microbial transglutaminase; transamidation; *Streptomyces mobaraensis*.

Introduction

Celiac disease (CD) is a chronic immune-mediated inflammatory disorder of intestinal mucosa, triggered by ingestion of wheat gluten and related proteins of rye and barley, in human leukocyte antigen (HLA)-DQ2 and/or -DQ8 positive individuals (Green, Lebwohl & Greywoode, 2015). Gluten is essentially composed of two protein fractions, gliadins and glutenins, characterised by high levels of glutamine (30–35%) and proline (10–15%). In the small intestine of CD patients, specific glutamine residues are converted to glutamic acid by tissue transglutaminase (tTG) (DiSabatino et al., 2012). After deamidation, gluten peptides increase their affinity binding for both DQ2 and DQ8 molecules (Molberg et al., 1998) and induce activation of intestinal specific CD4+ T cells. This causes alterations of duodenal mucosa, characterised by intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy (Di Sabatino et al, 2012; Molberg et al., 1998). Furthermore, the proline residues, resistant to intestinal proteases, ensure the survival of the immunostimulatory epitopes (Stamnaes & Sollid, 2015). The treatment of CD is currently based on a lifelong gluten-free diet to recover the intestinal mucosal integrity. However, gluten-free diet compliance is poor and alternative approaches have been proposed in order to improve the quality of life of CD patients (Crespo Pérez, Castillejo de Villasante, Cano Ruiz, & León, 2012). We developed an enzymatic approach that blocks the immunotoxicity of gliadin peptides by transamidation using food-grade microbial transglutaminase (mTG) (Gianfrani et al., 2007). In particular, we showed that, by treating wheat flour with mTG and lysine alkyl esters, the ability of gluten to induce the CD-specific immune response in human and murine experimental models was inhibited (Gianfrani et al., 2007; Lombardi et al., 2013). Most importantly, transamidated gluten reduced the number of clinical relapses in challenged CD patients with no changes of baseline values for serological and mucosal CD markers (Mazzarella et al., 2012). A two-step transamidation reaction was recently developed (Mazzeo et al., 2013) that was also able to modify the immunogenic epitopes of gliadin on a pilot scale level, while not influencing the main technological properties of semolina (Moscaritolo, Treppiccione, Ottombrino & Rossi, 2016). mTG from *Streptomyces mobaraensis* is the only cross-linking enzyme that is currently available for catalysing this type of covalent bond between proteins on a commercial scale. mTG-catalysed reactions can also be used to modify the functional properties of food proteins (Nielsen, 1995), such as texture, viscosity, foaming and emulsifying properties, and can lead to improvements in their nutritional value (Yokoyama, Nio, & Kikuchi, 2004). On a commercial scale, production of mTG is mainly carried out by submerged fermentation of *S. mobaraensis* (Yokoyama, Nio & Kikuchi, 2004). Further improvement of the large-scale production of mTG is continuously in progress, with the aim of lowering manufacturing costs. With the help of medium optimisation, a low-cost form of production may have the potential to provide a valid strategy for the industrial scaling up of the gluten detoxification process. On the basis of the above data, our aim was to set up an inexpensive treatment of wheat flour, by directly employing the filtered mTG-active supernatant of cultures from a food-grade *Streptomyces mobaraensis* strain, a natural mTG producer, and by evaluating the properties of the treated flour.

Our results indicated an anti-inflammatory activity for the treated flour, as a consequence of mTG treatment, without significantly altering its baking technological properties.

Materials and Methods

Mice

BALB/c mice and transgenic mice expressing the HLA-DQ8 molecule in the absence of endogenous mouse class II genes (Cheng et al., 1996) were reared for several generations on a gluten-free diet (Altromin-MT-mod, Rieper SpA, Bolzano, Italy) in pathogen-free conditions at our animal facility (accreditation n. 164/99-A). All procedures met the guidelines of the Italian Ministry of Health.

S. mobaraensis culture

S. mobaraensis DSM 40847 (German Collection of Microorganisms and Cell Cultures-DSMZ, Braunschweig, Germany) was allowed to grow on TBO sporulation agar medium (20 g/L tomato paste, 20 g/L oat flakes, and 20 g/L agar, pH 6.5) for up to 10 days. Spores (1×10^9) from fresh agar cultures were used to inoculate a basic medium at 180 rpm for 10 days at 30 °C. Up to 5% (v/v) of these pre-inocula were transferred to 50 ml of basic culture medium in 250-ml Erlenmeyer flasks and incubated at 30 °C, 180 rpm. The basic medium (BM) was a modification of those described by Zhang et al. (2012) and was altered by substituting 3% polypeptone with 1.5% meat peptone + 1.5% casein peptone. The addition of MgCl₂, an inducer of mTG, was also tested at different concentrations. Agitation and temperature values were selected on the basis of previous findings (Zhang et al., 2012). The scale-up process was conducted in a specifically designed pilot fermenter (16 L). The glass vessel was temperature controlled (30 °C) and equipped with a stirring shaft with Rushton-type blades. Stirring was conducted at an appropriate speed (150 rpm) to ensure distribution of nutrients/oxygen and avoiding mycelium desegregation. Sterile-filtered air was injected and controlled by a flowmeter (air flow range: 0-4.0 L/min).

Laser scanning fluorescence microscopy.

Cultures were stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Life Technologies Europe BV, Monza Italy), and observed under a Leica TCS-SP2-AOBS laser scanning microscope at wavelengths of 488 and 568 nm for excitation and 530 and 630 nm emission.

mTG assay

The enzymatic activity in culture supernatants was assayed according to the method described by Folk & Cole (1966), which is based on the chromogenic hydroxamate procedure using N- α -benzyloxycarbonyl-L-glutaminyglycine (Cbz-Gln-Gly) as a substrate. Activity in the mTG-active culture supernatant was measured at 525 nm. An enzyme unit is defined as the amount of enzyme which catalyses the formation of 0.5 mole of hydroxamate per min from N-CBZ-Gln-Lys and hydroxylamine at pH 6.0 at 37 °C.

Flour treatment and biochemical analysis

A total of 10 g of flour was resuspended in 8 volumes of 0.4 M NaCl and stirred for 10 min to extract albumins/globulins. The flour suspension was then centrifuged at 1,000 g for 10 min, and the supernatant was discarded. The recovered pellet was exhaustively washed with water to eliminate any residual soluble protein, and resuspended in two volumes of sterile filtered supernatant from *S.*

mobaraensis culture medium (M9 medium, Table 1), containing 20 mM lysine ethyl ester (K-C₂H₅; NutraBio.com, Middlesex, NJ, USA). Control was performed by resuspending flour in water containing 8 U/g mTG (ACTIVA®WM; 81-135 U/g; Ajinomoto Foods, Hamburg Germany) and 20 mM K-C₂H₅. The first reaction step was conducted for 2 h at 30 °C with stirring (100 rpm). The suspension was then centrifuged (1,000g, 10 min) to recover the supernatant containing the soluble protein fraction (spf) that was freeze-dried. A second reaction step was conducted for 3 h at 30 °C by resuspending the pellet with fresh culture supernatant or enzyme and K-C₂H₅ at the same concentrations. For biochemical analysis, a sample of 20 ml flour resuspension was recovered at the end of each step. After centrifugation at 3,000 g for 10 min, the residual gliadin and glutenin fractions were extracted from the pellet using a modified Osborne procedure (Kick, Belitz, Wieser & Kieffer, 1992). Protein content was assessed by Bradford analysis (Bradford, 1976).

Table 1

Medium optimisation for the production of mTG by *S. mobaraensis*

Component	Medium									
	BM	M1	M2	M3	M4	M5	M6	M7	M8	M9
0.1 M MgCl ₂	+	+	+	+	+	+	+	+	+	+
0.2% K ₂ HPO ₄	+	+	+	+	+	+				+
0.1% MgSO ₄ ·7H ₂ O	+	+	+	+	+	+				+
1.5% meat peptone	+									
1.5% casein peptone	+			+	+	+				
1% soluble starch	+	+	+	+	+	+		+	+	+
1.0 % fructose	+	+	+	+	+	+				+
1.0% glycerol									+	
1.0 % glucose										+
1.5% soya peptone			+			+				
1.5% vegetable peptone		+			+					+
11% whey							+	+	+	

Immunological analysis of treated flour

Six-week old DQ8 transgenic mice were primed by intraperitoneal injection with native gliadin or spf (100 ug) emulsified in Freund's complete adjuvant (Sigma) (day 0). Boosters containing the same amount of antigen in incomplete Freund's adjuvant were injected on days 7 and 14. Mice were sacrificed on day 21 to recover their spleens. Spleens were passed through a stainless steel wire mesh to dissociate cells. Erythrocytes were removed by treating the cell suspensions with a Tris-buffered ammonium chloride solution. For each sample, 1x10⁶ cells were incubated in 0.5 ml culture medium in 48-well flat bottom plates at 37°C for 72 h in the presence of native mTG or spf (100 ug/ml). The

supernatants were then collected and analysed for IFN-gamma and IL-10 protein levels by in-house sandwich ELISA (Lombardi et al., 2013).

Safety assessment of filtered culture supernatant from *S. mobaraensis* cultures

Sterile filtered mTG-active supernatant from *S. mobaraensis* cultures was administered to six-week old BALB/c mice as a substitute of drinking water for 15 days. Mice were daily monitored for external stress signals (mobility, diarrhoea, lethargy and rough hair coat), weight loss, and food and water intake.

Analysis of the baking characteristics of treated flour

A commercial preparation of bread wheat flour was resuspended in two volumes of sterile filtered mTG-active supernatant from *S. mobaraensis* culture medium (M9 medium, Table 1), and 20 mM K₂H₅PO₄. The first reaction step was conducted for 2 h at 30 °C and the resuspension was recovered by centrifugation (1,000g, 10 min). After an extensive washing of the reactor with tap water, a second reaction step was conducted for 3 h at 30°C with fresh mTG-active culture supernatant and enzyme and K₂H₅PO₄ at the same concentrations. Wheat flour resuspension was centrifuged (15,000g, 10 min) and dough was recovered. A bread baking procedure was adopted using 500 g of dough mixed with 3.0 g of table salt and 10.0 g of baker's yeast. The dough and bread weights (g), bake loss (%), height increase (cm) and specific volume were determined using standardized protocols (Leuschner, O'Callaghan & Arendt, 1997).

Statistical analysis

Statistical significance was determined by ANOVA or 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.

Results

Optimisation of the bacterial culture conditions

S. mobaraensis was cultivated in 250-ml shake flasks containing 50 ml of BM. We preliminarily determined the optimal concentration of the enzyme inducer $MgCl_2$ (Zhang et al., 2012) in BM. As shown in Fig.1 (upper panel), the highest mTG activity was detected after 150 h with a concentration of 0.1 M. Interestingly, any further increase of $MgCl_2$ resulted in a dramatic activity drop. Furthermore, $MgCl_2$ -induced mTG production largely occurred during the MII differentiation phase of multinucleated mycelium (Yagüe, López-García, Rioseras, Sánchez & Manteca, 2013) (Fig.1A, bottom panel and Fig. 1B). In order to develop an inexpensive fermentation strategy for large scale fermentations, variations of the BM medium were tested (Table 1). We found that M9 was a food-grade, low-cost medium, which also provides a good level of production of mTG activity (2.6 U/ml at 120 h; Fig.2).

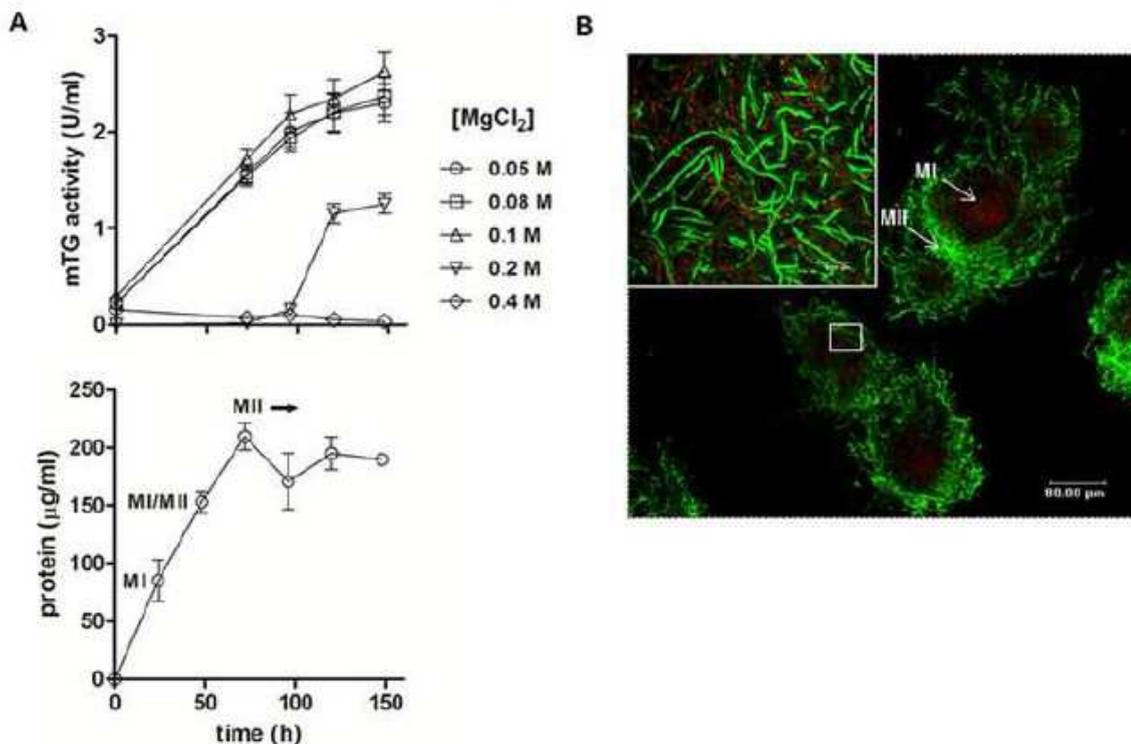


Fig.1. *S. mobaraensis* culture. **A)** Time course of mTG activity (U/ml, top panel) and cell growth (ug protein/ml, bottom panel) in basal medium (BM) at different concentrations of $MgCl_2$. Dots represent the mean + SD of triplicate cultures and are representative of three independent experiments. **B)** Confocal microscopy image of *S. mobaraensis* mycelium after 37 h of growth, showing MI and MII vegetative phases. The corner inset shows a high magnification (10X) of thramed area. Scale bar, 80 µm

M9 contains vegetable peptone as a substitute for meat and casein peptones (Table 1).

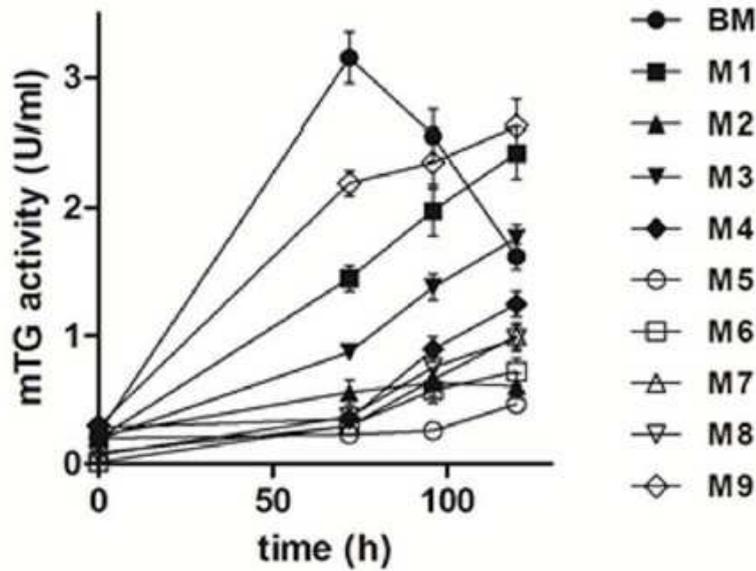


Fig.2. Time course of mTG activity in control medium (BM) and in the different tested media (see Table 1). Dots represent the mean + SD of duplicate cultures and are representative of three independent experiments.

Next, the culture was scaled up into a 16 L glass fermenter equipped to constantly monitor temperature and oxygen demand. The speed of paddle stirrers was set at 150 rpm to ensure an appropriate nutrients/oxygen distribution and avoiding mycelium disaggregation. Bacterial growth was determined in the absence of oxygen or with a low (1 L/min) or high (4L/min) oxygen supply. Intriguingly, the highest mTG activity was obtained in the absence of any oxygen influx (Fig. 3, top panel). On the contrary, the optimal growth condition was obtained with 1 L/min (Fig. 3, bottom panel). However, we were unable to reproduce mTG activity levels similar to those produced in flask. As a consequence of this outcome, the functional assessments described below were performed using mTG active culture supernatant from flask cultures.

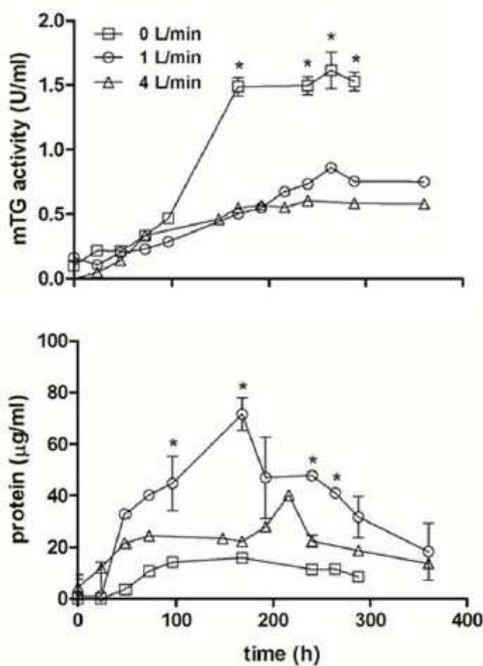


Fig.3. Time course of mTG activity (U/ml, top panel) and cell growth (µg protein/ml, bottom panel) in 16 L fermenter. Bacterial growth was performed in the absence of oxygen or with a low (1L/min) or high (4 L/min) oxygen supply. Dots represent the mean + SD of three independent experiments. *, $P < 0.05$.

Analysis of the prolamin fractions of wheat flour following treatment with *S. mobaraensis* culture supernatant

We have previously shown that wheat flour transamidation by mTG drastically increased both gliadin and glutenin solubilisation in water in low scale laboratory conditions (Mazzeo et al., 2013).

Herein, we tested this activity onto 1 L pilot scale; in particular, the catalytic activity of commercial mTG decreased both gliadin and glutenin yields in wheat flour to $23 \pm 0.7\%$ and $21.2 \pm 0.1\%$, respectively, after the second enzymatic step (mean + SD; Fig. 4). Notably, incubation of flour with filtered mTG-active culture supernatant from our *S. mobaraensis* log culture induced gliadin and glutenin solubilisation to levels comparable to those obtained with commercial mTG.

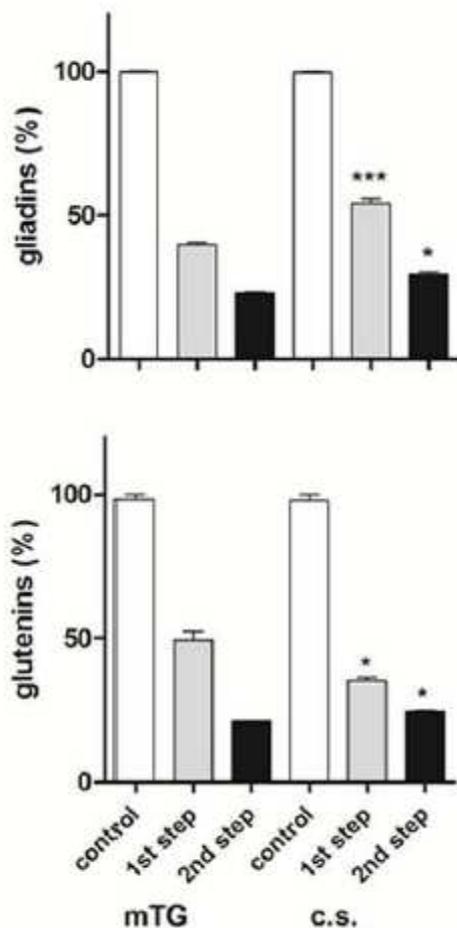


Fig.4. Residual yield of water-insoluble gluten proteins from the various treatment steps. Top panel, assessment of the residual alcohol-soluble gliadin fraction. Bottom panel, assessment of the residual alcohol-insoluble glutenin fraction. Columns represent the mean + SD of triplicate cultures and are representative of three independent experiments. *, $P < 0.05$; ***, $P < 0.001$; c.s., mTG-active culture supernatant of *S. mobaraensis*.

In particular, water-insoluble gliadin yield decreased to $29.3 \pm 1.2\%$, whereas glutenin was reduced to $24.5 \pm 0.6\%$ (mean + SD). In line with these results, a water-soluble protein fraction of gluten was produced following incubation with filtered mTG-active culture supernatant in the presence of lysine ethyl ester (spfcs).

Immunological activity of gluten solubilised following treatment with *S. mobaraensis* mTG-active culture supernatant

To evaluate the immune properties of spfcs, we used HLA-DQ8 transgenic mice, which only express the human MHC class II molecule that has been linked to CD (Cheng et al., 1996). Moreover, mice were from a colony reared on a gluten-free diet for several generations; accordingly, they lacked

oral tolerance toward gluten (Senger et al., 2005). To confirm immune cross-reactivity among wheat gliadin and spf, mice were immunised with native gliadin; spleen cells were then recovered and stimulated *in vitro* with native gliadin, spfcs or spf derived from mTG treatment (spfmTG). We found that both spfmTG and spfcs cross-reacted with gliadin by significantly inducing IFN-gamma secretion in sensitised spleen cells (Fig. 5A).

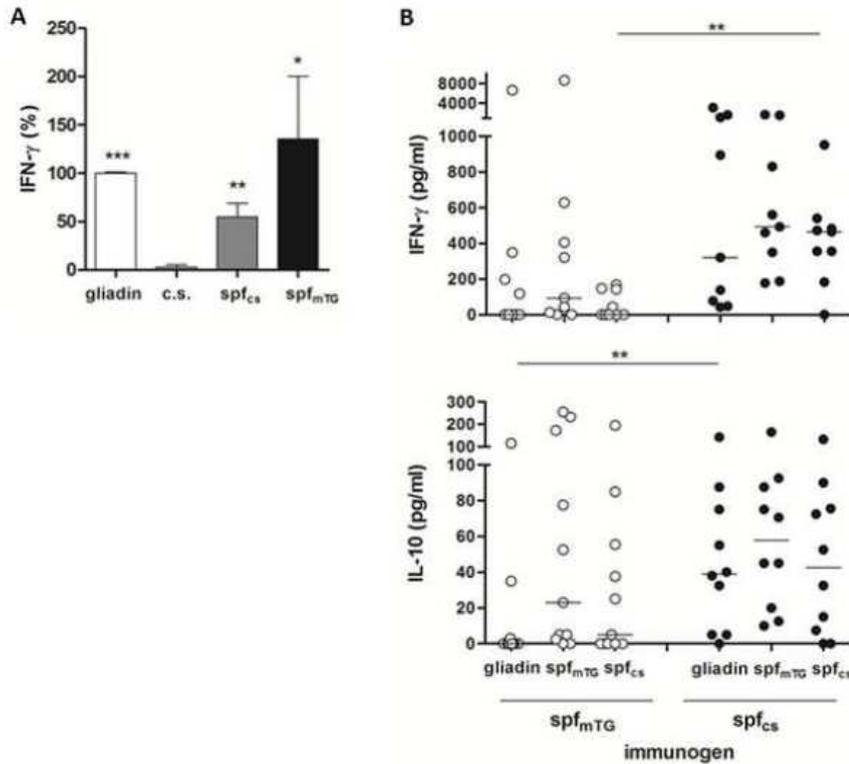


Fig.5 Immunogenic properties of treated flour. A, Gliadin-specific IFN- γ secretion of spleen cells from DQ8 mice immunised with native gliadin and challenged *in vitro* with spf from wheat flour treated with mTG-active culture supernatant (spfcs) or mTG (spfmTG). B, Antigen-specific IFN- γ and IL-10 secretions of spleen cells from DQ8 mice immunised with spfcs or spfmTG and stimulated *in vitro* with spf or native gliadin. Results represent one of two independent experiments and are expressed as mean value + SD. There were 8-10 mice in each group. **P<0.05.

Furthermore, spfcs was immunogenic, as spleen cells isolated from spfcs-sensitised mice produced IFN-gamma when challenged *in vitro* with the cognate antigen (Fig.5B, top panel). Incubation with an equivalent amount (in weight) of freeze-dried, filtered, mTGactive culture supernatant, as negative control, did not induce any response (data not shown).

Importantly, spleen cells from spfcs-sensitised mice induced a significantly higher IL-10 response when challenged *in vitro* with gliadin than spfmTG-specific spleen cells (Fig.5B, bottom panel).

Safety and technological assessments of filtered mTG-active culture supernatant from *S. mobaraensis* culture

A preliminary safety assessment was conducted by evaluating a 15 day oral challenge of undiluted sterile-filtered mTG-active culture supernatant in BALB/c mice. During the treatment, mice did not show any external stress signals. In detail, no significant alteration of mobility, diarrhoea, lethargy or development of rough hair coat was registered. Furthermore, the analysis of weight loss and off food/water intakes indicated no significant difference with control mice (Fig. 6). Dough was finally recovered from the flour suspension after treatment with filtered mTG-active culture supernatant to evaluate the baking properties of gluten.

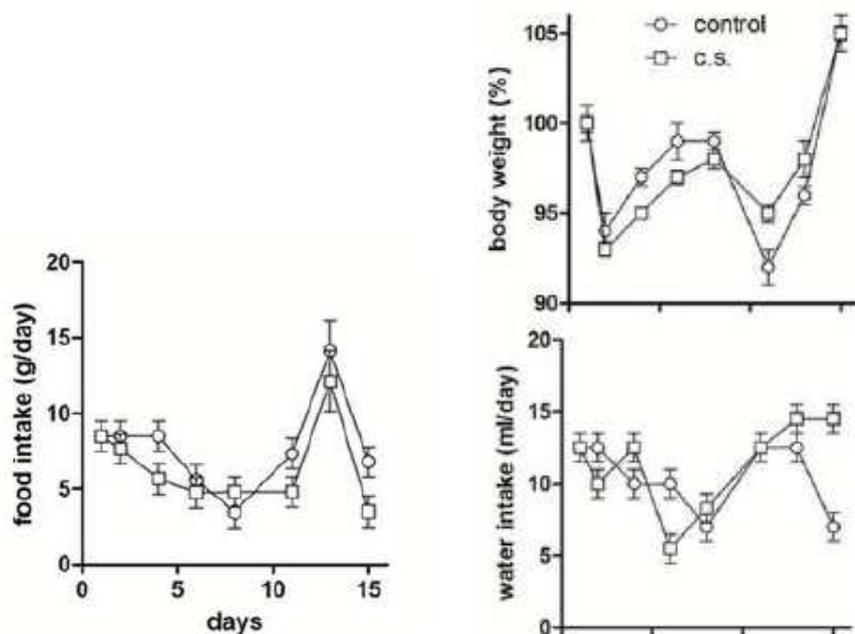


Fig.6. Safety assessment of mTG-active supernatant from *S. mobaraensis* culture. 6-12 wk-old BALB/c mice were administered with sterile-filtered culture supernatant from *S. mobaraensis* as substitute of drinking water. Body weight gains (top panel) water (middle panel) and food (bottom panel) intakes were measured dynamically for 15 days. Control mice were administered drinking water. Results represent one of two independent experiments and are expressed as mean value + SD. There were 8-10 mice in each group.

Interestingly, bread manufactured with supernatant-treated flour had only minor changes in the examined baking characteristics (Table 2). In addition, treated wheat bread had a brown crust colour and crumb structure, comparable to control bread. However, the wheat-like flavour was partially lost (not shown).

Table 2. Technical features of bread made from wheat flour treated with *S. mobaraensis* mTG-active culture supernatant.

Reaction conditions: culture supernatant	-	+	P-value
	K-C ₂ H ₅	+	
parameter	mean ± SD		
dough weight (g)	479± 10	450±1	0.04
bread weight (g)	451± 8	430± 5	0.01
bake loss (%)	6.0± 1.0	6.5± 1.0	0.2
height increase (cm)	5.0± 1.0	2.9± 1.0	0.1
specific volume	2.7± 0.1	2.3± 0.1	0.05

Discussion

The treatment of CD is currently based on a lifelong gluten-free diet to normalise the small intestinal mucosa. However, alternative approaches have been proposed in order to improve the quality of life of CD patients (Crespo Pérez, Castillejo de Villasante, Cano Ruiz, & León, 2012).

We have shown the inhibition of the gliadin inflammatory activity in different biological systems by enzymatic transamidation, using food-grade microbial transglutaminase (mTG) purified from *S. mobaraensis*, a transamidase of the endo- γ -glutamine: ϵ -lysine transferase type (Gianfrani et al., 2007; Mazzarella et al., 2012; Lombardi et al., 2013). The covalent attachment of amino acids by mTG is accepted in the food industry to also improve the nutritional quality and functional properties of food proteins. Moreover, the presence of ϵ -(γ -glutamyl)-lysine in gliadins is safe for humans, and the final catabolic step of transamidated gluten occurs in the kidneys, where this peptide provides a substrate for γ -glutamylamine cyclotransferase (Fink, Chung & Folk, 1980). Based on these findings, in this work, we explored the technological potential of a mTG-active culture supernatant from *S. mobaraensis* to directly reduce gluten toxicity, while improving the effectiveness of the process. The applicability of such an approach could be of strategic significance from the perspective of applying mTG-based gluten detoxification on an industrial scale. To address this issue, we preliminarily optimised flask cultivation conditions. mTG was synthesised as an inactive zymogen to avoid uncontrolled cross-linking of cellular proteins (Pasternack et al., 1998). Pro-mTG is secreted outside the cell wall of the microorganism at the beginning of fermentation and then transformed into an active enzyme by the action of proteases (Zhang et al., 2012).

Protease activities in MgCl₂ containing media were found to be much higher than those in the control medium. Furthermore, presence of 0.10 M MgCl₂ in the medium led to a significant mTG secretion (Zhang et al., 2012). In order to optimise the production of mTG from *S. mobaraensis*, we analysed different MgCl₂ concentrations in basal medium. In contrast with previous results, we also showed that concentrations lower than 0.1 M were sufficient to reach the highest level of mTG activity.

Importantly, MgCl₂ concentrations higher than 0.1 M were found to be detrimental for the enzyme activity titre. The latter finding could be related to an excessive induction of protease activities being able to reduce the enzyme half-life. More specific studies are required to address this matter.

From a technical point of view, the conditions that allow the highest transglutaminase activity represent the target of an optimisation process. However, economical aspects should also be considered. To address this issue we examined different raw materials, such as nitrogen, amino acids, vitamins and carbon sources. Interestingly, we found that vegetable peptone, but not soy peptone, could represent a valuable food grade substitute for meat and casein peptones, still enabling a high production of mTG. Culture scaling-up into a 16 L glass fermenter with M9 medium showed that mTG activity was mainly produced by *S. mobaraensis* when biomass reached the lowest growth rate under anaerobic conditions. These data suggest that growth and enzyme production were not associated, in agreement with previous results (Ando et al., 1989; Zhang et al., 2012). These results highlighted a possible new strategy to improve the industrial manufacturing of mTG, where, following mycelium growth, enzyme activity is triggered by a subsequent anaerobic step. However, we were unable to reproduce the same activity obtained in flask. As a result,

further work will be necessary to scale up the efficacy of the process. Gluten proteins are divided into two classes, based on their solubility in alcohol-water solutions:

gliadins (soluble) and glutenins (insoluble). Hydrated gliadins are the proteins that primarily contribute to dough viscosity and extensibility. Hydrated glutenins are both cohesive and elastic and are responsible for dough strength and elasticity. We have previously shown that both gliadin and glutenin yields decreased in wheat flour after a transamidation reaction that produced a water-soluble protein fraction with higher titre of these modified proteins (Mazzeo et al., 2013).

Importantly, mass spectrometry confirmed the presence of transamidated gluten in spf. Herein, we found that the solubility of both gliadins and glutenins was similarly changed following treatment with *S. mobaraensis* mTG-active culture supernatant of wheat flour in the presence of K-C₂H₅: about 70-75% of prolamins became soluble in water as a consequence of isopeptide bond formation. Importantly, this result was achieved by a lower level of mTG activity than for the control (2.6 vs. 4.8 U/ml), suggesting that other medium constituents might favourably influence this enzymatic catalysis. We previously showed that, in DQ8 transgenic mice, a model of gluten sensitivity, K-C₂H₅ cross-linking via mTG specifically affected gliadin immunogenicity, reversing the inducible inflammatory response (Lombardi et al., 2013). Therefore, we adopted the same model to analyse the phenotype of the gliadin-specific response induced by mTG-active culture supernatant of *S. mobaraensis*. Spleen cells isolated from mice immunised with native gliadin also recognised spfcs, confirming cross-reactivity between the two protein fractions. Notably, spfcs-specific spleen cells produced a significantly higher IL-10 response when challenged *in vitro* with gliadin, in comparison to spfmTG-specific spleen cells. This was an important output, leading to the consideration that the ability to revert the gliadin-inducible immune phenotype from inflammatory to anti-inflammatory could be exploited to restore gluten tolerance.

Dough recovered from the flour resuspension after the two-step reaction with filtered mTG-active culture supernatant contained most of the gluten that was soluble in water. Nevertheless, we could manufacture bread with only minor changes in the baking parameters, a result in agreement with our previous data obtained with mTG (Mazzeo et al., 2013). It is known that the physical properties of a dough arise from interactions between gluten proteins, particularly the disulfide-bonded glutenin macropolymer (Lindsay & Skerritt, 1999). Therefore, if soluble gluten still holds these features, this means that disulfide bonds are not changed during mTG-active culture supernatant treatment. In addition, a preliminary safety assessment in BALB/c mice indicated that the procedure was harmless.

In conclusion, wheat flour treatment based on the use of filtered *S. mobaraensis* mTG-active culture supernatant and K-C₂H₅ led to the acquisition of an immunomodulatory activity for gluten, without influencing the main baking technological parameters. Our data may have important implications for the management of CD, highlighting an inexpensive biotechnological strategy based on the use of *S. mobaraensis*.

Conflict of interest

The authors declare no financial or other conflict of interest.

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Chapter 3

“Effects of Two-Step Transamidation of Wheat Semolina on the Technological Properties of Gluten”

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Abstract

Celiac disease (CD) is an immune-mediated disorder caused by the ingestion of wheat gluten. A lifelong, gluten-free diet is required to alleviate symptoms and to normalize the intestinal mucosa. We previously found that transamidation reaction by microbial transglutaminase (mTG) was effective in down-regulating the gliadin-specific immune response in CD patients. In this study, the two-step transamidation protocol was adopted to treat commercial wheat semolina on a pilot scale. The effectiveness of the enzymatic reaction was tested by means of consolidated biochemical and immunological methods on isolated prolamins. We found that water-insoluble gliadin and glutenin yields decreased in wheat semolina to $5.9\% \pm 0.3\%$ and $11.6\% \pm 0.1\%$, respectively, after a two-step transamidation reaction. Using DQ8 transgenic mice as a model of gluten sensitivity, we observed a dramatic reduction in IFN- γ production in spleen cells challenged in vitro with the residual insoluble gliadin from transamidated semolina ($N = 6$; median values: 850 vs. 102; control vs. transamidated semolina, $p < 0.05$). The technological properties of treated wheat semolina were then tested by manufacturing classical pasta (spaghetti). Notably, the spaghetti manufactured with transamidated semolina had only minor changes in its features before and after cooking. In conclusion, the two-step transamidation reaction modified the immunogenic epitopes of gliadins also on a pilot-scale level without influencing the main technological properties of semolina. Our data shed further light on a detoxification strategy alternative to the current gluten-free diet and may have important implications for the management of CD patients.

Keywords:

celiac disease; transamidation; wheat semolina

Introduction

Celiac disease (CD) is an immune-mediated disorder caused in genetically susceptible individuals by the ingestion of wheat gluten and related prolamins present in barley and rye [1]. CD affects approximately 1% of the general population in developed and developing countries, with an increasing prevalence reported in Europe and the USA [2,3]. Currently, a lifelong, gluten-free (GF) diet is required to alleviate the symptoms of CD and to normalize the antibodies in the intestinal mucosa [3]. However, dietary compliance is poor, necessitating the development of alternative technological strategies to treat CD. Furthermore, gluten plays a key role in establishing the unique rheological properties and baking quality of wheat, which are partially restored in GF products. Moreover, to improve palatability, many GF products are manufactured with purified wheat starch, which invariably contains residual gluten. Gluten proteins are divided into two fractions according to their solubility in alcohol-water solutions: gliadins (soluble) and glutenins (insoluble). Both components contain high levels of glutamine (30%–35%) and proline (10%–15%) residues and very few negatively charged amino acids. These proteins undergo a process of selective deamidation in the small intestine of CD patients, during which specific glutamine residues are converted to glutamic acid by tissue transglutaminase (tTG) [4]. The presence of many proline residues in these proteins, which are resistant to digestive enzymes, ensures that many immunostimulatory epitopes survive digestion [5]. Notably, a previous study found that gliadin can be cleaved by bacterial prolyl endopeptidases (PEPs) into short peptides that then lose their activity [6]. Accordingly, PEPs have been evaluated as a technological tool for the preparation of detoxified gluten. One study reported that a 60-day diet of baked goods made from PEP-hydrolyzed wheat flour was not toxic to CD patients [7]. To improve the preservation of the gluten structure we tested a different enzymatic approach using the transamidation activity of food-grade microbial transglutaminase (mTG), a transamidase of the endo- γ -glutamine: ϵ -lysine transferase type [8]. Unlike tTG, mTG is a calcium-independent, low molecular weight protein, which has several advantages for food industrial applications [9]. This enzyme is commercially available as a dough improver that adds stability and elasticity to dough [10]. Importantly, the covalent attachment of amino acids by enzymatic procedures is also a generally accepted means of improving the nutritional quality and functional properties of food proteins. Previous studies have shown that the presence of the isopeptide linkages in gliadins does not impair their digestibility [11], indicating that this treatment is safe. The final catabolic step in gluten transamidation occurs largely in the kidneys, where ϵ -(γ -glutamyl)-lysine provides a substrate for γ -glutamylamine cyclotransferase [12]. Importantly, we found that the transamidation of gliadin following the treatment of wheat flour with mTG and lysine methyl ester caused a dramatic down-regulation in IFN- γ production in vitro in the intestinal T cells of CD patients [13]. Furthermore, we demonstrated that wheat flour, following transamidation using a new “two-step” procedure with lysine ethyl ester, was selectively associated with positive changes in the phenotype of the antigen-specific immune response in models of gluten sensitivity [14]. The present work investigated the reaction products of wheat semolina following transamidation using the two-step procedure on a pilot scale.

Materials and Methods

Quality Characteristics of Durum Wheat Semolina

The chemical and technological characterizations of semolina were performed by standard analyses: protein content (ICC 105/2 Kieldhal), gluten content % (ICC 137/1; 155; 158), yellow index (Minolta Chromameter CR-300, CEN standard method 15465), alveographic test (ICC 121), Braabender Farinograph (ICC 115/1). A commercial durum wheat was used for testing. Semolina was obtained by a pilot milling plant (Buhler MLU 202, Uzwil, Switzerland). Data are referred as mean of repeated analyses and differences between replicates were included within the specific ranges of each method.

Transamidation Reaction of Durum Wheat Semolina

Food-grade microbial transglutaminase (mTG) was from Ajinomoto Foods (Hamburg, Germany; ACTIVA[®]WM; 81–135 U/g); lysine ethyl ester (K-C₂H₅) was from NutraBio (NutraBio.com, Middlesex, NJ, USA). Semolina was suspended in two volumes of water containing 8 U/g mTG and 20 mM K-C₂H₅. Incubation was performed in a reactor plant Micro MFCS (BBraun AG, Melsungen, Germany) with a nominal capacity of 16 liters. The reactor plant was preliminary sterilized, then the temperature was decreased at 30 °C. The first step was conducted for 2 h at 30 °C and the suspension was recovered by centrifugation (1000× g, 10 min). After an extensive washing of the reactor with tap water, a second enzyme step was conducted for 3 h at 30 °C with fresh enzyme and K-C₂H₅ at the same concentrations. The suspension was finally centrifuged (15,000× g, 10 min) and dough recovered.

Biochemical Analysis of Transamidation of Wheat Semolina *Immunological Analysis of Transamidation of Wheat Semolina*

Transgenic mice expressing the HLA-DQ8 molecule in the absence of endogenous mouse class II genes [17] were reared for several generations on a GF diet (Altromin-MT-mod, Rieper SpA, Bolzano, Italy) in pathogen-free conditions at our animal facility (accreditation n. 164/99-A). All procedures met the guidelines of the Italian Ministry of Health. Six-week-old mice were primed by intraperitoneal injection with gliadins (300 µg) emulsified in Freund's complete adjuvant (Sigma) (day 0). Boosters containing the same amount of antigen in incomplete Freund's adjuvant were injected on days 7 and 14. Mice were sacrificed on day 21 to recover their spleens. Spleens were passed through a stainless steel wire mesh to dissociate cells. Erythrocytes were removed by treating the cell suspensions with a Tris-buffered ammonium chloride solution. For each sample, 5×10^5 cells were incubated in 0.2 mL culture medium in 96-well flat bottom plates at 37 °C for 96 h in the presence of gliadins (200 µg/mL). After 72 h, the supernatants were collected and analysed for IFN- γ protein levels by in-house sandwich ELISA.

Pasta Manufacturing Procedure

Semolina was used to produce pasta samples by a pilot plant (Namad Rome, Italy). The transamidated wet dough, showing an hydration index of 30%, was homogenized for 5 min at

room temperature in a kneader pre-mixer and then transferred to the mixing chamber under vacuum. In the next step, transamidated wet dough was pressed up to 100 bar through a screw (300 mm long and 45 mm diameter) at 30 °C. The dough was shaped into spaghetti (size \emptyset = 1.30 mm) using a bronze extruder. Then pasta was dried by a pilot plant drying device (Afrem—Clextal sas, Firminy, France) adopting the following temperature (°C)/relative humidity (rh) schedule: 77 °C/ 85% rh, 3 h; 70 °C/ 77% rh, 3 h; lowering from 70 °C to 35 °C/ 70% rh, 1 h; 35 °C/ 65% rh, 20 h; dried pasta was finally stored at room temperature under controlled atmosphere. Pasta cooking quality was evaluated by sensory analysis according to D'Egidio et al. [18]. Results related to the quality aspects were expressed as mean values of three determinations.

Statistical Evaluation

Statistical significance 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, USA). A *p*-value of 0.05 or less was considered to be significant.

Results

Qualitative Features of Semolina and Dried Pasta

The chemical and rheological characteristics were evaluated by classical methods: the protein content of semolina was $12.4\% \pm 0.1\%$ (d.m.) with a gluten content of $10.3\% \pm 0.1\%$ (d.m. basis); the yellow index (b) was 17.5 ± 0.1 and 15.7 ± 0.2 for semolina and dried pasta, respectively; and the rheological characteristics gave results of an alveographic test of W 210 (10^{-4} Joule) and p/L 4.5 and a farinograph braabender with an absorption of 55% and stability 5.0 min.

Pilot-Scale Production of Transamidated Semolina

We empirically determined that the best reactor performance could be achieved by enzymatically treating a maximum of 6.0 kg of durum wheat semolina in a final volume of 13.5 L. Accordingly, semolina was slowly suspended in 10.0 L of 20 mM K-C₂H₅ water solution and the suspension was transferred into the reactor. ACTIVA[®]WM was gently added under stirring conditions. Subsequently, the mixing speed was increased and we found that 450 rpm was needed to obtain a uniform distribution of the enzyme. After centrifugation the pellet was suspended in 20 mM K-C₂H₅ (12.0 L final volume) to perform the second step. By adopting this approach, we obtained a yield of 9.6 kg of transamidated dough.

Analysis of Transamidated Prolamins

The production of isopeptide bonds from the catalytic activity of mTG dramatically decreased the gliadin yield to $29.3\% \pm 1.9\%$ and $5.9\% \pm 0.3\%$ after the second step (mean \pm SD; Figure 1). On the contrary, the glutenins yield was fairly affected after the first enzyme step ($86.6\% \pm 1.6\%$), and it decreasing to $11.6\% \pm 0.1\%$ after the second step. Next, we focused on the immunological effects of gliadins extracted from semolina following a two-step transamidation process. To determine possible modifications in the T cell-mediated response, we used HLA-DQ8 transgenic mice, which only express the human MHC class II molecule that has been linked to CD [17]. Following immunization with gliadins, spleen cells were recovered and stimulated *in vitro* with different gliadin preparations. The immune response was analyzed by evaluating the IFN- γ expression. Results shown in Figure 2 indicated that spleen cells from immunized mice induced significant cytokine protein levels after a 72 h culture when stimulated with native gliadin. Notably, when gliadin-specific spleen cells were stimulated with residual insoluble gliadin isolated from semolina subjected to a two-step transamidation, the production of IFN- γ was dramatically blocked.

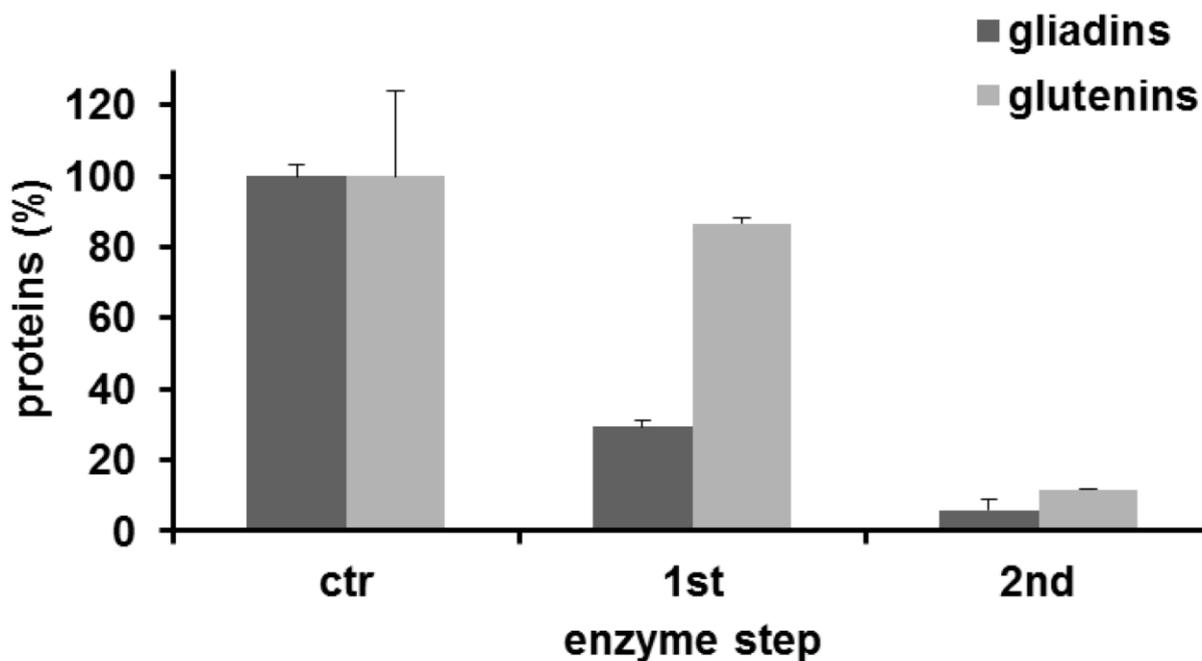


Figure 1. Assessment of the residual gliadin and glutenin protein fractions following the transamidation reaction, purified according to the modified Osborne procedure. Each bar represents values (means \pm SD) calculated as the percentage of control (untreated semolina) of triplicate experiments.

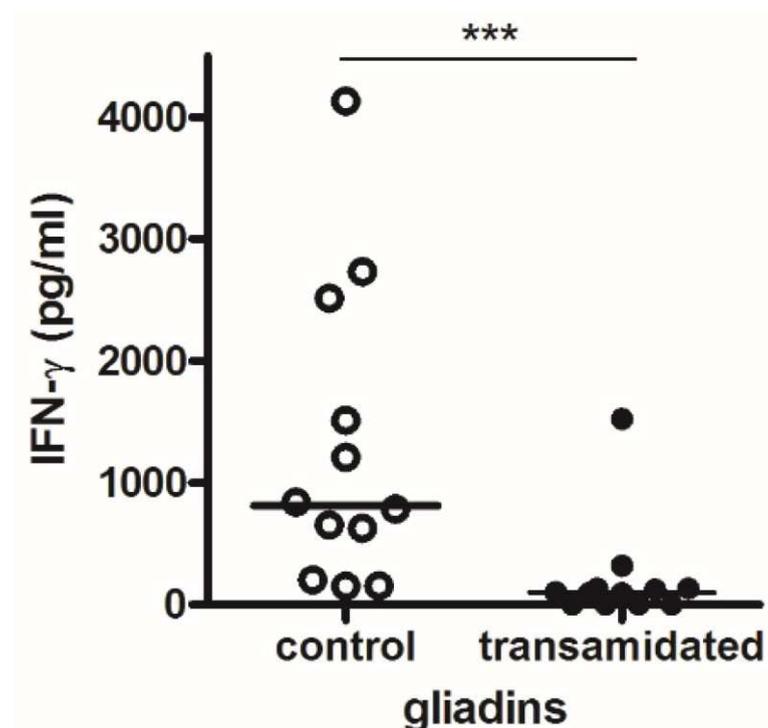


Figure 2. Antigen-specific IFN- γ expression assessed after culturing spleen cells from immunized mice for 72 h ($N = 12$). Each dot represents values (pg/mL) from a single mouse calculated as the difference between the means of triplicate cultures containing antigen and triplicate cultures with medium alone. Control is the cytokine response to native gliadin. *** $p < 0.001$.

The Rheological Properties of Transamidated Semolina

After enzyme processing, transamidated wet dough showed a hydration index of 50% with a protein content that decreased to 7.2% (d.m.). As shown in Figure 3, we found a complete loss of classical rheological parameters in the transamidated product. Nevertheless, by performing a

preventive drying treatment, which was needed to lower hydration to 30%, wet dough could be normally processed for pasta manufacturing.

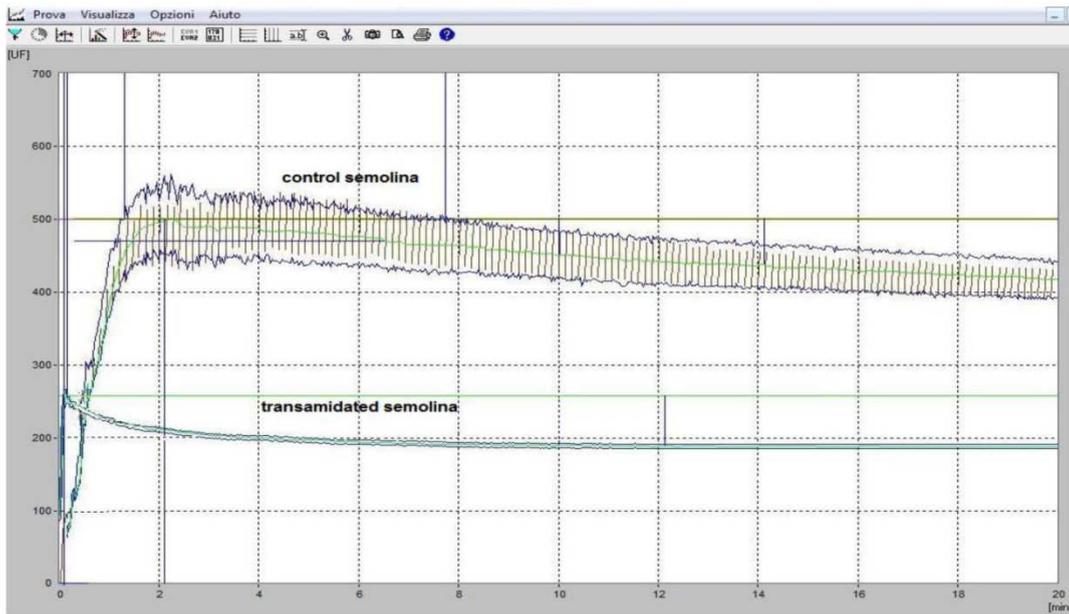


Figure 3. Farinograph profile of control and transamidated semolina.

The Technological Properties of Transamidated Semolina

After cooking, the water uptake in transamidated pasta was lower than in the control sample (200 g and 280 g wet weight, respectively, Table 1). Notably, the main cooking features were found unchanged in the spaghetti manufactured with transamidated semolina in comparison with commercial pasta (Table 1).

Parameters	Control Pasta	Experimental Pasta
Ø (mm) fresh pasta after extrusion	nd	1.6 (±0.0)
Ø (mm) raw dried pasta	1.3 (±0.0)	1.3 (±0.0)
cooking time until disappearance of the nucleus (min)	7	6
pasta weight after cooking (t 0') (g)	280 ± 0.6	200 ± 0.5
pasta weight after cooking (t 9') (g)	270 ± 0.3	193 ± 0.2
springiness	70 (±5)	70 (±3)
firmness	75 (±5)	75 (±5)
stickiness (t 9')	70 (±5)	70 (±5)
taste (t 9')	70 (±5)	90 (±3)
appearance (t 9')	70 (±5)	80 (±3)
flavor (t 9')	70 (±5)	75 (±5)

Table 1. Structural characteristics of pasta after cooking.

Discussion

This study showed that the pilot-scale two-step transamidation of wheat semolina was able to completely block the immune recognition of wheat gliadins *in vivo*. Importantly, this treatment did not hamper the main technological properties of gluten, as good quality dried pasta was produced. CD is an inflammatory autoimmune disease of the small intestine [1] that affects genetically susceptible individuals. A major hallmark of CD is inappropriate intestinal T cell activation triggered by peptides from wheat gliadins and glutenins, and by related prolamins from barley and rye. To date, several gliadin epitopes that elicit a T cell response have been identified, and most of these were recognized following tTG-mediated deamidation of specific glutamine residues [4,19]. Based on these findings, we previously reported that transamidation of gliadin by mTG suppressed the immune response of intestinal T cell lines from CD patients [13]. tTG and mTG have very different structures; furthermore, mTG has developed a calcium-independent catalytic mechanism [20]. However, we showed similar site-specificity for their catalytic activity [13]. It must be underlined that this detoxification strategy does not require a complete knowledge of all the toxic sequences present in gluten. This is an important clue considering that gluten is characterized by an intrinsic complexity, which contains hundreds of related proteins present either as monomers or linked by inter-chain disulphide bonds [15]. Hydrated gliadins are the proteins that primarily contribute to dough viscosity and extensibility. Hydrated glutenins are both cohesive and elastic and are responsible for dough strength and elasticity. In this study, we confirmed that the solubility of both gliadins and glutenins was drastically changed following mTG treatment of wheat semolina in the presence of $K-C_2H_5$: more than 94% gliadins and 88% glutenins, respectively, became soluble in water as a consequence of isopeptide bond formation. Most importantly, wheat semolina transamidation mediated by mTG modified the immunogenicity of gliadins *in vitro*, which is instrumental for generating celiacogenic epitopes. Furthermore, the transamidated forms were characterized by a complete loss of immune cross-reactivity toward anti-native gliadin antibodies [21]. In particular, R5-ELISA, a commercially available immunoassay used to detect residual gluten in GF foods, indicated that the transamidation of wheat flour in the presence of $K-C_2H_5$ causes effective epitope masking to occur in gliadin components [20]. In a first dietary intervention study, the reintroduction of gluten after a one-step treatment reduced the number of clinical relapses in challenged CD patients [22]. Nevertheless, the one-step reaction was not found sufficient in eradicating the gluten activity in all examined CD patients. Whether the herein-reported upgraded reaction is effective in fully blocking relapses in treated CD patients is currently under investigation. On the basis of the above reported data, dough recovered from the durum wheat semolina suspension after the two-step reaction contained most of the gluten that was transamidated. Nevertheless, we produced pasta with no changes in its technological and organoleptic parameters. It is known that the physical properties of a dough arise from interactions between gluten proteins, particularly the disulphide-bonded glutenin macropolymer [23]. Our data suggested that transamidated gluten can still hold most of these features by considering that disulphide bonds are not involved.

Conclusions

We demonstrated that the detoxification protocol of durum wheat semolina on a pilot scale, based on the use of food-grade mTG and lysine ethyl ester, largely produced transamidated water-soluble gliadins and glutenins; furthermore, it was selectively associated with a full reduction of the gliadin-specific immune response in wheat semolina. Notably, the enzyme treatment preserved the main technological properties of gluten. Our data may have important implications for the management of CD patients by shedding further light on an innovative enzymatic strategy as an alternative to the gluten-free diet.

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Author Contributions

M.R. conceived and designed the experiments; L.T., A.O. and S.M. performed the experiments; S.M. and M.R. analyzed the data; M.R. and S.M. wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CD	coeliac disease
GF	gluten free
K-C ₂ H ₅	lysine ethyl ester
PEPs	prolyl endopeptidases
mTG	microbial transglutaminase
tTG	tissue transglutaminase

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Chapter 4

“Immune-modulating effects in mouse dendritic cells of *Lactobacilli* and *Bifidobacteria* isolated from individuals following omnivorous, vegetarian and vegan diets”

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Abstract

Lactobacilli and *Bifidobacteria* play a primary role in modulation of gut immunity. By considering that microbiota composition depends on various factors, including diet, we asked whether functional differences could characterize faecal populations of *Lactobacilli* and *Bifidobacteria* isolated from individuals with different dietary habits. 155 healthy volunteers who followed omnivorous, ovo-lacto-vegetarian or vegan diets were recruited at four Italian centres (Turin, Parma, Bologna and Bari). Faecal samples were collected; *Lactobacilli* and *Bifidobacteria* were isolated on selective media and their immunomodulatory activity was tested in mouse dendritic cells (DCs). Pre-incubation with lactobacilli increased LPS-induced expression of the maturation markers CD80 and CD86, whereas pre-incubation with *Bifidobacteria* decreased such expression.

Analysis of the cytokine profile indicated that strains of both genera induced down-regulation of IL-12 and up-regulation of IL-10, whereas expression of TNF- α was not modulated. Notably, analysis of anti-inflammatory potential (IL-10/IL-12 ratio) showed that lactobacilli evoked a greater anti-inflammatory effect than did *Bifidobacteria* in the omnivorous group ($P < 0.05$). We also found significantly reduced anti-inflammatory potential in the bacterial strains isolated from Bari's volunteers in comparison with those from the cognate groups from the other centres. In conclusion, *Lactobacilli* and *Bifidobacteria* showed a genus-specific ability of modulating *in vitro* innate immunity associated with a specific dietary habit. Furthermore, the geographical area had a significant impact on the anti-inflammatory potential of some components of faecal microbiota.

Keywords: omnivorous diet, vegetarian diet, vegan diet, microbiota, immunity, dendritic cells.

Introduction

Several endogenous and environmental factors can impact both the composition and functionality of gut microbiota throughout life [1]. In particular, substantial evidence indicates that diet is instrumental in the structure and functionality of gut microbiota [2-6]. Epidemiologic evidence also suggests that modulation of immune response mechanisms in the gut can contribute to the development of gastrointestinal disorders and allergic diseases. In this context, *Lactobacilli* and *Bifidobacteria* are considered key players because they constitute important members of the normal intestinal microbiota in animals and humans, particularly *Bifidobacteria* in infants [7,8]. The current knowledge of mechanisms by which intestinal microbiota influences both regulatory and effector components of the immune system highlights the importance of enterocyte and dendritic cell (DC) interactions. DCs are considered to be gate-keepers of the immune system, and their contact with microbiota is crucial for proper development of gut immunity [9]. This contact is essentially achieved by interacting directly with bacteria that have gained access to lymph nodes and Peyer's patches via M cells. Furthermore, subepithelial DCs may sample the microbiota by passing their dendrites between epithelial cells into the gut lumen [10]. However, the precise mechanisms by which intestinal microbiota can influence the development and function of DCs remain to be further elucidated. Additionally, the composition of a healthy microbiota, which is required to confer immune protection, is not known, nor is the influence of dietary habits to improve the function of the microbiota. Herein, we addressed these issues by establishing whether *Lactobacilli* and *Bifidobacteria* species and strains arising from specific diets actually translate to the modulation of immune function. 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 [11, 12] to determine their role in modulating *in vitro* immune markers of mouse dendritic cell functions. Our data revealed genus-specific effects of selected bacteria on the maturation of DCs, as well as on inducible immune mediators produced by DCs *in vitro*, thus establishing a differential degree of modulation of the phenotype of DCs. Importantly, the latter parameter was found to be dependent on dietary habit.

Materials and Methods

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 for more than 1 year were recruited at 4 Italian centres (Bari, Bologna, Parma and Turin) [11-13] (<https://clinicaltrials.gov>; ClinicalTrials.gov Identifier: NCT02118857;MRMOVVD), as indicated in Table 1. The research was conducted according to the Declaration of Helsinki. Informed consent was obtained from all subjects. The compliance to the declared diet type was verified by means of a 7-day weighed food diary, completed every day for a total of 7 days [11]. Three faecal samples/volunteer (ca. 15 g) were collected for 3 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.

Table 1. List of mesophilic *Lactobacilli* and *Bifidobacteria* isolates from human faecal samples in this study

Recruitment Centre	Bacteria	Diet Regimen		
		Omnivorous (n*; code**)	Vegetarian (n*; code**)	Vegan (n*; code**)
Turin	<i>Lactobacilli</i>	15 (1-15)	12 (16-24; 26-28)	13 (30-42)
	<i>Bifidobacteria</i>	15 (1-15)	13 (16-23; 25-29)	11 (30-33; 35; 36; 38-42)
Parma	<i>Lactobacilli</i>	10 (43-52)	15 (53-67)	12 (68-79)
	<i>Bifidobacteria</i>	8 (45-52)	11 (53; 54; 56-60; 62; 65-67)	9 (68; 71; 73-79)
Bari	<i>Lactobacilli</i>	15 (80-103)	12 (104-107; 109-116)	10 (120-129)
	<i>Bifidobacteria</i>	15 (80-103)	11 (105-113; 115; 116)	12 (118-125; 127-129)
Bologna	<i>Lactobacilli</i>	15 (130-144)	14 (145-158)	12 (159-170)
	<i>Bifidobacteria</i>	2 (6; 139)	1 (146)	2 (161; 166)
Total	<i>Lactobacilli</i>	55	53	47
	<i>Bifidobacteria</i>	40	36	34

*: n, number of subjects.

** : code number, arbitrarily assigned to identify subjects.

*Isolation and growth of *Lactobacilli* and *Bifidobacteria**

10 g of faeces from each volunteer was homogenized 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 aliquots of each dilution were included into Rogosa Agar (Oxoid) with 21 mM acetic acid or spread onto Bifidobacterium Agar (Becton Dickinson, Milan, Italy) [12-14]. 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. For each faecal sample, 10 random colonies were picked from appropriate plate dilutions for analysis *in vitro* [13, 15, 16]. Cell morphology and cell motility of selected colonies were evaluated for genus confirmation [17].

Bifidobacteria were cultured in trypticase-phytone-yeast extract (TPY; Oxoid) at 37°C under anaerobic conditions and *Lactobacilli* in the Man, Rogosa and Sharpe medium (MRS; Oxoid) at 30°C under aerobic conditions; cells were collected during exponential growth phase. Randomly selected cultures were finally checked by PCR-DGGE and sequencing of amplicons [13]. Cell concentration was evaluated by measuring optical density at 600 nm and converting this value to the corresponding CFU ml⁻¹ value. Bacteria were irradiated with 2800 Gy (Gray) of γ -irradiation by Gamma-Cell 1000 (MDS Nordion, Canada) to prevent their proliferation before being used as stimuli for DCs.

Mice

BALB/c mice were maintained under pathogen-free conditions at the animal facility of the Institute of Food Sciences. Mice were used at 6-12 weeks of age and were euthanized by inhalation of anaesthesia with isoflurane. These studies were approved by the National Institutional Review Committee.

Isolation of bone marrow-derived dendritic cells

Murine DCs were generated according to a previously published method [18]. In brief, bone marrow cells from the femurs and tibiae of mice were flushed, and bone marrow cell aliquots (2x10⁶) were diluted in 10 ml of RPMI 1640 medium supplemented with 25 mM HEPES, antibiotics (penicillin 100 IU ml⁻¹, streptomycin 100 IU ml⁻¹), 10% foetal calf serum and 20 ng ml⁻¹ granulocyte-macrophage colony-stimulating factor (GM-CSF) (culture medium) before being seeded in 100-mm petri dishes (Falcon, Heidelberg, Germany). On day 3, 10 ml of culture medium was added and on day 7, 10 ml of the culture medium was replaced with freshly prepared medium. On day 9, non-adherent DCs were harvested by gentle pipetting. Cell aliquots (1x 10⁶ ml⁻¹) were then placed in 24-well plates and incubated in culture medium with 5 ng ml⁻¹ GM-CSF. Cell viability was microscopically evaluated by dye-exclusion test using Nigrosin (1% solution) and >90% live cells were found in all experiments.

Microbial challenge

DCs (1x 10⁶ ml⁻¹) were incubated for 24 h with irradiated bacteria resuspended in complete RPMI medium at a 30:1 bacteria (CFU):DC (n) ratio. Following incubation, cells were treated with 1 μ g ml⁻¹ LPS for 6 h (LPS pulse) to induce the maturation of DCs and cultured for 0-24 h in complete RPMI medium. Cells were collected for RNA analysis. Spent media was 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 24 h of bacterial incubation.

FACS analysis

DCs were stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated Abs (BioLegend, San Diego, CA, USA) against CD11b, CD11c, CD80 and CD86. Cell staining was analysed using a CyFlow Space flow cytometer (Partec, Munster, Germany) and FlowJo software (Tree Star Inc., Ashland, OR, USA). For each Ab, an isotype control of the appropriate subclass was used.

Analysis of cytokine production

Total RNA was extracted from DCs using the TRIzol Reagent (Life Technologies Italia, MonzaMB, 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. cDNA was prepared from 1 μ g 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 μ l, containing iQTM SYBR Green Supermix (Bio-Rad), 0.2 μ M of each primer and cDNA. The reaction conditions for 39 cycles were 95°C for 30 s, 54.6°C (L-32), 60°C (IL-12, TNF- α), or 66.0°C (IL-10) for 30 s, and 72°C for 40 s. The relative gene expression levels of TNF- α , IL-12p40 and IL-10 were calculated using the $\Delta\Delta$ CT method and presented as fold change in gene expression after normalization to the L-32 housekeeping gene. The following primer sequences were designed and used in this study: L-32, forward 5'-AGCAGAGCTGGAGTCGCTTT-3', reverse 5'-GGAGCTGCCATCCAAAAGATACTA-3'; TNF- α , forward 5'-CATCTTCTCAA AATTCGAGTGACAA-3', reverse 5'-TGGGAGTAGACAAGGTACAACCC-3'; IL-12p40, forward 5'-GGAAGCACGGCAGCAGAATA-3', reverse 5'-AACTTGAGGGAGAAGTAGGAATGG-3'; IL-10, forward 5'-GACAATAACTGCACCCACTTC-3', reverse 5'-AGCTGGTCCTTTGTTTGAAGAAA-3'.

Supernatants from DC cultures were analysed for IL-12, TNF- α and IL-10 protein levels by sandwich-type ELISA. First, 100 μ l of capture antibody solution (BioLegend) 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 μ l of PBS supplemented with 2% 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 overnight at 4°C. After incubation, three washing steps with PBS supplemented with 0.05% Tween-20 were performed, and 100 μ l of biotinylated antibody solution was added to the wells and incubated for 2 h at room temperature. After three washes, streptavidin-horseradish peroxidase conjugate (1:2000 dilution; BioLegend) was added to the wells and incubated for 1 h at room temperature. Finally, after washing, 100 μ l of 63 mM Na₂HPO₄, 29 mM citric acid (pH 6.0) containing 0.66 mg ml⁻¹ 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⁻¹.

Statistical analysis

Statistical significance 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.

Results

Modulation of maturation markers by diet-selected Lactobacilli and Bifidobacteria

To preliminarily examine the effects of bacterial pre-incubation on DC maturation, *Lactobacilli* and *Bifidobacteria* isolated from individuals undergoing different diet regimens were evaluated (*Lactobacilli* from subjects n. 3, 18 and 42; *Bifidobacteria* from subjects n. 14, 21 and 39 of Table 1). DCs were mainly CD11b-CD11c+ (data not shown) with low expression of the costimulatory molecules CD80 and CD86 (Fig. 1, iDCs). Incubation with the TLR4 ligand LPS for 6 hours resulted in increased expression of CD80 and CD86 surface maturation markers, although to different degrees in each independent experiment, as a consequence of heterogeneity in the iDC population (Fig. 1, mDCs). Pre-incubation with *Lactobacilli* further increased the number of CD80+CD86+ cells (Fig. 1, subjects n. 3, 18 and 42). On the contrary, pre-treatment with all three *Bifidobacteria* isolates caused a reduction in LPS-inducible expression of CD80 and CD86 (Fig. 1, subjects n. 14, 21 and 39). Next, we analysed the whole bacteria collection by expressing the number of double positive cells in terms of % of the positive control (mDCs).

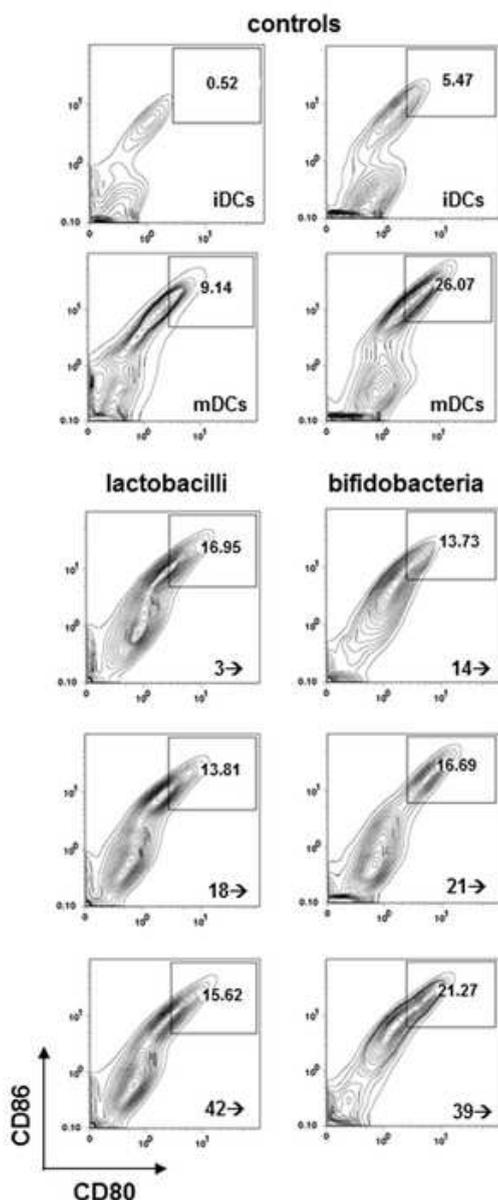


Figure 1. FACS analysis of DCs from BALB/c mice. Immature DCs (iDCs) were challenged with irradiated *Lactobacilli* (from subjects n. 3, 18 and 42 of Table 1) or *Bifidobacteria* (from subjects n. 14, 21 and 39 of Table 1) and then subjected to a 6-h LPS pulse to induce DC maturation (mDCs).

Both iDCs and treated or untreated mDCs were double stained for CD80 and CD86. Data were collected from ungated cells and are representative of three independent experiments.

The results shown in Fig. 2 indicated that the response varied widely both for *Lactobacilli* (Fig. 2a) and *Bifidobacteria* (Fig. 2b). Nevertheless, it was statistically confirmed that pre-treatment with *Lactobacilli* mainly increased the LPS-induced expression of maturation markers, whereas pre-incubation with *Bifidobacteria* decreased such expression. In addition, we reported that when increases of CD80 and CD86 were induced by *Bifidobacteria* isolates, they never reached the higher levels generated by *Lactobacilli* (Fig. 2a, b).

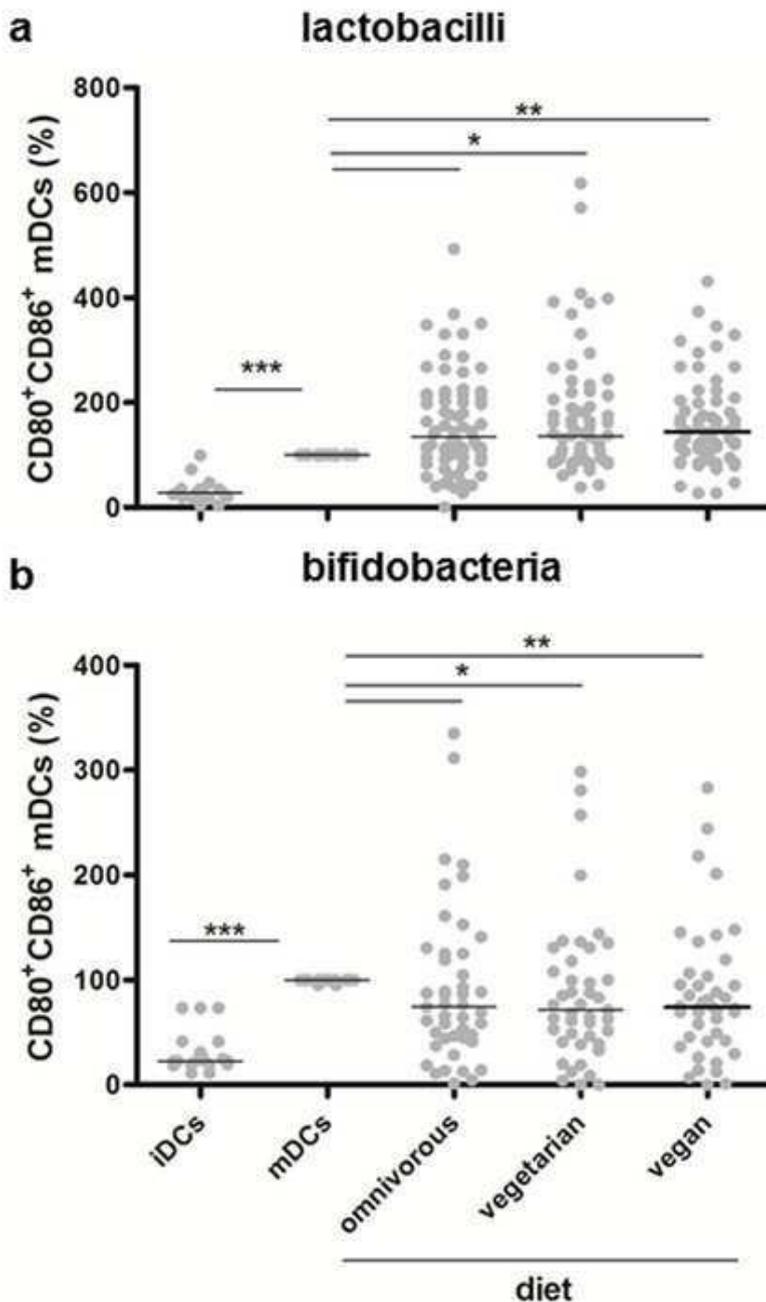


Figure 2. Expression of the co-stimulatory markers CD80 and CD86 on the surface of mDCs. mDCs were pre-treated with irradiated *Lactobacilli* (a) or *Bifidobacteria* (b) isolates from adults who followed an omnivorous (n= 55, 40; a, b), ovo-lacto-vegetarian (n. 53, 36; a, b) or vegan (n. 47, 34; a, b) diet. Data were collected from ungated cells, and bars represent medians. *, $P < 0.05$; **, $P < 0.01$. Importantly, the examined diet regimens were not translated into changes in modulatory activity of either *Lactobacilli* or *Bifidobacteria*. Furthermore, no differences were found by examining these data on the basis of their geographical origin (data not shown).

Setup of the cytokine analysis in vitro

Real-time PCR reactions were performed under gene-specific conditions to amplify IL-12p40, IL-10 and TNF- α cDNAs, with L-32 as a housekeeping gene. Kinetic analysis of cytokine transcription induced by a LPS pulse in DCs showed that in this model, the examined transcripts promptly peaked at the end of the pulse (IL-12 and TNF- α) or 1 h later (IL-10); then, they were

rapidly down-regulated following removal of the mitogen (Fig. 3a). Therefore, cytokine mRNA levels were definitively assessed 1 h after the LPS pulse and compared to cognate protein levels, which were analysed in the supernatants of parallel cultures 24 h later. To preliminarily estimate the effects of bacterial pre-incubation of iDCs on cytokine expression, we tested *Lactobacilli* isolates collected for three consecutive weeks from subjects n. 3, 18 and 42, who were subjected to different diet regimens. IL-12 mRNA levels were differentially regulated by the bacterial samples, but the final outcome was suppression of protein expression in all cases (Fig. 3b, upper panel), suggesting stringent post-transcriptional control of this cytokine by *Lactobacilli*. On the contrary, IL-10 was significantly up-regulated by all samples at the transcriptional level (Fig. 3b, middle panel). TNF- α mRNA was significantly down-regulated even when basal expression of the protein was generally unaffected by *Lactobacilli* challenge (Fig. 3b, bottom panel). By comparing the samples collected in different weeks, we could detect quantitative differences in regulation only for IL-10.

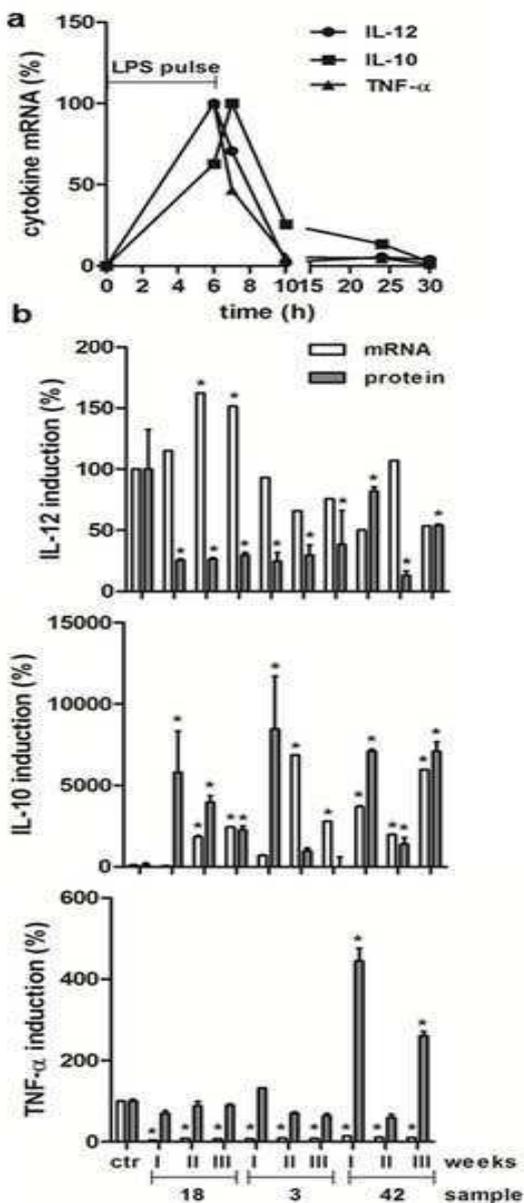


Figure 3. Setup of cytokine expression by DCs. a) Kinetic analysis of IL-12, IL-10 and TNF- α transcription in iDCs in response to LPS pulse; values were calculated as fold change in gene expression and expressed as % of maximum induction. b) Comparison between mRNA and cognate protein levels for IL-12 (upper panel), IL-10 (middle panel) and TNF- α (bottom panel) of DCs pretreated with *Lactobacilli* from subjects n. 3, 18 and 24 collected for three weeks. Cells and culture supernatants were collected as indicated in the Materials and Methods section and analysed for cytokine mRNAs and secreted protein expression by using real-time PCR and sandwich-type ELISA, respectively; values are expressed as % of control (unchallenged mDCs); columns represent the mean \pm SD and are representative of three independent experiments. *, $P < 0.05$.

Modulation of the cytokine profile by diet-selected *Lactobacilli* and *Bifidobacteria*

On the basis of the setting up analysis, bacterial collections were screened by determining cytokine protein levels, and data were expressed as % of the positive control (mDCs). Then, results from each independent experiment were pooled and reported in Fig. 4 and Fig. 5. It was confirmed that maturation of iDCs caused significantly increased expression of IL-12 (Fig. 4a, b) and TNF- α (Fig. 5a, b). On the contrary, analysis of regulatory IL-10 in a larger sample size indicated that an increase in cytokine protein levels was not consistently obtained by LPS pulse (Fig. 4c, d), reflecting a higher sensitivity of this cytokine to cell heterogeneity of iDCs in different experiments.

Pre-incubation with both *Lactobacilli* and *Bifidobacteria* collections generated a wide range of values in the cytokine response. Nevertheless, we found that both genera induced a statistically relevant down regulation of IL-12 expression that resulted independently of the dietary regimen (Fig. 4a, b).

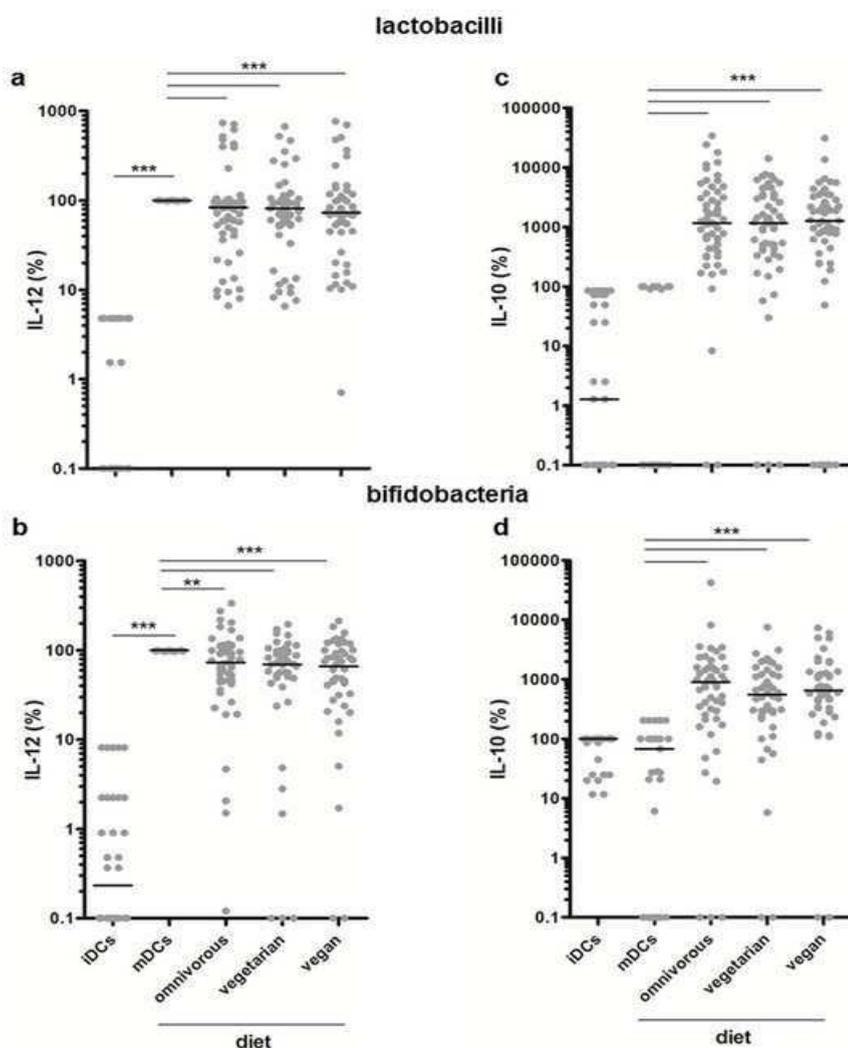


Figure 4. Protein expression of IL-12 and IL-10 by mDCs. mDCs were pre-treated with irradiated *Lactobacilli* (a, c) or *Bifidobacteria* (b, d) isolates from adults who followed an omnivorous (n. 55,40; a and c, b and d), ovo-lacto-vegetarian (n. 53, 36; a and c, b and d) or vegan (n. 47, 34; a and c, b and d) diet before LPS pulse. Culture supernatants were collected

after 24 h and analysed for IL-12 (a, b) and IL-10 (c, d) expression by sandwich-type ELISA. Data were expressed as % of control (unchallenged mDCs); bars represent medians. **, $P < 0.01$; ***, $P < 0.001$.

On the other hand, pre-incubation with both *Lactobacilli* and *Bifidobacteria* isolates significantly upregulated IL-10 expression, once again independently of diet (Fig. 4c, d). Yet, expression of the pleiotropic cytokine TNF- α was not significantly modulated by pre-incubation with *Lactobacilli* (Fig. 5a) or *Bifidobacteria* (Fig. 5b).

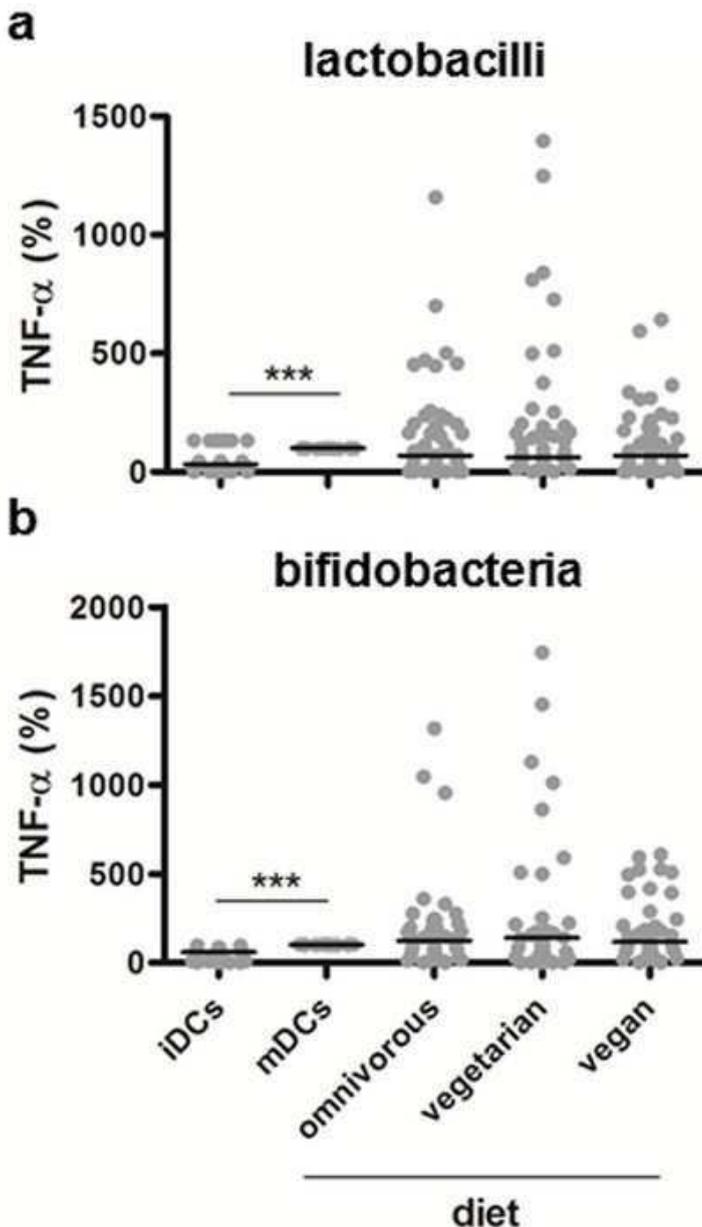


Figure 5. Protein expression of TNF- α by mDCs. mDCs were pre-treated with irradiated *Lactobacilli* (a) or *Bifidobacteria* (b) isolates from adults who followed an omnivorous (n= 55,40; a, b), ovo-lacto-vegetarian (n=53,36; a, b) or vegan (n=47,34; a, b) diet before LPS pulse. Culture supernatants were collected after 24 h and analysed by sandwich-type ELISA. Data were expressed as % of control (unchallenged mDCs); bars represent medians. ***, $P < 0.001$.

We have shown that, although statistically relevant, both the IL-12 and IL-10 responses varied widely; therefore, to more stringently analyse the impact of dietary regimen, we calculated anti-inflammatory

potential in terms of the IL-10/IL-12 ratio from each microbial sample. The results reported in Fig. 6 indicated that most of the examined samples had a ratio >1, thus confirming the overall anti-inflammatory activity for both *Lactobacilli* and *Bifidobacteria*. Importantly, a statistically significant higher anti-inflammatory potential was observed for *Lactobacilli* (median >10) in comparison with *Bifidobacteria* in the omnivorous group.

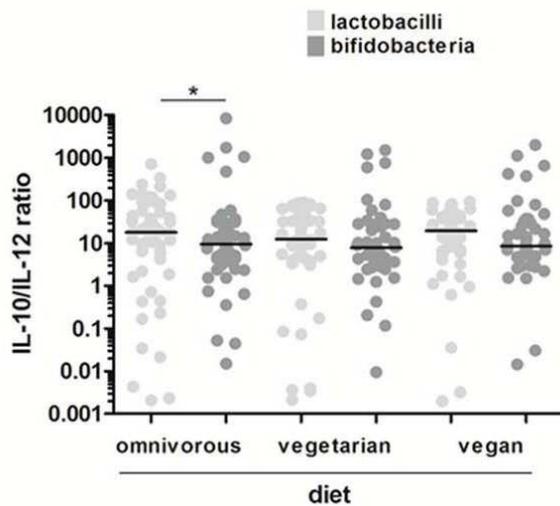


Figure 6. Anti-inflammatory potential of diet-selected *Lactobacilli* and *Bifidobacteria*. Cytokine values reported in Fig. 4 were expressed as IL-10/IL-12 ratios for each *Lactobacilli* and *Bifidobacteria* population from adults who followed an omnivorous, ovo-lacto-vegetarian or vegan diet. Bars represent medians. *, $P < 0.05$.

Interestingly, by further examining these data on the basis of geographical origin, we found reduced IL-10/IL-12 ratios in the isolates collected from Bari in comparison with the cognate groups from all other centres (Fig. 7a, b); specifically, the Dunn's post-hoc test analysis revealed significant differences between isolates from the vegan group recruited in Turin and those from the vegetarian group in Bari among *Lactobacilli* (Fig. 7a) and between isolates from omnivores recruited in Turin and Bari among *Bifidobacteria* (Fig. 7b)

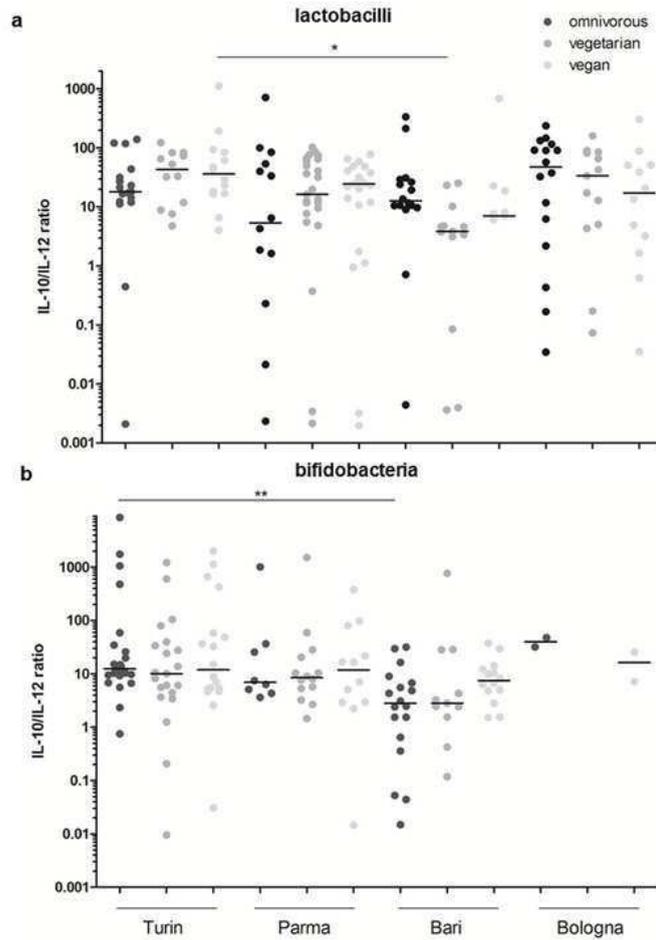


Figure 7. Anti-inflammatory potential of *Lactobacilli* and *Bifidobacteria* analysed across diet and geography. Cytokine values reported in Fig. 4 were expressed as IL-10/IL-12 ratios for *Lactobacilli* and *Bifidobacteria* populations from adults recruited at four Italian centres who followed an omnivorous, ovo-lacto-vegetarian or vegan diet (see Table 1). Bars represent medians. *, $P <$

Discussion

In this study, we found a genus-specific ability to modulate *in vitro* mechanisms of innate immunity by faecal populations of *Lactobacilli* and *Bifidobacteria* that was also influenced by the dietary habit. Gut microbiota shapes host immunity by inducing maturation of the gastrointestinal lymphoid tissue. In addition, other mediators, such as polysaccharide A and short-chain fatty acids (SCFAs), have been shown to stimulate Treg-cell differentiation. In particular, SCFAs can induce IL-10 and retinoic acid from DCs, which play a major role in mucosal tolerance [19]. Interestingly, a significant association between consumption of agrarian-based diets and increased levels of faecal SCFAs has been previously found in the individuals herein examined [11]. Furthermore, the culturable lactic acid bacteria (LAB) loads were found to be lower in the vegan and ovo-lactovegetarian groups [12]. On the contrary, no significant difference between the ovo-lacto-vegetarian and omnivorous groups was observed in relation to the culturable *Bifidobacteria* load, although such load was significantly lower in the vegan group [12]. It is well known that the composition and density of microbiota fluctuates with age and can also be influenced by various factors, including diet. Nevertheless, by two years of age, the gut microbiota is essentially dominated by members belonging to the Firmicutes and Bacteroidetes phyla and begins to resemble an adult microbiota [20]. Among the factors, diet clearly influences the developing gut microbiota: after the introduction of solid food, the diversification of *Bacteroides* spp. and *Clostridium* spp. rapidly increases, whereas the proportion of *Bifidobacterium* spp. stabilizes [20]. *Lactobacilli* numbers found in stool samples seem to be more specifically related to the ingestion of fermented foods. Importantly, strong evidence suggests a primary role of both *Lactobacilli* and *Bifidobacteria* in modulation of immune mechanisms in the gut. Therefore, we addressed this issue by evaluating whether, beyond differences in numbers and species [12], genus-specific functional differences could characterize these bacteria isolated from the screened groups. For each faecal sample, 10 random colonies were picked for analysis from adequate plate dilutions. It has been reported that colonies provide an adequate representation of the major bacterial strains cultured on a selective medium [15, 16]. We found that immunological outcomes widely varied within each group, suggesting that no bias was introduced by the strategy of bacteria isolation that we adopted. Nevertheless, due to the large number of individuals included in the examined groups, most data were found to be statistically significant. Specifically, we showed that bacteria belonging to the *Lactobacillus* genus further increased the number of CD80+CD86+ mDCs, whereas this number was decreased by *Bifidobacteria*. To the best of our knowledge, this is the first work showing that the two genera may differentially alter the level of DC activation. Both CD80 and CD86 bind to CD28 and, with a lower affinity, to its homolog CTLA-4 (cytotoxic T-lymphocyte antigen 4) on T cells. CD28-CD80/86 ligation enhances T-cell proliferation, whereas CTLA-4-CD80/86 interaction inhibits T-cell responses [21]. Consequently, we speculated that the reduced expression of CD80 and CD86 mediated by most of the screened *Bifidobacteria* favours the latter interaction, and this could play a role in maintaining immune tolerance through interaction with CTLA-4. Clearly, the

increased number of CD80+CD86+ mDCs generated by *Lactobacilli* tend to preclude a crucial role for CTLA-4 in lactobacilli-mediated immune homeostasis in the gut. Notably, the evaluation of anti-inflammatory potential, expressed in terms of the IL-10/IL-12 ratio generated from each sample, showed higher values for *Lactobacilli* than for *Bifidobacteria* in the omnivorous group.

Taken together, they are suggestive of genus-specific mechanisms involved in DC education. A possible outcome of this differential activity could be the recruitment of different regulatory cells.

Interestingly, oral administration of *B. breve*, but not *L. casei*, in mice has recently been found to induce IL-10-producing CD4+ T cells that possessed properties of Tr1 cells [22], suggesting that a specific *Bifidobacterium* strain could maintain intestinal homeostasis through the induction of intestinal Tr1 cells, differently from *Lactobacilli*. Further studies are then required to determine if this property could be considered genus-specific.

It is known that long-term dietary intake influences the structure and functionality of gut microbiota in humans [23]. In the populations examined herein, in agreement with differences in counts for LAB and *Bifidobacteria* [12], the long-term dietary regimens did generate differences in characterized immune features in the studied isolates. Furthermore, we found reduced anti-inflammatory potential in the isolates collected in Bari in comparison with those from the groups at other centres. This finding was well in line with our previous results obtained in Caco-2 cells: *Lactobacilli* collected from the vegetarian group recruited in Bari induced a significant increase in IL-8 expression [13]. By considering sample homogeneity for age, BMI and sex ratio at the different recruitment centres [11], genetic or unselected environmental factors could still influence how and from where a gut microbiota/microbiome is acquired.

In conclusion, by examining the faecal *Lactobacilli* and *Bifidobacteria* isolates in groups with large sample sizes, we highlighted for the first time the existence of genus-specific differences in their immune-modulatory activity on DCs. In agreement with diet-related variations in plate counts of culturable LAB and *Bifidobacteria*, we also found an association between crucial immune features of bacterial populations and a specific dietary habit. Finally, the geographical area had a significant impact on the anti-inflammatory potential of members of these bacterial genera.

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Conflict of interest

The authors have no financial or personal conflicts of interest.

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Chapter 5

“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.

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] and [3]. Changes in diet, use of antibiotics and intestinal colonisation by helminths can modify intestinal microbial communities [4] and [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 Caco2 cells.

Materials and methods

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] and [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.

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).

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] and [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.

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.

***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% CO₂. 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).

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. Cells were collected for RNA, NF- κ B and RedOx status analyses.

Spent media were centrifuged at 10,000g 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.

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 μg 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- β), or 62.0°C (TSLP) for 30 s, and 72°C for 40 s. The relative gene expression levels of TGF- β , IL-8, TSLP and IL-10 were calculated using the $\Delta\Delta\text{CT}$ 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- β 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 Maxisorp; 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 and incubated for 1 h at room temperature. Finally, after washing, 100 μl of 63 mM Na_2HPO_4 , 29 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.

Analysis of NF- κ B and redox status

Cytoplasmic and nuclear protein fractions were prepared from Caco-2 cells and used for phospho-NF- κ B and NF- κ B immunodetection, respectively [14]; phospho-NF- κ B p65 (Ser536) (93H1) rabbit monoclonal antibody (#3033) and NF- κ B 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 to the previously published protocols [15]. Glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GSR) activities were spectrophotometrically evaluated in cytoplasmic extracts and their activities, upon normalisation to protein content, were expressed as IU or as nmoles NADPH mg⁻¹min⁻¹[16] and [17].

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).

Results

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 were then calculated and are 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 amplicons. PCR-DGGE 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.

Table 2.

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

Media	Vegetarian (mean \pm SD)	Vegan (mean \pm SD)	Omnivorous (mean \pm SD)
Rogosa agar 30 °C	4.62 \pm 1.41 ^b	4.10 \pm 1.73 ^a	4.72 \pm 1.69 ^b
Bifidobacterium agar modified	8.76 \pm 1.07 ^b	8.38 \pm 1.33 ^a	8.73 \pm 1.13 ^b

Differing superscript letters indicate statistically significant differences ($P < 0.05$).

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- β , 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 6 h, 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- β 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).

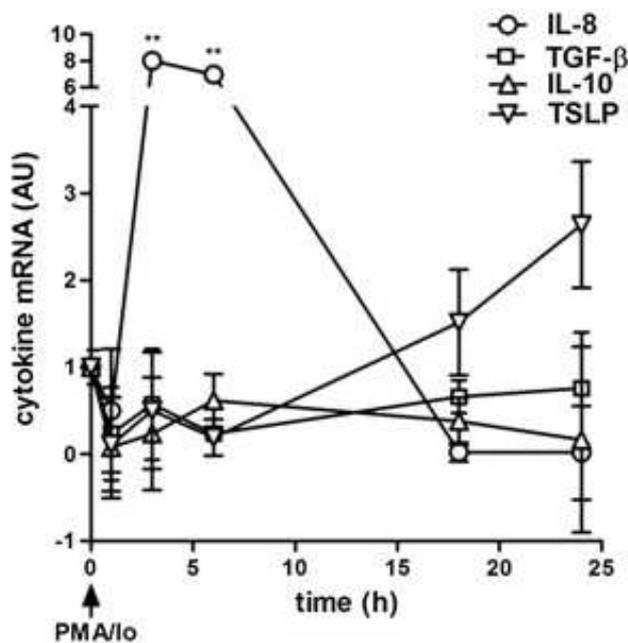


Fig. 1. Setup of cytokine transcription by Caco-2 cells. Kinetic analysis of IL-8, IL-10, TGF- β and TSLP transcription in Caco-2 cells in response to PMA/Io stimulation; values were calculated as fold change in gene expression (absolute units, AU). Cells were collected at 0, 3, 6, 17 and 24 h after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

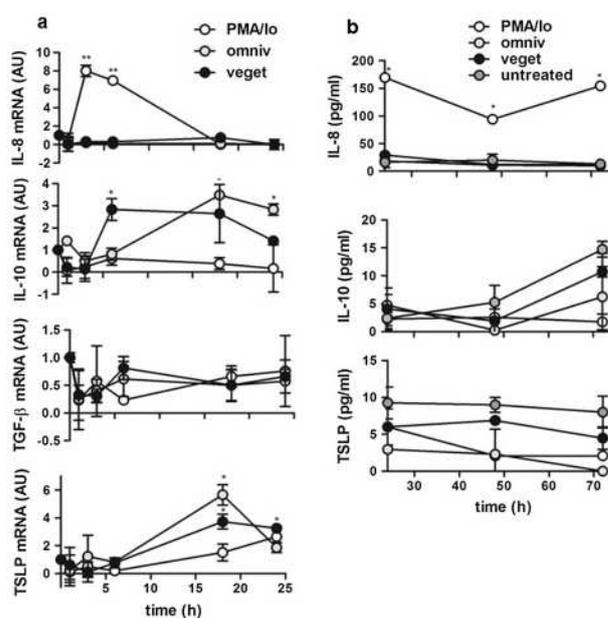


Fig. 2. Comparison between mRNA (a) and cognate protein levels (b) for IL-8, IL-10, TGF- β and TSLP of Caco-2 cells pre-treated with PMA/Io or *Lactobacilli* from two randomly selected *Lactobacilli* populations from a vegetarian (veget) and an omnivorous subject (omniv) collected in Turin and Bari, 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 \pm SD and are representative of three independent experiments. **, $P < 0.01$.

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).

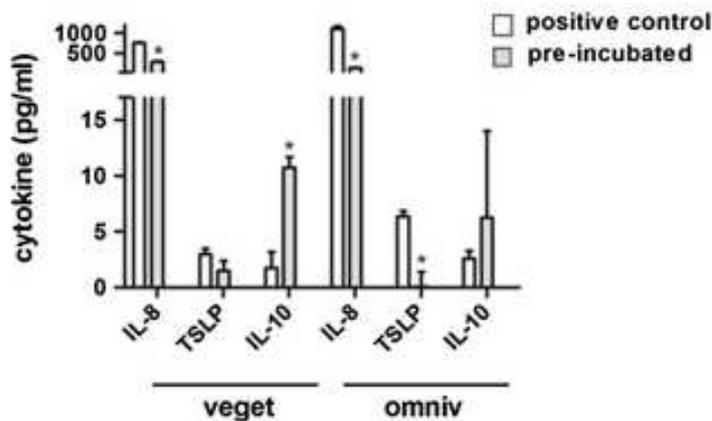


Fig. 3. Protein levels for IL-8, IL-10, TGF- β and TSLP secreted by Caco-2 cells pre-treated with *Lactobacilli* from test samples for 72 h and then pulsed for 24 h with PMA/Io. Culture supernatants were collected and analysed by using sandwich-type ELISA. Columns represent the mean \pm SD and are representative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

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 are reported in Fig. 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 and b). Furthermore, analysis of regulatory TSLP in a larger sample size confirmed that this cytokine was not modulated by PMA/Io (Fig. 4c and 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 and 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 and b) and TSLP responses (Fig. 5c and 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.

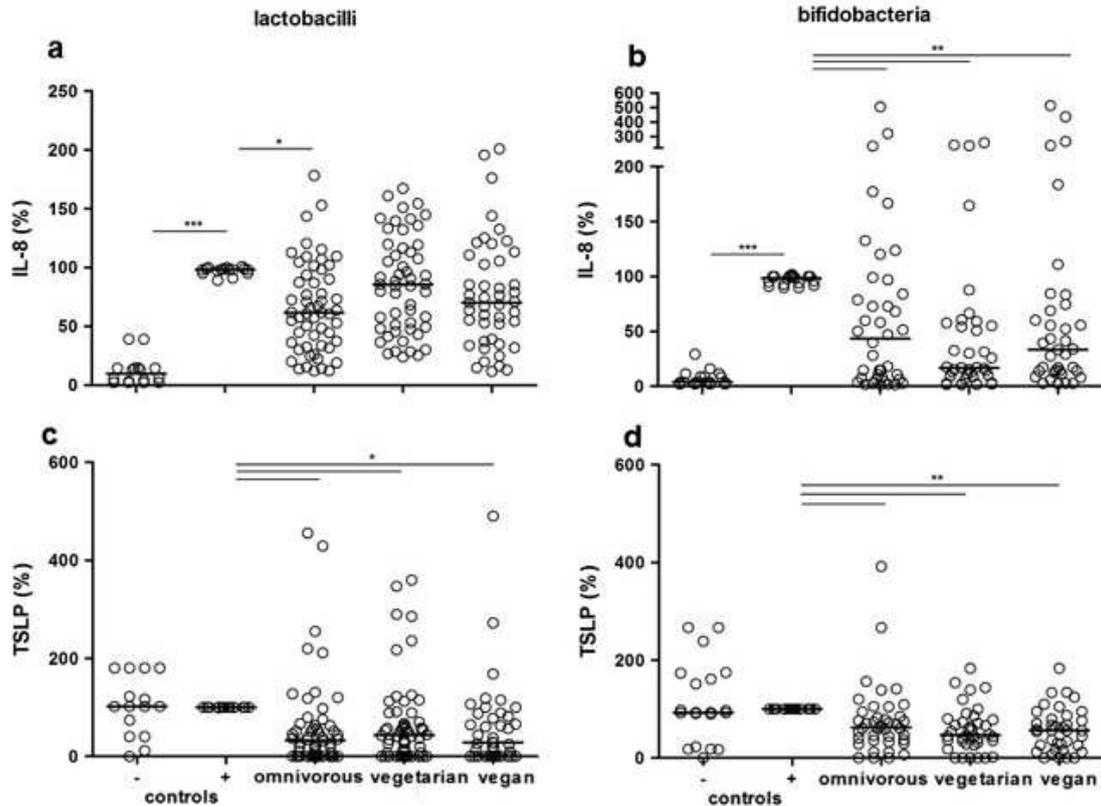


Fig. 4. Protein expression of IL-8 and TSLP by stimulated Caco-2 cells. Caco-2 cells were pre-treated with irradiated *Lactobacilli* (a, c) or *Bifidobacteria* (b, d) isolates from adults who followed an omnivorous, ovo-lacto-vegetarian or vegan diet (Table 1) before PMA/Io stimulation. Culture supernatants 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

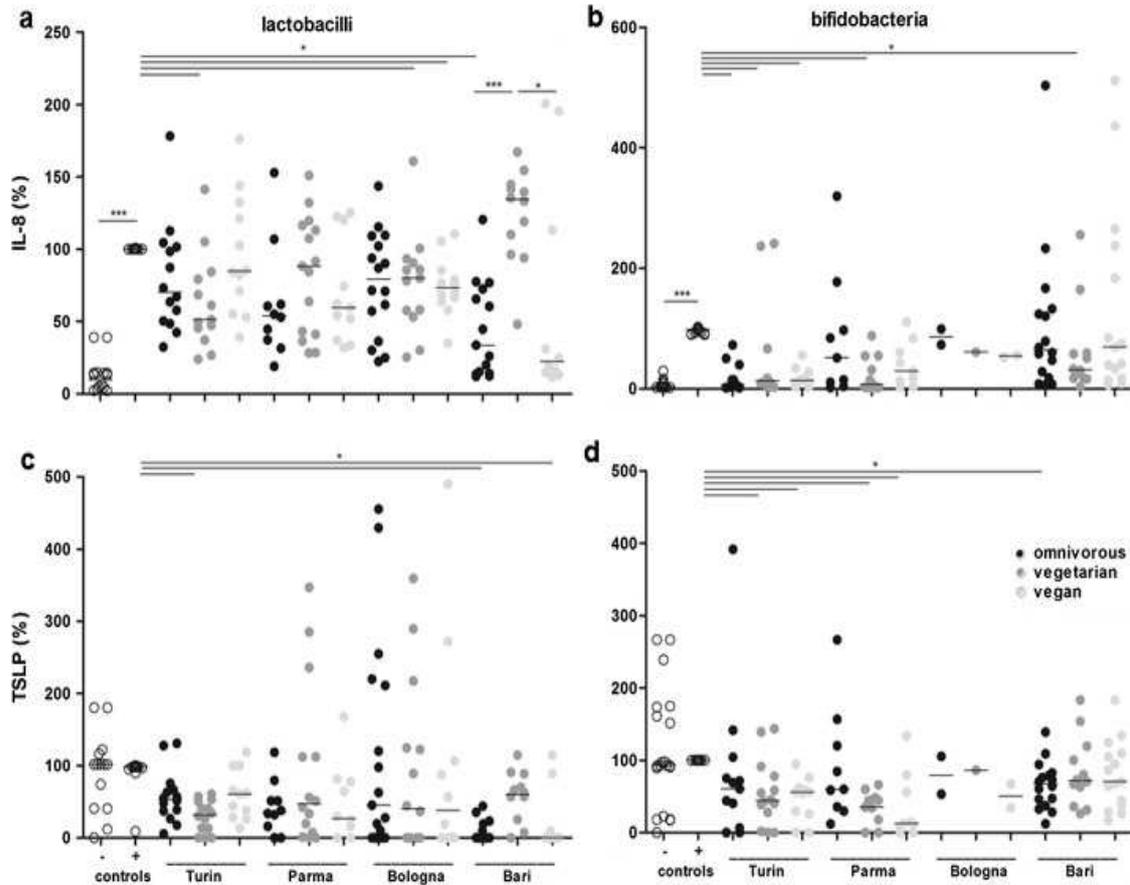


Fig. 5. Immunomodulatory potential of *Lactobacilli* and *Bifidobacteria* analysed across diet and geography. Cytokine values were reported for *Lactobacilli* and *Bifidobacteria* populations from adults recruited at four Italian centres who followed an omnivorous, ovo-lacto-vegetarian or vegan diet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

Mechanisms of IL-8 down-regulation

It is known that the activation of NF- κ B 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 pro-inflammatory effect associated with PMA/Io exposure of Caco-2 cells was indicated by a slight increase in nuclear phospho-NF- κ B levels (Fig. 6A). Interestingly, the examined *Lactobacilli* were found to be 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 in intracellular pool of oxidized glutathione (GSSG). As shown in Fig. 6B, the significantly higher GSH and GSSG yields,

measured in Caco2 cells pre-treated 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).

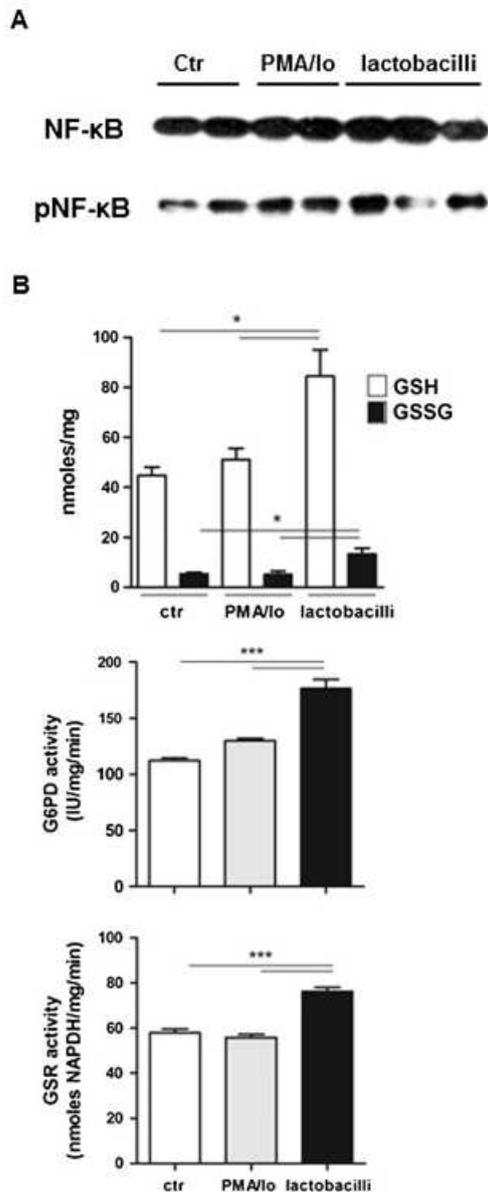


Fig. 6. Mechanisms underlying the immunomodulatory activity of *Lactobacilli*. A. Western blot analysis of cytoplasmic (upper panel) and nuclear extracts (lower panel) for NFkB and phospho-NFkB, respectively. B. Intracellular GSH and GSSG concentrations to evaluate cellular RedOx status (upper panel); 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 \pm SD from triplicate analysis on three different cultures. ctr, control (untreated Caco-2 cells); *, $P < 0.05$.

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 pro-inflammatory 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] and [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] and [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- β , IL-10 and TSLP. Caco-2 cells were stimulated with a combination of phorbol myristate acetate (PMA) and ionomycin (Io). Ionomycin causes disruption of Ca²⁺ 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 κ B (NF- κ B) pathway [18] and [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, enterocyte-derived 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 in [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 pre-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- κ B was not involved in the herein reported down-regulation 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 conflict of interests.

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Conclusions

Main findings of this Doctoral thesis, previously described in five chapters, respond to the common objective of progressing in the understanding of the role of GRAS bacteria and their metabolites in modulating the immune system.

In the first chapter we analyzed two different media to develop a new methodology for metabolite isolation by studying three different strains of *Lactobacillus paracasei*. We adopted the new approach of growing bacterial strains in media generally used for the culture of DCs, RPMI and X-VIVO 15. Previous studies (Zagato et al., 2014) demonstrated that a commonly used medium for the growth of *lactobacilli* (i.e. MRS) has by itself an effect on cytokine production of DCs. Therefore, in this study we found that RPMI1640 and the serum-free X-Vivo 15 were suitable for bacterial growth. Importantly, these data strongly suggest the existence of soluble metabolites that were functionally active (immunomodulatory) only in a serum-free medium. Particularly, the addition of artichoke phenolic extract (APE) to the RPMI 1640 medium of strain IMPC 2.1 revealed an interesting property: the increasing production of the anti-inflammatory cytokine IL-10 associated with a decreasing secretion of the pro-inflammatory IL-12. The probiotic strain *Lactobacillus paracasei* IMPC 2.1 has been used for the development of innovative and patented functional vegetable products (Lavermicocca et al, 2009). Studies demonstrated that IMPC 2.1 is able to reach in adequate amounts the gut when delivered by vegetable matrices such as artichokes and table olives (Lavermicocca et al, 2005, Riezzo et al, 2012, Valerio et al, 2010 and Valerio et al, 2011). So we may suppose that this anti-inflammatory effect is not APE-specific but is due to the interaction between APE and the strain metabolism. Consequently, this primary study paves the way for developing functional food manufactured with bacterial metabolites.

The possible role of a microbial culture filtrate in modulating adverse immune responses was further analysed in Chapters 2 and 3 by focusing on Coeliac disease (CD). To date, the only available treatment for CD is a long-life gluten free diet. In recent years, an innovative strategy to detoxify wheat flour was developed at the CNR (Mazzeo MF, et al 2013). This strategy encompasses an enzymatic treatment of wheat flour/semolina with a microbial transglutaminase (mTG) secreted by *Streptomyces mobarensis*, that was found effective in masking the toxic sequences of gluten, thus blocking the inflammatory response to this food molecule in CD patients. Herein, we set up an inexpensive treatment by employing culture supernatant of *S. mobaraensis*, without any substantial purification step. In particular, we found a food-grade medium that still gave a good production of mTG. Also, the set-up of growth condition on a 16L fermenter showed a not previously reported result: a higher production of mTG was obtained in anaerobic conditions, when *S. mobaraensis* is in a secondary development phase (MII). However, MII differentiation is not sufficient to guarantee secondary metabolite production, as it can also be regulated by environmental signals, including components of the culture medium, such as nitrogen (Aharonowitz, 1980), carbon (Sánchez et al., 2010) and phosphate (Chouayekh & Virolle, 2002; Martín, 2004). Furthermore, two proteases are involved in the processing of protransglutaminase into the mature active enzyme (Zotzel et al. 2003a, b). The presence

of MgCl₂ in the medium is also instrumental (L. Zhang et al 2011). In addition, the expression of secondary metabolic gene clusters is controlled by many different families of regulatory proteins (Mervyn J Bibb 2005). By considering all these findings, we can explain our data assuming that the anaerobic condition caused MII differentiation that promoted, in the presence of adequate MgCl₂, the biosynthesis of secondary metabolites, more specifically a better concentration of mTG.

Treatment of wheat flour with culture supernatant (in the presence of lysine ethyl ester as amine group donor) confirmed the effectiveness of the supernatant to induce the biochemical modifications previously reported for purified mTG (Mazzeo MF, et al 2013), i.e. gliadin and glutenin solubilisation. Safety assessment in mice revealed no toxicity of the produced sterile-filtered supernatant. Notably, cytokine analysis of splenocytes from immunized DQ8 transgenic mice (a model of gluten sensitivity) gave an important outcome: the ability of gluten that was isolated from treated wheat to revert the gliadin-inducible immune phenotype in these cells, from inflammatory to anti-inflammatory. This was an important result in the perspective to restore gluten tolerance in CD patients.

The possibility to influence the human immune response by using GRAS bacteria remains an attractive perspective, by also considering the vast literature data on the role of intestinal microbiota in shaping mucosal immunity. In the past 10 years, our understanding of the composition of the adult gut microbiota has undergone a significant change. More recently, a large number of studies has been focused on the development of microbiota composition since in newborn. In particular, family habits and the geographical location represent relevant environmental factors influencing infant gut microbiota colonization (J. M. Rodríguez, et al. 2015; Yatsunenkov T, Rey FE, 2012). The last chapters of my thesis were dedicated to this topic. In particular, we focused on the relationship between the immunomodulatory abilities of *Bifidobacteria* and *Lactobacilli* found in the intestine of healthy individuals and food habits. To address this issue we analyzed a large number of *Bifidobacteria* and *Lactobacilli* isolated from faecal samples of volunteers who followed omnivorous, vegetarian or vegan diets. Bacteria were tested by adopting two cellular models: mouse dendritic cells (DCs), the principal immune cells that drive the phenotype of the adaptive immune response, and Caco-2 cells, a model of human enterocyte. Our data indicated, for the first time, a main difference between the two genera: *Lactobacillus* genus increased the number of costimulatory molecules on DCs, whereas this number was decreased by *Bifidobacteria*. Notably, the evaluation of the anti-inflammatory potential induced in CD4s, expressed in terms of the IL-10/IL-12 ratio generated from each sample, showed higher values for *Lactobacilli* than for *Bifidobacteria* in the omnivorous group. This difference between the two genera was already reported in literature (S. Y. Zanjani, et al 2016) where the application of *Lactobacillus acidophilus* in the production of probiotic products was found useful in reducing damage caused by lead exposure in comparison to *Bifidobacterium lactis*, suggesting that the *Lactobacillus* have greater immunomodulatory potential. Also in our study we found a reduced anti-inflammatory potential in the isolates collected in Bari in comparison with those from other Italian centres. Therefore we highlighted that the geographical area associated with the different kind of diet had a significant impact on the anti-inflammatory potential of members of these bacterial genera. By also analyzing the effects on Caco-2 cells we

underscored a peculiar immune-modulatory activity profile for *Lactobacilli* associated with a specific dietary habit. Therefore, the geographical area had a significant impact on the anti-inflammatory potential of members of the *Lactobacillus* genus.

In the light of all the above reported work, future activities could be dedicated to identify and to isolate potential beneficial strains from the *Lactobacilli* and *Bifidobacteria* collected populations, as well as cognate metabolites. This work is mandatory to better understand if the immunomodulatory properties are associated to a single metabolite or synergy of different strains is required to develop functional food and nutraceuticals with specific biological activities.

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