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Probiotic Lactobacillus strains attenuate oxysterols-induced alteration of human intestinal membrane permeability

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Abstract

The intestinal membrane is an important structure which carries out central functions such as nutrient absorption and excretion and secretion of several products and acts as a barrier to protect the human body from potentially harmful compounds arriving from diet; foods are sources of both potentially dangerous and potentially protective molecules. When a noxious stimulus occurs, it can alter membrane balance and functionality, mostly by altering the tight junctions, increasing its permeability, or causing a shift on microbiota composition and thus sustaining inflammation. Inflammation and oxidative stress have been linked to the loss of intestinal integrity, a crucial event in the initiation and progression of pathological intestinal disorders such as inflammatory bowel diseases (IBD) and cancer. Oxysterols are cholesterol oxidative products, which have been reported to act negatively on intestinal membrane, causing an increase in its permeability and a local inflammation. On the other hand, several studies reported probiotics and short chain fatty acids (SCFAs), produced by the microbiota, as able to exert anti-inflammatory properties and to improve gut barrier permeability.

In this context, the aim of this research project was to evaluate the capacity of two probiotic strains of *Lactobacillus* (*Lactobacillus plantarum 299v*® (DMS 9843) and *Lactobacillus casei DG*® (CNCMI1572), used as bacterial extract or live culture, to protect intestinal epithelium against the alteration of permeability induced by oxysterols and to investigate the mechanism of action in relation to tight junctions modulation and cellular signaling. A preliminary study was also conducted to investigate any modifications induced by oxysterols or probiotics on the metabolic activity of the gut resident bacteria.

To achieve this objective, monolayers of differentiated Caco-2 cells have been used as in vitro model of intestinal barrier, and a batch culture system, to mimic the colonic environment. The alteration of cell monolayers permeability, treated with oxysterols alone or

together with the bacterial extract or live culture was evaluated through the measurement of transepithelial electrical resistance (TEER), in relation to the modulation of tight junctions, occludin, zonulin and JAM-A, linked to MAPKs, p38 and ERK1/2 activation.

Batch culture system have been inoculated with a human faecal sample in a basal media added with oxysterols and/or the bacterial extract or the live culture of the two different strains of the lactobacillus in order to evaluate, possible changes in microbiota SCFAs production.

Our results provide, for the first time, evidence of the ability of *Lactobacillus spp.* probiotics to protect intestinal cells against the pro-inflammatory effect of oxysterols, *in vitro*.

We observed a protective effect turned toward one key inflammatory mechanism such as the alteration of the intestinal permeability, caused by the oxysterols-induced TJs disruption, due, at least in part, to the modulation of MAPK/p38 pathway. The similar efficacy exerted by the bacterial extracts and the pure cultures, suggest a promising effect of both probiotic strains tested as bacterial extracts in the treatment of intestinal inflammation, avoiding the stimulation of the immune system, which is a side effect of the use of the commercialised pure cultures. Regarding batch cultures, we did not detect any significant interaction among oxysterols, probiotics and intestinal microbiota metabolic activity in the experimental condition used; this part of the research project is a preliminary study which we aim to enrich with further investigations.

Taken together our data strengthen the link between diet and intestinal inflammation and encourages us to continue studying probiotics as a useful tool in the prevention and management of the most common intestinal pathologies linked to the inflammatory process.

1. Introduction

1.2 The intestinal mucosa

The intestinal mucosa is a separating layer between the inside and the outside of the human body, essential for the maintenance of the gut homeostasis and to prevent intestinal inflammation. It represents the first line of host defense against the external environment and against both commensal bacteria (in order to block them in the lumen where they exert their roles) and invading enteric pathogens **(Martens et al., 2018)**; it absolves important functions like nutrient absorption, excretion and secretion of different products, barrier against pathogens **(Shimizu et al., 2007)**.

The key role of the intestinal mucosa is guaranteed by the correct functioning of all the elements which compose it, a mixture of cells and molecules acting together to protect the body against pathogens and contaminants, mostly introduced through diet. Cells like intestinal enterocyte (IECs), goblet cells (which secrete mucins) and immune cells, cellular junctions, like adherent and tight junctions (TJs), an overlaying layer of mucus, antimicrobial peptides (AMPs) and a population of residential bacterial, called microbiota, are organized into layers which, all together, form the intestinal mucosal barrier **(Martens et al., 2018)**.

The mucosa is also the primary site in which the *mucosa-associated lymphoid tissue* (MALT), present in all the body mucosae, is exposed to, and interacts with, the external environment **(Turner, 2009)**. In the intestine in particular, this structure is called GALT (*gut-associated lymphoid tissue*).

The GALT represent the innermost layer, accountable for the immune responses and the tolerance mechanism. More precisely, cells involved in the immunological reactions find place part in the layer under the epithelium, part are intra-epithelial, isolated or aggregated in *Peyer's patches* (PP); functions like induction of immune tolerance or defense against pathogens result from the complex interplay between immune cells located in the lymphoid follicles and the follicle-associated epithelium **(Jung et al., 2010)**. This crosstalk seems to be

regulated by pathogen recognition receptors, especially Nod2 *(Nucleotide binding oligomerization domain containing 2)*, which, among all, is implicated in pathology such as *irritable bowel syndrome* (IBS). Although TLR *(Toll-like receptor)* exerts a limited role in PP homeostasis, Nod2 regulates the number, size, and T-cell composition of PPs, in response to the gut flora. In turn, T-cells, in particular CD4+, present in the PP are able to modulate the paracellular and transcellular permeability **(Barreau et al., 2010; Jung et al., 2010)**. Dendritic cells and M-cells are protagonists of the GALT functions: they act as *antigen-presenting cells* (APC), with the role to recognize antigens and refer this message to T-lymphocytes **(Corr et al., 2008)**. If T-reg are activated, the tolerance mechanism works, otherwise lymphocytes start the production of cytokines leading to inflammation.

Over the GALT, resides a physical barrier constituted by cells, including IECs, and proteins assembling the TJs. The connection of IECs to each other through TJs is important in order to maintain the correct membrane permeability: selected nutrients are able to pass the barrier and arrive in the blood through transcellular or paracellular mechanism depending on the molecule, while bacteria and pathogens are blocked.

In this layer, in particular in the intestinal villi of the small intestine, it is also possible to find *Paneth* cells, specialized in the production of antimicrobial enzymes called defensin, lysozyme and phospholipase A with the role of preserving the organism against pathogens **(Ouellette, 2010; Bevins et al., 2011)**, endocrine cells (with some differences between small and large intestine), and goblet cells (both in the small and in the large intestine).

Goblet cells can produce and secrete mucins, large and highly glycosylated polymers that form the mucus layer covering the intestinal mucosa **(Rodríguez-Piñeiro et al., 2012)**. Mucus has the role to protect the intestinal mucosa against mechanical disturbances and to provide an environment for bacteria to interact with the host without necessarily eliciting an immune response **(Rodríguez-Piñeiro et al., 2013).** Moreover, secretory immunoglobulin A

(IgA) and AMPs are released into the mucus as a defense against pathogens and potentially harmful commensal bacteria **(Martens et al., 2018)**.

The most exposed layer, which is directly in contact with the intestinal lumen, is formed by commensal bacteria and their products and is called microbiota.

Lastly, under all these layers resides a vascular barrier which consists of the vascular endothelium and the enteric glial cells that surround it, major components of the enteric nervous system **(Ghosh et al., 2020)**.

Despite all the defensive mechanisms, the gut lumen and so the intestinal barrier is exposed to oxidative stress **(Barker, 2014)**, toxics, molecules that can trigger the immune system, pathogen bacteria as well as commensal bacteria deriving both from endogenous and exogenous environment, for instance from diet. This is especially true for the colon, where residence time of luminal contents is prolonged and endogenous antioxidant capacity is lower as compared to the small bowel **(Amidon et al., 2015)**.

When pathogens, toxins or a diet full of fat, sugars and modified and refined foods occur, the defensive mechanisms of the intestinal barrier could be lost and the permeability tends to increase; when the permeability rises, the selective mechanism of passage is compromised, and undesirable products can pass through the membrane leading to a low-grade inflammation and to a translocation of toxins and bacterial components such as LPS (*lipopolysaccharide*) through the endothelium. When the intestinal membrane loses its function as physical barrier and increases its permeability, creates the basis for the development of a syndrome known as *Leaky gut syndrome*, where TJs are not able to work properly, leading to a more permeable epithelium which is linked to several intestinal and systemic diseases, such as type 2 diabetes, atherosclerosis, heart disease, heart failure, neurological diseases, and IBDs **(Citi, 2018; Ghosh et al., 2020)**. A leaky gut is represented by a wrong connection between

IECs, thus allowing potentially dangerous products to pass towards the barrier; this is mostly due to a malfunctioning of the TJ which, in a healthy status, connect cells to one another.

1.2.1 Tight junctions

One of the mechanisms allowing the intestinal membrane to absolve its protective role are TJs. The intestinal epithelium, as well as all epithelia and endothelia, has the role to separate the internal environment of the body from the potentially dangerous external one. Two ways can be used by nutrients in order to pass from the luminal content to the blood circulation: the first is passing through enterocytes in a way called *trans-cellular* (hydrophobic molecules), the second one is an intercellular way, named *para-cellular* (hydrophilic molecules). The latest is regulated by proteins known as *carriers* **(Anderson et al., 2001)**. In this context, TJs act like gates which choose substances to block and to let enter the barrier, selecting those molecules based on charge and size. TJs, which are essential for establishing the barrier function across a cell layer, work together with other types of cell junctions: adherents junctions (AJs) and desmosomes, which mechanically link cells by bridging the cytoskeleton of adjacent cells, and communicating junctions, or gap junctions (GJs), which chemically and electrically couple neighbouring cells **(Wei et al., 2013)**. AJs are involved in cell surface adhesion **(Chattopadhyay et al., 2013)**, communication between cells, regulation of cytoskeleton and thus their presence is essential for the organization of a tissue; they are also important in order to form the TJs and to connect them to the cytoskeleton **(Meng et al., 2009)**. Most studied AJs include cadherins and catenins **(Gumbiner, 2005).** The others, GJs, are complexes of proteins with the prevalent function to permit exchange of metabolites and electrical signals between cells **(Nielsen et al., 2012)**.

The TJs, together with the actin-rich AJs, form a functional unit called the apical junction complex **(Mitic et al., 1998)**, which constitutes a regulated barrier in the paracellular pathway, creating a defence mechanism against toxic, pathogens and antigens and thus protecting the host against pathologies and allergies; moreover, they are also involved in basic cellular processes like the regulation of cell growth and differentiation **(Balda et al., 1998)**.

The apical junction complex is formed by the synergy of trans-membrane proteins like occludin **(Furuse et al., 1993),** claudins, **(Furuse et al., 1998),** junctional adhesion molecules (JAM) **(Martin-Padura et al., 1998)** and tricellulin **(Ikenouchi et al., 2005)**.

In particular, transmembrane proteins such as occludin, claudin and JAM seal the paracellular space between adjacent epithelial cells, while plaque proteins, such as the zonula occludent (ZO) family, act as adaptors that connect transmembrane proteins to the peri-junctional actomyosin ring **(Ulluwishewa et al., 2011)**.

1.2.1.1 Occludin

Occludin is a 65 kDa trans-membrane protein, existing in 2 isoforms both composed of 4 trans-membrane domain, three cytoplasmatic and two extracellular **(González-Mariscal et al., 2008)**. Both its ammino (*N*-) and carboxy (*C*-) terminal domains are localized in the cytoplasm and the C-terminal is the most involved in the assembling of TJs and in particular in the communication of ZO-1 which is a link between occludin and the actin filaments **(Furuse et al., 1994)**. Thus, the regulation of the functions of occludin as a TJ resides in this domain, and, in particular, in its phosphorylation/dephosphorylation on threonine and serine residues **(Feldman et al., 2005; Mitic et al., 2000)**. Several reports have described the regulation of occludin by protein kinase C (PKC) and casein kinase (**McKenzie et al., 2006**); indeed, phosphorylation appears to be a key mechanism for regulating the biological function of occludin **(Feldman et al., 2005)**. In a healthy epithelium, occludin is highly phosphorylated in these residues and this is a key point for the maintenance of a good operation of the TJs **(Lee, 2015)**.

The N-terminal domain is also important for the organization of the TJs; indeed, truncated mutant may be unable to fully oligomerize with the other TJ proteins, with the result of a disruption of the TJs sealing properties **(Bamforth et al., 1999)**.

Occludin also exhibits a MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain, a four-transmembrane structural motif common among junctional proteins involved in membrane apposition and fusion events **(Cummins, 2012)**.

Apart from dephosphorylation, an activity of degradation of occludin into inactive fragments, can be directed by metalloproteinases (MMP); this leads to a derangement of TJs, which increase the permeability of the epithelium. Numerous evidences report the connection between MMP and occludin, both in vitro and in vivo **(Cummins, 2012).**

1.2.1.2 Zonulin

The family of zonulin proteins is composed of three molecules, *ZO-1, ZO-2, ZO-3* **(Lopez-Bayghen et al., 2006).** ZO-1 is the most studied in the gut and has a molecular mass of 220 kD. ZO proteins belong to the large family of membrane-associated guanylate kinase (MAGUK)-like proteins comprising a number of subfamilies based on domain content and sequence similarity. ZO-1 and ZO-2 were the first junctional components known to directly interact with occludin **(Furuse et al., 1994)**, thus they are involved in the formation of TJs complex, supporting trans-membrane proteins like occludin, claudin and JAM-A. On the other hand, the role of this protein family is not only confined to the formation of the TJs, but they are also involved in transduction of signalling and in the modulation of transcription **(Bauer et al., 2010)**.

The ZO proteins have 3 PDZ domains, 1 SH3 domain, 1 GUK domain and a proline-rich region located at the C-terminus in ZO-1 and ZO-2 and between the second and third PDZ domains in ZO-3 **(Gonzalez-Mariscal et al., 2000)**. ZO-1 PDZ-domains allows the association with JAM-A in the TJs, which is mediated through PDZ domain 3. The SH3 and GUK domains, along with others, play a key role in the assembly and localization of TJs **(Fanning et al., 2007)**. The N-terminal domain is a binding site for proteins that makes up TJs such as occludin, while the C-terminal domain interacts with the actin associated with cytoskeletal proteins **(Fanning et al., 2002)**.

1.2.1.3 Junctional adhesion molecule

Junctional adhesion molecule (JAM) is an integral membrane protein, of about 32 kDa, considered to be an Ig-like protein, that was found to be selectively concentrated at intercellular junctions of endothelial and epithelial cells **(Martìn-Padura et al., 1998)**. It exists in three variants (*JAM-1/2/3*), characterized by 2 extracellular domains, one transmembrane domain, and one intracellular C-terminal domain **(Cunningham et al., 2000)**. JAMs have a PDZ-binding region, which is important for the building of the TJs; indeed, all three JAMs associate with ZO-1 in a PDZ-domain-dependent manner **(Severson et al., 2009).**

JAM members are expressed in various cell types including epithelial, endothelial, and immune cells, and exhibit different expression patterns in both a tissue- and cell type-specific manner. In intestinal epithelial cells, JAM-1, also known as JAM-A, is expressed, and involved in TJs regulation **(Lee, 2015)**. In vivo, the function of JAM-A in colonic mucosa has been examined using JAM-A knockout mice, which have increased intestinal permeability, inflammation and cellular proliferation compared to wild-type controls **(Severson et al., 2009)**.

1.2.1.4 Tight junctions and inflammation: loss of the normal intestinal permeability in the gut

Disruption of the intestinal TJ barrier, followed by permeation of luminal harmful molecules, induces a perturbation of the mucosal immune system, and inflammation, which can act as a trigger for the development of intestinal and systemic diseases. In particular, the cytokine-mediated dysfunction of the TJ barrier, resulting in immune activation and tissue inflammation, is thought to be important in the initiation and/or development of several intestinal and systemic diseases; in contrast, some growth factors play important roles in protection and maintenance of TJs integrity. Negative regulators include Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), Interleukin (IL)-1β (IL-1β), IL-6, IL-17, while positive regulators are IL-10, epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) **(Lee, 2015)**. The production of different cytokines in inflamed intestinal mucosa is thought to be responsible for the downregulation of TJs; for instance, the expression of claudins, that are important sealing proteins in the tight junctional complex, is decreased by TNFα, IFNγ, IL-1β, IL-6, IL-9, and IL23 thus causing an alteration of epithelial permeability *in vitro* and *in vivo*. These cytokines interact with their enterocyte receptors and induce intracellular signalling cascades resulting in the altered activity of various transcriptional factors in the nucleus (such as Hopx, Hnf4α, Klf4, Tcf712, Hif1β) **(Lechuga et al., 2017)**. Furthermore, it has been reported that IFN-γ decreases barrier function in T84 cells by diminishing ZO-1 expression levels; TNF-α is also involved in the decrease of epithelial barrier function and TJ complexity in HT-29 cells and, as well as IFN-γ, in the down-regulated expression of the human occludin promoter **(Mooren et al., 1998; Youakim et al., 1999; Mankertz et al., 2000)**.

Regarding the gut, treatment of intestinal epithelial cells with monoclonal JAM-A antibodies inhibits the resealing of the TJs, as indicated by delays in transepithelial electrical resistance (TER) recovery and occludin assembly **(Lee, 2015)**; it has been reported that JAM-

A knockout mice exhibit higher permeability to dextran and myeloperoxidase activity in the colon compared to wild type mice. In addition, the colonic injury and inflammation induced by dextran sodium sulfate are more severe in the JAM-A knockout mice than in wild type mice **(Lee, 2015)**. A study on a mouse model of colitis induced by dextran sodium sulfate (DSS) evidenced colonic inflammation, a suppression of the expression of antioxidant enzymes and TJs as well as an activation of NF-κB and nuclear factor erythroid 2-related factor 2/Kelch-like ECH-associated protein 1 (Nrf2/Keap1) signaling pathways **(Yin et al., 2015)**.

In human trials, IBD patients demonstrate increased intestinal paracellular permeability **(Lee, 2015)**; this event follows the delocalization and/or the decrease of proteins forming the TJ complex. For example, dysregulation of the zonulin pathway has been associated with a leaky gut, due to increased intestinal permeability in the pathogenesis of gastrointestinal disorders such as coeliac disease, non-coeliac wheat sensitivity, IBS, and IBD **(Ajamian et al., 2019)**. Das et al., on a study on biopsies from patients with Crohn's disease (CD) or ulcerative colitis (UC), found a reduced expression of ZO-1 in UC and CD compared to controls **(Das et al., 2012)**. In UC, the mechanism besides the diarrhoea has been linked to an altered TJ structure connected to a leak that can reduce net ion transport, so contributing to the diarrhoea **(Schmitz et al., 1999)**. On HT-29/B6 cell monolayers, IL-13 was identified as an important effector cytokine in UC that impairs epithelial barrier function by affecting epithelial apoptosis and TJs **(Heller et al., 2005)**.

An alteration in TJs has been also studied in celiac disease, demonstrating that TJs structure presents a leak in patients with celiac disease, correlated with a deregulation of the genes coding for the proteins forming the TJs **(Schulzke et al., 1998; Jauregi-Miguel et al., 2014)**. A study conducted in 2016 evidenced an alteration of TJs even in first-degree relatives (FDRs) of celiac disease patients; the intestinal permeability was significantly higher in FDRs than in controls, with an under expression of TJs ZO-1 and occludin, while no significant

difference in the serum zonulin level was detected between the two groups **(Mishra et al., 2016)**.

1.2.2 MAPK

Structural proteins involved in the formation of TJs are strictly regulated by different upstream signals such as MAPKs *(mitogen-activated protein kinases)*, PKC, myosin light chain kinase, and Rho GTPases and from molecules produced as second messengers such as c-AMP and Ca^{2+}; this regulation is important for the maintenance of the function and integrity of the junctions (**Stein et al., 1997; Matter et al., 2003; Ulluwishewa et al., 2011**).

In this context, MAPKs have largely been studied because of their multiple function inside the cell (**Cargnello et al., 2011**); these proteins are able to respond to numerous stimuli arriving from inside or outside the cell, such as stress and inflammation. Their stimulation leads to a cascade of responses which ends with a signal to activate or deactivate gene transcription in order to control a large number of fundamental cellular processes, including growth, proliferation, differentiation, motility, stress response, survival and apoptosis (**Owens et al., 2007; Plotnikov et al., 2011**).

The cascades are evolutionary conserved and involve multiple phosphorylations, from MAP3K (*MAPK kinase kinase*) to MAP2K (*MAPK kinase*) and then MAPK. The phosphorylation determines the activation of the protein and it is reversible. Indeed, numerous factors are known to influence the spatiotemporal regulation of MAPK signalling including the density and rates of internalization of cell surface receptors, the association of MAPK signalling components with scaffold proteins, and the balance between kinase activators and inactivators (**Owens et al., 2007**). To the last group belong protein phosphatases, able to dephosphorylate MAPK, named MKPs (*MAP kinase phosphatases*) which can be divided into three classes due to their preference for tyrosine residue, serine/threonine residue or both tyrosine and threonine residues (**Farooq et al., 2004**).

The mammalian family of MAPKs includes 14 families, divided into 7 groups; the best known and most studied are *extracellular signal-regulated kinase 1/2* (ERK1/2), p38 α-β-γ-δ, and *c-Jun amino (N)-terminal kinase 1/2/3* (JNK1/2/3) **(Kim et al., 2010; Cargnello et al., 2011)**.

ERK1 and ERK2 are related protein-serine/threonine kinases that participate in the MAPK/ERK pathway, also known as *Ras-Raf-MEK-ERK* signal transduction cascade **(Roskoski, 2012)**. This cascade participates in the regulation of a large variety of processes including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription; they are mostly involved in the activation of immediate early gene products such as *c-Fos* and *c-Myc*, which in turn induce late-response genes that promote cell survival, cell division, and cell motility **(Roskoski, 2012)**. ERK1/2 are activated by different stimuli such as inflammatory cytokines, osmotic stress, derangement of microtubules **(Raman et al., 2007)**, insulin and growth factors **(Boulton et al., 1990)**.

p38 is a MAPK which belongs to the group of SAPK (*Stress-Activated Protein Kinase*) due to its strong activation in response to cytokines and environmental stresses **(Johnson et al., 2002)**. p38 is activated in immune cells by inflammatory cytokines and has an important role in activation of the immune response. p38 MAPK is activated by many other stimuli, including hormones, ligands for G protein–coupled receptors, and stresses such as osmotic shock and heat shock **(Johnson et al., 2002)**, but also oxidative stress, UV ray, hypoxia, ischemia, IL-1 and TNF-α **(Cuadrado et al., 2010)**. p38 is able to stimulate the production of pro-inflammatory cytokines through the modulation of transcription factors like NF-kB **(Karin, 2006)**, or by modulation of transduction and stability of mRNA **(Buxade et al., 2008; Ronkina et al., 2008)**. This action on inflammatory response is most attributed to the isoform p38α **(Kim et al., 2008)**.

Moreover, p38α is involved in the prevention of carcinogenesis through the induction of apoptosis and the ability to arrest cellular growth in response to oxidative stress **(Dolado et al., 2007)**.

JNK 1/2/3 are a group of MAPKs that are activated in response to stimuli similar to those activating p38 **(Cargnello et al., 2011)**. Inflammatory cytokines, oxidative stress, as well as environmental stress in general and growth factors are able to stimulate JNK activation which leads to the stimulation of proteins involved in the regulation of cellular proliferation, differentiation and death **(Kumar et al., 2015)**.

All these three forms of MAPKs are able to respond in some way to the environmental stresses, especially oxidative stress and to participate to the inflammatory process; moreover, studies reported their influence on TJs, linking the activation of MAPKs by inflammatory and oxidative stimuli to the reorganization of TJ complexes. As intestinal membrane is the major separating layer between the inside and the outside of the body **(Martens et al., 2018)**, it is easy to understand how much this important structure is exposed to reactive oxygen species and pathogens present in this site, derived both from endogenous and exogenous elements, for example from diet; these elements can be involved in the alteration of TJ, leaded by the stimulation of MAPKs.

In the context of the alteration of membrane permeability functions, prominent to an increase of inflammation and oxidative stress, dietary habits play a massive role in terms of source of both protective and potentially dangerous products. Lipid oxidation product, such as oxysterol, for example, assume a central role as they are protagonist of modern diet, especially the *Western diet*, usually rich in refined sugars and carbohydrates and fat foods **(Statovci et al., 2017)**.

These products have been demonstrated to induce oxidative stress and consequent inflammation, thus damaging the intestinal membrane structure, and leading to an increase in its permeability, through a mechanism involving MAPKs **(Gonzalez-Mariscal et al., 2008)**.

1.3 Oxysterols

Cholesterol (cholest-5-en-3β-ol) is the most significant and the best-known zoosterol that can be found in all mammals' cells (including neurons); is a crucial constituent of cell membranes, where it works as a precursor of bile acids, D_3 vitamins, and steroid hormones, controls membrane fluency and permeability **(Hur et al., 2007; Otaegui-Arrazola et al., 2010)** and is an element forming lipoproteins, that are engaged in transport and metabolism of fats in organisms **(Brzeska et al., 2016)**. It is composed of three regions (Figure 1): a hydrocarbon tail (also called the lateral chain), a ring structure region with four hydrocarbon rings (A, B, C, and D), and a hydroxyl group.

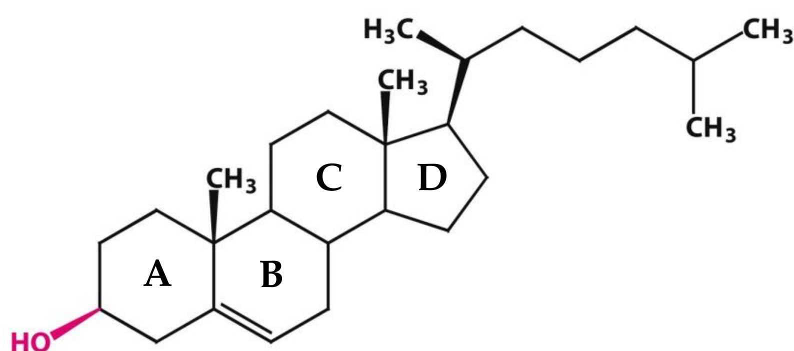

Figure 1. Molecular structure of cholesterol

Cholesterol molecules present in lipid bilayers and in foods are largely susceptible to oxidation. Generally, oxidation of sterols produces the so-called sterol oxidation products (SOP) or oxysterols (OS). In particular, oxidation of cholesterol produces cholesterol oxidation products (COPs), while oxidation of phytosterols produces phytosterol oxidation products (POPs) **(Brzeska et al, 2016)**. Cholesterol oxidation products known as oxysterols, as well as phytosterol oxidation products also considered to be oxysterols **(Guardiola et al., 2002)**, are a

group of sterols similar in structure to cholesterol (cholest-5-en-3b-ol), but containing an additional hydroxyl group, ketone group or epoxide group on the sterol nucleus or a hydroxyl group on the side chain of the molecule (**Morel et al., 1996; Linseisen et al., 1998; Brown et al., 2009**). In humans, these molecules have been identified in various tissues and biological fluids, in an amount and typology that reflects the diet, lifestyle, enzymatic activities and redox status **(Vaya et al., 2011)**. In addition, these molecules are also present in foods due to lipid oxidation reactions, which are the consequence of production processes and storage including heating, contact with oxygen and exposure to sunlight **(Lizard, 2016)**.

The most prominent oxysterols found in foodstuff, plasma and tissues are: cholest-5-en-3β,7α-diol (7α-hydroxycholesterol, 7α-HC; formed via the unstable compound 7α-hydroperoxycholesterol), cholest-5-en-3β,7β-diol (7β-hydroxycholesterol, 7β-HC; formed via 7b-hydroperoxycholesterol), 5-cholestane-3β,5α,6β-triol (cholestanetriol, CT), cholest-5-en-3β-ol-7-one (7-ketocholesterol, 7-KC), 5,6α-epoxy-5α-cholestan-3β-ol (cholesterol-α-epoxide, α-CE), 5,6β-epoxy-5β-cholestan-3β-ol (cholesterol-β-epoxide, β-CE), cholest-5-en-3β,20α-diol (20α-hydroxycholesterol, 20-HC), cholest-5-en-3β,22-diol (22-hydroxycholesterol, 22-HC), cholest-5-en-3β,25-diol (25-hydroxycholesterol, 25-HC), and cholest-5-en-3β,26-diol (26-hydroxycholesterol, 26-HC) **(Griffiths et al., 2017)**.

1.3.1 Endogenous and exogenous oxysterols

The biological source of oxysterols is cholesterol, which, via numerous chemical reactions, is transformed into its oxidized derivatives. These products have been found in human body where they arrive both by exogenous sources, mainly diet, and endogenous sources. Endogenous oxysterols are produced by either non-enzymatic (auto-oxidation) or enzymatic reactions (**Kulig et al., 2016**); more frequently they derive from non-enzymatic routes than from the enzymatic ones **(Brzeska et al., 2016).** Enzymatic reactions generally involve the side chain of the molecule (i.e., 24-HC, 25-HC and 27-HC); non-enzymatic reactions involve the sterol nucleus, mainly the 7-position, due to the presence of a susceptible double bond between carbon C5 and C6 (i.e., 7-KC and 7α/β-HC). However, there are exceptions to this rule; for example, 25-HC and 7α-HC can be produced by both enzymatic and non-enzymatic routes **(Gill et al., 2008)**.

1.3.1.1 Enzymatic reactions

Enzymatic oxidation of cholesterol occurs in the liver by several cytochrome P-450 isoenzymes **(Linseisen et al., 1998).** Important reactions are those that drives cholesterol degradation to bile acids. Cholesterol-7α-hydroxylase (CYP7A1) in the liver catalyses the rate-limiting step in the degradation of cholesterol to bile acids; in addition, hepatic cholesterol 26-hydroxylase, also denoted as sterol 27-hydroxylase (CYP27A1), when attacking the 25-pro-S methyl group (C-27) **(Björkhem, 1992)**, is implicated in bile acid biosynthesis **(Linseisen et al., 1998)** driving the production of both 27-hydroxycholesterol and its terminal oxidation product 3β-hydroxyl cholestenoic acid **(Pikuleva et al., 1998)** converted in chenodeoxycholic coenzim A by the oxysterol-7α-hydroxylase (CYP7B1) **(Yantsevich et al., 2014; Kakiyama, 2019)**. CYP7B1 catalyses the introduction of a 7α-hydroxyl group into 27-hydroxycholesterol and 25-hydroxycholesterol **(Jones, 2012)**. Outside the liver, activity of the 27-hydroxylase is detectable in a variety of cells including fibroblasts, vascular endothelial cells, macrophages, brain and kidney cells **(Babiker et al., 1997; Xie et al., 2003)**; similarly, oxysterol 7α-hydroxylase is also widely expressed in different cells and tissues. 24- and 25-hydroxylase activities were also found in mammalian liver **(Smith, 1996)**; enzymes 24-hydroxylase (CYP46A1) and 25-hydroxylase ($CH_{25}H$) lead to the formation of 24-HC and 25-HC **(Meaney, 2013)**. 24-hydroxylase is mostly expressed in the neurons of the central nervous system, but absent from other tissues **(Russell et al., 2009)**, while data in the literature reports that expression of the cholesterol 25-hydroxylase ($CH_{25}H$) gene is extremely limited; it has been reported that it is induced following treatment of murine bone marrow derived macrophages with lipopolysaccharide, in a TLR-4 dependent manner **(Diczfalusy et al., 2009)**. In the liver it is also possible to find the activity of CYP3A4 that catalyses the formation of both 25-HC and 4β-hydroxycholesterol **(Honda et al., 2011)**.

Biosynthesis of C21-steroid hormones requires 20- and 22-hydroxylation of cholesterol, forming 20α-hydroxycholesterol, 22-HC, and cholest-5-ene-3b, 20, 22-triol **(Linseisen et al., 1998).**

The enzymes regulating the formation of oxysterols are not universally present in different tissues and cells, with the consequence that organs are enriched with particular oxysterols, typically in relation with the relative expression of enzymes. For example, the brain contains relatively high amounts of 24S-hydroxycholesterol (24S-HC) and its generative enzyme, cholesterol 24-hydroxylase (CYP46A1), is highly expressed in central nervous system neurons **(Meaney, 2013)**.

1.3.1.2 Non-enzymatic reactions

Non-enzymatic reactions (cholesterol auto-oxidation) can occur both in human body and in food. The common oxysterols found in food 7α-HC, 7β-HC, α-CE, β-CE, CT, 7-KC, 20-HC, 25-HC are almost the same as that detected in plasma **(Paniangvait et al., 1995)**. Non-enzymatic oxidation is primarily responsible for the generation of certain oxysterols such as 7-KC and 7β-HC **(Poli et al., 2013)**. Reactive oxygen species (ROS), including alkoxy and peroxyl radicals, can oxidize cholesterol molecules **(Griffiths et al., 2016; Poli et al., 2013)**. Cholesterol can also be oxidized by the leukocyte-H_2O_2-HOCl system during inflammation **(Poli et al., 2013)**.

Cholesterol autoxidation is known to proceed via two distinct pathways, a free radical pathway driven by a chain reaction mechanism (type I autoxidation) and a non-free radical pathway (type II autoxidation). Type II autoxidation reactions of cholesterol occurs where oxygen levels are high, and singlet oxygen and ozone are the non-radical molecules involved in the mechanism. These reactions of cholesterol cause the formation of four products: 5α-, 5β-, 6α- and 6β-hydroxycholesterol preceded by their respective hydroperoxide intermediates **(Miyoshi et al., 2014; Girotti et al., 2019)**. Type I photo-oxidation reactions occur where O_2 levels are relatively low. The abstraction of a C7 allylic hydrogen from the cholesterol molecule gives rise to two epimeric ring hydroperoxides: 3β-hydroxycholest-5-ene-7α-hydroperoxide (7α-OOH) and 3β-hydroxycholest-5-ene-7β-hydroperoxide (7β-OOH) (**Teng et al., 1973; Girotti et al., 2017**; **Girotti et al., 2019**). Abstraction could be accomplished by a strong oxidant such as the radical anion of a proximal photosensitizer or a reactive oxygen species that it generates, for example hydroxyl radical ($HO^{\cdot}$) **(Foote, 2012)**. Rapid addition of O_2 to the resulting C7 radical, followed by hydrogen abstraction from another cholesterol or proximal other lipid, gives 7α-OOH **(Rodriguez-Estrada et al., 2014; Girotti et al., 2019)**. Since it is

very unstable, it progressively epimerizes into 7β-OOH, which is thermodynamically more stable. The 7α-OOH and 7β-OOH rapidly transform themselves into other oxysterol species such as 7α-OH, 7β-OH, 5α, 6α-epoxycholesterol (α-epox), 5β, 6β-epox and 7-KC **(Schroepfer, 2000)**. 7β-OH is generated via either a cholesterol auto-oxidation processing or an enzymatic pathway involving the type I 11β-hydroxysteroid dehydrogenase expressed in various tissues including vascular cells **(Garenc et al., 2010).**

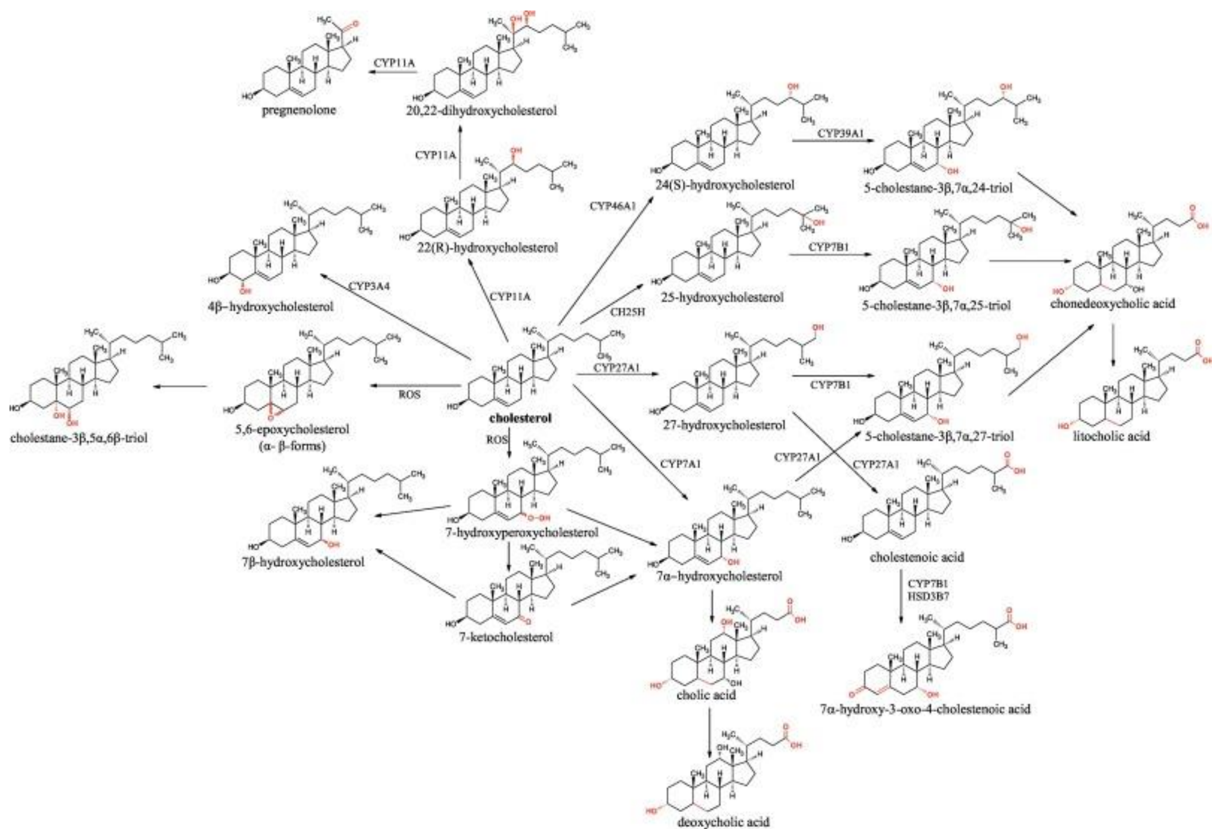

Figure 2. Main products of enzymatic or auto-oxidation of cholesterol found in humans **(Zmysłowski et al., 2019).**

1.3.2 Absorption

Pathophysiological biochemical activity of oxysterols may be even twice as high as that of cholesterol itself **(Poli et al., 2013)**. Research on rats showed that 92% of oxysterols that are produced or arrived in the lumen, are absorbed, while in the case of their parent compounds absorption is only 75%. Indeed, oxysterols are more easily absorbed than cholesterol and have faster plasma clearance **(Vejux et al., 2008)**. Once they arrive in the lumen, the absorption process is the same as the one of cholesterol, but, as they are more polar than cholesterol itself, they can stay on the top layer of chylomicrons and they can be more easily released to the tissues **(Emanuel et al., 1991)**. In the plasma, oxysterols are transported by lipoproteins and their levels are higher in low-density lipoproteins (LDL) than in high-density lipoproteins and very-low-density lipoproteins. Some studies have also shown that oxysterols can be transported by albumin **(Guardiola et al., 2002)**. Due to their higher water solubility, oxysterols such as 25-OH-cholesterol, 27-hydroxycholesterol and 24(S)-hydroxycholesterol, but not cholesterol, have been shown to cross the blood–brain barrier, thereby connecting the peripheral with the cerebral sterol metabolism **(Björkhem, 2002; Björkhem et al., 2009)**. Despite their importance, knowledge about intracellular location of oxysterols, kinetics and transport of oxysterols, is still scarce **(Nåbo et al., 2018)**.

1.3.3 Oxysterols in food and diet

Exogenous oxysterols are introduced through diet. Healthy human plasma contains an average of 12.6 mg/L of oxysterols; ingestion of foods containing oxysterols increases these levels in plasma and leads to deleterious health effects **(Khan et al., 2015)**. For this reason, the interest for the content of oxysterols in the diet has increased in the last years. Food containing cholesterol, mainly meat, eggs, milk and derivate, are exposed to the risk of oxysterols formation, depending on the amount of cholesterol and on treatment they are subject to: food processing, like heating and cooking (mostly drying, frying, steaming and canning **(Maldonado-Pereira et al., 2018)**) **(Lee et al., 2006; Domínguez et al., 2014; Freitas et al., 2015)**, dehydration **(McCluskey, 1997)**, storage and packaging **(Lee et al., 1996)**, and irradiation **(Lee et al., 2001)** can dramatically trigger oxysterols accumulation in animal based foods **(Maldonado-Pereira et al., 2018)**. Non-enzymatic oxidation of cholesterol through reactive oxygen species occurs by agents that can lower the energetic requirements for the reaction to arise, such as heat, light, and metal ions **(Maldonado-Pereira et al., 2018)**.

Several studies have been recently conducted to evaluate if and how food processing methods act on the increase in oxysterols concentration. Khan et al, reported that microwaving and oven grilling resulted in higher production of oxysterols in processed meat as compared with other cooking methods and that refrigerated storage tended to significantly increase the oxysterols content; this increase was found to be lower in fresh products than in cooked products **(Khan et al., 2015)**. In another study regarding the presence of oxysterols in diverse type of milk (UHT, powder milks, condensed milk, and evaporated milk), two different heating modes, microwave-assisted and conventional heating, have been mimicked to study their influence on the formation of oxysterols in milk, reaching the conclusion that conventional heating seems to be healthier than microwave one, since short periods of microwave irradiation (60 seconds) can lead to levels of oxysterols similar to conventional heating for 5 min

(Calderón-Santiago et al., 2012). Innosa et al., studied the presence of 7-ketocholesterol, the most representative of oxysterols family, in eggs and derivative obtained through dissimilar procedures of cooking, finding that the uncooked matrices, as fresh yolks and raw dough, showed undetectable levels of 7-KC, while in homemade cakes at different cooking times the oxysterol was detected, confirming the key role of heating in the formation of this compound **(Innosa et al., 2019)**. A particular problem is represented by ready-to-eat meals, because these foods usually undergo several processing stages, from heating to pasteurization temperatures, followed by freezing, as part of the storage procedure which lasts for days before they are re-heated or microwaved. It has been found that refrigeration as well as re-heating increase the formation of oxysterols **(Min et al., 2016; Maldonado-Pereira et al, 2018)**.

Some studies showed an important role of polyunsaturated fatty acids, in particular, linoleic acid **(Barriuso et al., 2016; Innosa et al., 2019)**, and antioxidants, like astaxanthin **(Carballo et al., 2018),** in protecting cholesterol from oxidation, in cooking foods. For example, quercetin treatment reduced oxysterol content after 7 days of refrigerated storage of fresh meat **(Andrés et al., 2014)**.

This may suggest that diets based on foods containing important amounts of antioxidant and 'good fats', like oleic acid, can be preventive in the onset of the pathologies related with oxysterols increase. Indeed, antioxidants and good fats are able to protect cholesterol present in foodstuff against oxidation, thus reducing the amount of cholesterol oxidative products introduced with diet. This result is also achieved by a dietary regimen with low amounts of oxysterols themselves. An example of this is represented by Mediterranean diet; on the other hand, the *Western diet* and all the diets rich in animal products and low in vegetables and fruit can increase oxysterol related risks. Some studies reported for example that a vegan nutrition, with no animal-based foods, reversed chest pain in patients with angina **(Ellis et al., 1977)** and that a diet including small amounts of low-fat egg whites and non-fat dairy along with exercise,

relaxation techniques, and abstinence from smoking, caused the regression of coronary artery blockage, compared to a control group who followed a diet with 30% calories from fat and whose coronary blockages worsened **(Ornish et al., 1990)**.

Brown made a summary about the correlation between diet and cardiovascular risk linked to the presence of oxysterols in foodstuffs; in his reviews he takes into consideration some studies about vegan, vegetarian, Polynesian, Mediterranean, very low-fat, customary fat, unsaturated fat, or saturated fat diets, concluding that the common atheroprotective factor in all these plant-based dietary patterns is that they all have reduced amounts of oxysterols **(Brown, 2019)**.

From this brief summary about diets, it is clear that the amount of oxysterols that arrives in the intestinal lumen, is strictly connected to the food habits, as different type of nutrition can lead to different levels of oxysterols in the intestine and so at systemic level.

1.3.4 Physiological functions and pathological aspects of oxysterols

1.3.4.1 Physiological functions

Oxysterols have been reported to be important products in the regulation of physiological processes occurring in the human body: as regulators of the expression of genes involved in lipid and sterol biosynthesis like hydroxy-methylglutaryl-CoA reductase (HMGR), and low-density lipoprotein (LDL) receptor **(Pandak et al., 2019)**; as regulator of the synthesis of other lipids, for example sphingomyelin **(Nåbo et al., 2018)**; as substrates for the formation of bile acids (through CYP7A1 enzyme); as regulators of cholesterol metabolism through the mediation of reverse cholesterol transport (acting on HDL lipoprotein) **(Garenc et al., 2010)**. They are also involved in bile acids synthesis and in the activation of cholesterol transporters (ATP-binding cassette) and Apo-E **(Hong et al., 2014)**. Moreover, they are known to trigger liver X receptor (LXR) in order to regulate fatty acid synthesis and cholesterol efflux and to stimulate the peroxisome proliferator-activated receptor gamma (PPARγ) to increase fat movement out of cells **(Pandak et al., 2019)**. Regarding LXR, oxysterols are reported to be able to bind this receptor and so activate it. This action increases the expression of sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate response element (ChRE)-binding protein; while both of these proteins directly activate fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC-1), and stearoyl-CoA desaturase 1 (SCD-1), resulting in an increase in triglycerides synthesis, ChRE-BP can also indirectly act for the same purpose through the regulation of LPK (*liver-type pyruvate kinase*), a key enzyme in the glycolytic pathway, that allows the production of acetil-CoA, important substrate for the synthesis of fatty acids **(Joseph et al., 2002; Mutemberezi et al., 2016)**. Oxysterols can also control fatty acids production by inducing the interaction between INSIGN *(insulin induced gene protein)* and SCAP *(SREBP cleavage-activating protein)* and so the activation of SREB-P **(Radhakrishnan**

et al., 2007). Binding of oxysterols to the LXR transcription factors can stimulate expression of proteins, such as ATP binding cassette transporter 1 (ABCA1) or the related ABCG1 and Idol, an E3 ubiquitin ligase that mediates degradation of the LDL receptor **(Howe et al., 2016)**.

For this reasons, human organs and tissues are provided of enzymes that are able to generate these products, and mechanisms to regulate their concentration; the acidic pathway for the synthesis of bile acids is a way to regulate oxysterols levels, as they are produced as intermediate of the formation of bile acids **(Pandak et al., 2019)**.

1.3.4.2 Pathological effects

Human body possesses enzymes involved in the production of oxysterols, as they shown physiological functions but, if their concentration increases, they may present a pathological effect. Oxysterols can be generated through auto-oxidation of cholesterol, which is not driven by enzymes; these mechanisms can occur both in foodstuff and in human body and they can favour the development of major diseases like cardiovascular diseases, dementia, osteoporosis, and some cancers **(Zarrouk et al., 2014),** as well as intestinal diseases such as IBD **(Testa et al., 2018)**. Some oxysterols, especially ketosterols, are cytotoxic and mutagen **(Adachi et al., 2001).** They can act as trigger in cell death **(Massey, 2006; Kulig et al., 2016; Nåbo et al., 2018)** that can occur through several mechanisms, like gene expression regulation **(Kulig et al., 2016)**, apoptosis and autophagy **(O'callaghan et al., 2001; Olivier et al., 2017)** or necrosis and could be related to the production of reactive oxygen species **(Lizard et al., 1998; Nury et al., 2015; Levy et al., 2018)**. This effect has been confirmed in various cell lines including human adipose tissue mesenchymal stem cells **(Levy et al., 2014; Silva et al., 2017)**, smooth muscle cells, fibroblasts, vascular endothelial cells **(Guardiola et al., 1996; Levy et al., 2018)** and several cancer cell lines **(Kloudova et al., 2017; Warns et al., 2018)**.

Oxysterols may also trigger the atherosclerotic plaque formation; in fact, while dietary cholesterol and oxysterols are rapidly metabolized by the liver into bile acids, the transient increase of these molecules in the plasma can favour their accumulation in the arterial wall. Sato et al., and Gargiulo et al., show that oxysterols are also involved in the acceleration of the plaque destabilization and rupture in mice feed with a high fat diet added with oxysterols, more than in the ones feed with a high fat diet **(Sato et al., 2012, Gargiulo et al., 2017)**.

In demyelinating or non-demyelinating neurodegenerative diseases, increased levels of 7-KC, 7β-OH and 24(S)-hydroxycholesterol (24S-OHC) can be observed in brain lesions **(Nury et al., 2015)**.

Oxysterols have been suggested to play a pivotal role in inflammation and neuroinflammation **(Kim et al., 2006; Guillemot-Legris et al., 2016)**. They are recognized as strongly enhancing inflammatory reactions, by inducing both expression and synthesis of inflammatory cytokines, including TNF-α, IL-1β and IL-6, chemokines such IL-8, monocyte chemotactic protein-1 (MCP-1) and monocyte inflammatory protein-1β (MIP-1β), and adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin **(Vejux et al., 2009)**. The expression of these and other inflammatory mediators is closely dependent on the activity of NF-κB, a transcription peptide demonstrated to be strongly up-regulated by oxysterols, through activation of the protein kinase C-extracellular signalling-regulated kinase 1/2 (PKC-ERK1/2) pathway **(Leonarduzzi et al., 2005)**. Inflammation is undoubtedly a major driving force for the progression and complication of major chronic diseases. The recruitment and activation of phagocytes, characteristic of inflammatory reactions, lead to an increased steady-state level of radical and non-radical reactive oxygen species (ROS) that in turn amplify inflammation, giving rise to a vicious circle that sustains and expands the inflammatory process. Indeed, the inflammation process determines a shift of the biochemical redox equilibrium in cells and tissues towards an excess of oxidative reactions, and oxidative stress is the main endogenous mechanism of non-enzymatic generation of oxysterols **(Poli et al., 2013)**; the reciprocal ability of inflammation and oxysterols to up-regulate one another, tends to amplify the overall inflammatory process **(Poli et al., 2013)**.

1.3.5 Oxysterols damage on intestinal membrane

Cholesterol oxidation products can be introduced from diet, thus arrive in the intestine, where they are able to cause an alteration of the intestinal membrane permeability **(Deiana et al., 2017)**. That leads to a leaky gut, strictly connected to gastro-intestinal and systemic pathologies.

The use of oxysterols in a concentration of 60 µM, that is reported to be similar to that introduced through diet **(Mascia et al., 2010)**, has been reported to be the highest concentration of mixture able to induce strong pro-inflammatory action and cell layer permeability alteration without exerting cytotoxic effect **(Deiana et al., 2017)**.

Studies reported that oxysterols are able to cause an alteration in apoptotic pathways in Caco-2 differentiated cell monolayers through a massive production of reactive oxygen species, and an activation of the enzyme NOX-1, thus determining a reduction of the mitochondrial potential, a release of cytochrome c, and induction of caspase 3 **(Biasi et al., 2009)**. Through the activation of NOX-1, oxysterols can also stimulate the production of IL-8, which is strongly involved in inflammation **(Mascia et al., 2010);** moreover, 25-OH can enhance the action of the proinflammatory cytokine IL-1β **(Bai et al., 2005)**.

In 2019, Willinger, reported that oxysterols action through the G protein-coupled receptor 183 (GPR183) directs the migration of innate lymphoid cells, which is essential for the formation of lymphoid tissue in the colon and that the interaction of GPR183 with oxysterols regulates intestinal inflammation **(Willinger, 2019)**.

On the other hand, studies have been conducted in order to find dietary compounds able to block the damaging action exerted by oxysterols on gut membrane, due to their capacity to cause oxidative stress and inflammation. For example, a recent study demonstrated the ability of theobromine to counteract the decrease of monolayer permeability induced by oxysterols in differentiated Caco-2 cells. Oxysterols induced inflammation was characterised by IL-8 and

MCP-1 overproduction and intestinal barrier damage, related to an alteration of TJs claudin 1, occludin and JAM-A protein levels, MMP−2 and −9 activation and anti/pro-apoptotic protein changes. The observed protection was linked to the ability of theobromine to inhibit the production of cytokines and MMPs responsible for TJs loss and apoptosis in intestinal cells **(Iaia et al., 2020)**. Extra-virgin olive oil polyphenols have been also linked to a reduction of inflammatory and oxidative stress induced by oxysterols on Caco-2 cell monolayers **(Serra et al., 2018)**. Moreover, in 2019, *Rossin et al.* reported the beneficial potential of specific cocoa bean shell extracts fractions, rich in (−)-epicatechin and tannins,-able to counteract oxysterol-induced inflammation **(Rossin et al., 2009)**.

1.4 Probiotics

The other side of a modern diet, considered a carrier of potentially dangerous molecules, is the increased availability of foodstuffs naturally rich or enriched with components that can exert a beneficial action on human body. Among them, especially in the last years, an important consideration has been given to probiotics **(Abdelazez et al., 2018).**

The definition of probiotics comes from the FAO ("*Food and Agriculture Organization of the United Nations*")/WHO ("*World Health Organization*") and was reformulated with minor changes from an ISAPP expert panel in 2013 as follows: 'live microorganisms that, when ingested in adequate amounts, confer a health benefit to the host' **(Hill et al., 2014)**. The word probiotic (from the latin *pro* and the greek *βιοσ* literally meaning "for life") was introduced by the German scientist *Werner Kollath* in 1953 to designate "active substances that are essential for a healthy development of life" **(Gasbarrini et al., 2016)**. In 1965 two scientists, *Lilly* and *Stillwell* used this word to describe molecules that, produced by a bacterium, allow the growth of another **(Lilly et al., 1965)**. Since then, the definition and the idea of probiotics has changed several times as studies were increasing. The first who talked about bacteria as positive microorganisms, that are resident in the intestine and that can be introduced with food, was a Russian biologist working at the *Pasteur Institute* in Paris, *Elie Metchnikoff*, who conducted studies about longevity. He observed that Balkan rural population lived longer than people in other countries and this was correlated to a frequent consumption of fermented dairy foods such as yogurt; this finding made him state a hypothesis on the beneficial effect of some bacteria, most of all Lactobacilli. Nevertheless, this assumption has found a correlation in researches conducted in the same institute by *Tissier*, who found that the intestine of healthy new-born was populated by *Bifidobacteria*, absent in the intestine of new-born with diarrhoea **(Tissier, 1900; Metchnikoff, 1907; Capurso, 2016)**.

The definition implies that to be considered as probiotics, microorganisms need to show some characteristics, such as resistance to gastric acidity and bile acids, in order to resist to the digestion process and arrive alive in the human gut, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, ability to reduce pathogen adhesion to surfaces, bile salt hydrolase activity, resistance to spermicides (applicable to probiotics for vaginal use) **(FAO/WHO, 2002)**.

The use of probiotics is very ancient and traditionally they have been introduced in the human body through fermented food, mainly dairy products and vegetables that have been consumed for centuries, with the purpose to increase the healthy state, with particular attention to gastrointestinal tract **(Zucko et al., 2020)**. Human gastrointestinal tract contains species that are considered probiotics; in fact, most of the probiotic bacteria were originally isolated from healthy humans **(Krishna Rao et al., 2013)**. Even if fermented foods and drinks are still one of the main sources of probiotics, nowadays they are also diffuse as supplements in a form of capsules or pills **(Gosálbez et al., 2015)**, becoming an important ingredient for industries to produce both supplements and probiotic enriched foods **(Granato et al., 2020; Zucko et al., 2020)**.

The inclusion of probiotics in dietary supplements primarily utilizes probiotics in the freeze-dried powder format (capsules, tablets, powder in stick packaging, sachets) and are usually stored at ambient conditions; dietary supplement products should deliver the probiotic count declared on the label throughout the shelf life of the product in order to ensure that the consumer receives the adequate dose of probiotics to affect the targeted structure, function, health claim or the otherwise suggested health benefit **(Fenster et al., 2019)**.

On the other hand, industries are not only focused on the production of dietary supplements containing probiotics, but also on the enrichment of common foodstuff **(Hilton, 2017)** with variable types and doses of them. The enriched food may naturally contain

probiotics, as yogurt **(Aryana et al., 2017)**, milk, cheese **(Fernández et al., 2015)** and ice cream **(Alamprese et al., 2005; Bakr Shori et al., 2018)** or may lack them, like chocolate, crackers, breakfast cereal, snacks, chips, peanut butter, and crispy granola bars **(Fenster et al.,, 2019)**.

Nevertheless, probiotics are nowadays used to produce pharmaceutical products and infant products **(Sanders et al., 2014; Al-Tawaha et al., 2018)**.

Despite the safety and the good properties that probiotics have shown, first during the history and second when they have been tested during studies, it is also true that some strains have been reported to have adverse effects, most correlated with commercial formula, especially in patients with previous diseases **(Sanders et al., 2014; Zucko et al., 2020)**.

Indeed, as WHO/FAO stated, probiotics may theoretically be responsible of effects like systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals, and gene transfer **(Marteau, 2001)**; regarding this last point it is important to consider the potential to carry and spread antibiotic resistance genes, which is a crucial safety assessment often ignored **(Saarela, 2019)**. All the side effects are more likely to appear in people belonging to the group of at-risk populations, as immunocompromised patients, or subjects with abnormal gastrointestinal mucosal barrier, or recovering from surgical procedures **(Didari et al., 2014)**.

It is also important to underline that changes that industries make in order to respond to the commercial needs can significantly alter the properties and the safety of probiotics. For example, *Sanders* at al. report, in their review, that growth conditions and substrates, cryoprotectants, food formula, processing conditions, and storage conditions may affect probiotic properties as scientists seek to optimize processes, viability, and function. Such modifications concomitantly may generate detectable differences in genes (mutations, genome rearrangements), gene expression patterns, or metabolic output (**Sanders et al., 2014)**.

Doron and Snydman, following the theoretical possible side effect list reported by WHO/FAO, analysed the literature reporting cases of probiotic side effects in human trials. In particular, they reported cases of fungemia, sepsis, endocarditis, D-lactic acidosis and worsened of mortality in patients with severe pancreatitis; on the other hand, they also stated that a safety use of probiotic has been reported in solid organ transplant recipients, and other immunocompromised hosts and that there is no evidence reported about the excessive immune stimulation in susceptible individuals, and transfer of antimicrobial resistance **(Doron et al., 2015)**. Yet, *Suez et al.*, investigated the effects of probiotic supplementation after antibiotic treatment and found that probiotics perturbed rather than aided microbiota recovery back to baseline **(Suez et al., 2018)**. Antibiotic therapy significantly enhanced gut mucosal colonization by probiotics, which then delayed indigenous gut mucosal reconstitution for up to five months **(Suez et al., 2018; Zucko et al., 2020).**

Regarding gastrointestinal disorders, several studies reported no effect or side effect of probiotics IBS. A meta-analysis conducted by *Niu et al.*, comparing 35 randomized control trials about probiotic use in IBS, reported a lower incidence of persistence of symptoms, a beneficial effect on global symptoms and the abdominal pain score, and flatulence score, but also a higher incidence of any adverse event **(Niu et al., 2020)**.

About IBDs, a meta-analysis of probiotics in remission of IBD by *Ganji-Arjenaki* and *Rafieian-Kopaei* has found a significant effect in patients with ulcerative colitis and children with IBD, while effect was not significant for Chron's disease **(Ganji-Arjenaki et al., 2018; Zucko et al., 2020)**.

Minor side effects are normally reported in some cases of use of different strains of probiotic; these include abdominal cramping, nausea, soft stools, flatulence, and taste disturbance **(Doron et al., 2015)**.

In conclusion, nowadays the safety of probiotics is still insufficiently explored **(Dore et al., 2019)** and, considering what it is known so far, evaluate the risk-benefit ratio before prescribing the probiotics is recommended **(Didari et al., 2014)**. However, it is now well recognized that the specific health claims associated with probiotics are highly strain specific, therefore, their safety needs to be assessed very comprehensively at strain level on case-to-case basis **(Pradhan et al., 2020)**.

1.4.1 Lactobacilli

Nowadays microorganisms recognized as probiotics are in a large and increasing number, however, among this multitude, the most common belong to genera like *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Pediococcus* **(Sieber et al., 2004; O'Shea et al., 2012)**. Among them, species belonging to the genera *Lactobacillus* are the most studied and used in commercial products **(Ashraf et al., 2014)**.

Lactobacilli populate a variety of habitats where carbohydrate-based substrates are available, such as plants, plant-derived matrices, silage, fermented foods (e.g., dairy products, mainly yogurts, cheeses and kefir, fermented dough) milk, wine, meat, fruit, vegetables, cereals, spoiled foods, organic matrices and sewage **(Hammens et al., 2006, Rossi et al., 2016)**. However, *Lactobacillus* species have been isolated from the entirety of the human and animal GI tract (from oral cavity to faeces) as well as the skin and urogenital tract. This genus is estimated to constitute 6% of the total bacterial cell numbers present inside the human duodenum and approximately 0.3% of all bacteria in the colon **(Heeney et al., 2018)**. Over more than 200 species of *Lactobacillus* known so far, just about 50 have been speculated to be resident of the human gastrointestinal (GI) tract; a study conducted by *Rossi et al*., reported that among 86 strains of *Lactobacillus*, belonging to 52 different species and found in stool samples of human volunteers, 43 seemingly occupied the GI tract as true residents, since were detected in a time span of almost 2 years **(Rossi et al., 2016)**. Lactobacilli inhabiting the GI tract include *L. casei*, *L. plantarum*, *L. fermentum*, and *L. rhamnosus*, while the most common isolates from the stomach mucosa are *L. antri*, *L. gastricus*, *L. kalixensis*, *L. reuteri*, and *L. ultunensis* **(Turroni et al., 2014; Zhang et al., 2018)**.

The genus *Lactobacillus* taxonomically belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae* **(Zhang et al., 2018)**. It is composed of

about 237 species **(Sousa et al., 2019)** of facultative, anaerobic, catalase-negative, *Gram*-positive, non-spore-forming rods **(Kleerebezem et al., 2009)**.

Lactobacilli, Enterococci and Streptococci are included in the functional group of Lactic Acid Bacteria (LAB), since they catabolize hexoses to lactic acid through obligate or facultative homolactic or heterolactic fermentation, the latter yielding also two-carbon products (ethanol and/or acetic acid) and CO_2 **(Rossi et al., 2016)**. The most studied LABs belong to different species of the genera *Lactobacillus*, *Weissella*, *Carnobacterium*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Tetragenococcus*, *Bifidobacterium* and *Leuconostoc*.

The phylogenetic tree based on 1,418 bases pairs of 214 species of the genus *Lactobacillus* lists the formation of six groups. The main LABs are allocated into group 1 (*L. rhamnosus, L. reuteri, L. cellobiosus, L. casei, L. paracasei, L. curvatus and L. fermentum*), group 2 (*L. florum, L. fructivorans, L. kunkeei, L. diolivorans, L. hilgardii, L. plantarum, L. brevis and L. buchneri),* group 3 *(L. mali, L. oeni, L. nagelii and L. vini*), and group 6 (*L. gasseri, L. lactis, L. acidophilus, L. delbrueckii, L. bulgaricus and L. jensenii*) **(Sousa et al., 2019)**.

1.4.1.1 Metabolism of LAB

Depending on the monosaccharide substrate which they mainly use as a source of sustenance and, consequently, based on the final fermentation products, Lactobacilli are divided into two groups: homofermentative and heterofermentative (these last, obligatory and facultatively). Homofermentative Lactobacilli are exclusively classified as obligatory (group I); since they possess a key enzyme for the glycolytic pathway (fructose-1,6-diphosphatase aldolase) they can use hexoses sugars via the *Embden-Meyerhof-Parnas* (EMP) pathway, producing, as secondary metabolite, only lactic acid (>85% of the final products); on the contrary, they are unable to ferment pentose sugars and gluconate, due to the absence of the phospho-ketolase enzyme, present instead in heterofermentative (**Pessione, 2012; Salvetti et al., 2012; Abdel-Rahman et al., 2013; de Mesquita et al., 2017**).

All heterofermentative possess an enzyme inducible from pentoses, phospho-ketolase, that allows them to ferment, not only hexoses, but also pentose sugars, producing lactate, acetate and ethanol **(Abdel-Rahman et al., 2013)**.

In particular, Lactobacilli belonging to the group of obligatory heterofermentative (group III), can ferment hexoses carbohydrates, with the production of lactic acid, acetate (aerobiosis) or ethanol (anaerobiosis) and CO_2, through the phospho-gluconate and phospho-ketolase pathways. These micro-organisms own enzymes able to act on 6-phosphate glucose to form 6-phosphogluconate, followed by ribulosium 5-phosphate and xylulose 5-phosphate; from xylulose 5-phosphate, a phospho-ketolase allows the formation of glyceraldehyde 3-phosphate (which then follows the same glycolytic pathway forming pyruvate and then lactate) and acetyl-phosphate (which is transformed into acetic acid (aerobiosis condition) or ethanol (anaerobic condition) and CO_2. On the other hand, they do not have the fructose-1,6-diphosphatase aldolase, so they are not able to ferment sugars through the EMP pathway.

Facultatively heterofermentative lactobacilli (Group II) can switch between homo and hetero-fermentative metabolism, depending on sugar availability in the environment, as they possess both fructose-1,6-diphosphate aldolase and phospho-ketolase; they are capable of fermenting exogenous sugars with predominantly lactate creation (about 90% of the final products) via the EMP pathway, such as homofermentative; though, in the presence of a limited concentration of glucose they can exploit the phospho-gluconate pathway and produce lactate, acetate (aerobiosis) or ethanol (anaerobiosis) and formic acid. Differently from obligatory heterofermentative, this group does not generate CO_2 as final product of pentose fermentation **(de Mesquita et al., 2017)**.

About disaccharides, the most commonly used substrate by Lactobacilli is lactose, formed by a molecule of galactose and a molecule of glucose. Some LAB species are known to partially ferment lactose, due to their incapacity of fermentation with galactose **(Iskandar et al., 2019).** Lactose utilization occurs through two different pathways. The first involves a carbohydrate-specific permease **(Barrangou et al., 2003)**; lactose is imported in the cell where an intracellular β-galactosidase hydrolyses it into galactose, which is metabolized by *Leloir pathway*, and glucose, that enter the glycolysis **(Premi et al., 1972)**. The second pathway of lactose utilization occurs through the use of lactose-specific phosphoenolpyruvate-dependent phosphotransferase system transporters (PTSs), that phosphorylate carbohydrates as they enter the cell **(Reizer et al., 1997)** with the formation of lactose phosphate, then hydrolysed by phospho-β-galactosidase. The hydrolysis of lactose phosphate results in glucose and galactose 6-phosphate (further metabolized by the tagatose pathway **(Kandler, 1983; Francl et al., 2012)**. The end-product of the *Leloir pathway* is Glucose-1P, whereas the Tagatose-6P end products are triose-3 phosphates (Glyceraldehyde-3Phosphate (GPDH) and Dihydroxyacetone Phosphate (DHAP)). All these end-products can subsequently enter the glycolysis pathway **(Iskandar et al., 2019)**.

In the *Leloir pathway*, galactose and lactose are internalized by a permease (galP). Lactose is then hydrolysed via a β-galactosidase (lacZ or lacLM) into glucose and β-galactose. The resulting galactose is subsequently metabolized into glucose-1P by the action of galM (galactose mutarotase), galK (galactose kinase) and galT/galE (galactose-1-phosphate uridyltransferase, UDP-glucose-4-epimerase). Glucose-1P is then transformed into glucose-6P, an intermediate of the glycolysis, by the action of the phosphoglucomutase (encoded by pgm). In the Tagatose-6P pathway, lactose-6P is hydrolyzed by a phospho-β-galactosidase (encoded by lacG) into glucose and galactose-6P. This latter is then transformed into 2 trioses (GPDH and DHAP) via a series of 3 reactions encoded by lacAB (galactose-6-phosphate isomerase), lacC (tagatose-6-phosphate kinase) and lacD (tagatose-1,6-diphosphate aldolase) **(Iskandar et al., 2019).**

1.4.1.2 Effect of probiotics on the gut health

As already mentioned above, the interest on probiotic use is still growing because of their numerous potentialities on human health, especially with regard to the gastro-intestinal tract.

One of the most useful benefit of the probiotic resides in their positive effect on the restoration of natural microbiota after pathologies or antibiotic therapy, which lead to an alteration in gut bacterial ecosystem, also known as dysbiosis. Gut microbiota dysbiosis is a condition related with the pathogenesis of intestinal illnesses (IBS, celiac disease, and IBDs and extra-intestinal illnesses (obesity, metabolic disorder, cardiovascular syndrome, allergy, and asthma), thus it is particularly important to be able to treat it effectively **(Hemarajata et al., 2013)**. Among the innovative therapeutic strategies used for this purpose, there is the administration of probiotics, prebiotics, and synbiotics as well as the phage therapy, the faecal transplantation or the bacterial consortium transplantation **(Gagliardi et al., 2018)**. Another function of probiotics is to counteract the activity of pathogenic intestinal microbiota, introduced from contaminated food and environment: probiotics may effectively inhibit the development of pathogenic bacteria, such as *Clostridium perfringens*, *Campylobacter jejuni*, *Salmonella Enteritidis*, *Escherichia coli*, various species of *Shigella*, *Staphylococcus*, and *Yersinia* **(Markowiak et al., 2017)**.

Moreover, probiotics can help in the lactose digestion: individuals with lactose maldigestion can tolerate lactose present in yogurt to a much greater extent than the same amount of lactose in raw milk, and one of the mechanisms of action to explain this finding is the high level of lactase produced by probiotic lactic acid bacteria, which is released within the intestinal lumen when these bacteria are lysed by bile secretions. Lactase then acts on the ingested lactose, thus relieving maldigestion symptoms **(Tuohy et al., 2003)**.

Regarding the most diffuse intestinal pathologies, IBDs, a recent review reports that to date, the use of probiotics seems to have no consistent benefit in treating CD, while, although more evidence is needed in the evaluation of probiotics efficacy, promising results have been obtained in UC **(e Silva et al., 2020)**. Indeed, the positive effects exerted by probiotics on human heath are strain specific.

Focusing on IBS, Dale et al., reported that, even though the exact mechanisms of probiotics in the human body are not fully understood, probiotic multi-strain supplements, but not mono-strain ones, are thought to improve IBS symptoms through manipulation of the gut microbiota **(Dale et al., 2019)**. As authors reviewed, different studies reported divergent effects on the symptoms improved by probiotic supplementation: some evidence found a general improvement in IBS symptoms, whereas others reported improvement in specific symptoms like abdominal pain and bloating **(Dale et al., 2019)**.

As reported by *Laliani et al.* in their review **(Laliani et al., 2020)**, recent researches have revealed intriguing results suggesting the bacterial potential in cancer therapy, which could be considered as an alternative anticancer approach that has promising results on tumour cells *in-vivo*. Moreover, with genetic engineering, some natural or genetically modified bacterial strains can directly target hypoxic regions of tumors and secrete therapeutic molecules leading to cancer cell death. Additionally, stimulation of immune cells by bacteria, bacterial cancer DNA vaccine and antitumor bacterial metabolites are other therapeutic applications of bacteria in cancer therapy **(Laliani et al., 2020)**.

As know so far, mechanisms explaining the improvement of human health due to probiotics are not yet fully understood. Possible mechanisms of action are their ability to produce antimicrobial compounds, enhancement of the epithelial barrier through attachment, competition for pathogenic binding sites, or modulation of the immune system **(Bermudez - Brito et al., 2012)**. Anyway, the positive effect of a probiotic on a healthy or pathological gut

environment depends on the tested strain; indeed, more specific properties of two species belonging to the mostly studied group of probiotics, *Lactobacilli*, are reported below.

1.4.1.3 *Lactobacillus plantarum*

Among the lactobacilli group, one of the most studied species and extensively used in food industry as probiotic microorganism and/or microbial starter is *Lactobacillus plantarum* **(Behera et al., 2018)**. It is a group of optional heterofermentative bacteria that can adapt to a great variety of environments. As all members of Lactobacilli, *L. plantarum* requires at least one fermentable carbohydrate as an energy source, from which it is able to produce lactic acid as the main final product; however, since it is optional heterofermentative, it can also produce acetic acid, ethanol and carbon dioxide. This specie can switch from a heterofermentative metabolism (via phospho-gluconate) to a homofermentative one (via *Embden-Meyerhof-Parnas* pathway). *L. plantarum* grows at temperatures between 15 and 45 degrees, which makes this specie extremely heat tolerant. The presence of particular enzymes or the production of some bacterial molecules, allows this microorganism to survive adverse conditions that may be present in the gastrointestinal, vaginal and urogenital tracts **(Al-Tawaha et al., 2018)**, as well as osmotic and oxidative stress **(Ferrando et al., 2015)**. For example, among the peculiarities of *L. plantarum,* it has developed enzymatic adaptations to deal with oxidative stress: enzymes such as catalase, peroxidase and reductase that, together with the high intracellular concentration of manganese ions inside the bacterial cell, are essential to limit the damage exerted by oxygen radicals, allowing this microorganism to exert anti-oxidant properties **(Al-Tawaha et al., 2018).**

L. plantarum group (LPG) comprises five closely taxonomical species: *L. paraplantarum*, *L. pentosus*, *L. fabifermentans*, *L. xiangfangensis*, and *L. plantarum (subsp. plantarum and subsp. Argentoratensis)* **(Behera et al., 2018)**. *Lactobacillus plantarum* is commonly found in the human gastrointestinal-tract **(de Vries et al., 2006)** and in other matrices, mainly fermented food systems, including pickles, sauerkraut, Korean kimchi, brined

olives, sourdough, Nigerian Ogi, and other fermented fruits and vegetables and also some cheeses, fermented sausages, and stockfish (unsalted fish) **(Behera et al., 2018).**

Microorganisms included in the *L. plantarum* group are generally recognize as safe (GRAS), since they have a long history of natural occurrence and safe use in a variety of food products, with just a few studies that report adverse effects, among the hundreds reports of its safe use **(de Vries et al., 2006)**. For this reason, as well as for the positive properties they have shown, they are largely used in foodstuff and traditionally applied in the conservation of a variety of fermented food products.

Regarding the production of fermented foods, due to its antimicrobial/antifungal and antioxidant activities **(Behera et al., 2018)** as well as its ability to resist adverse condition such as osmotic and oxidative stress **(Ferrando et al., 2015)**, *L. plantarum* is a widely used strain in the food industry. For example, the osmotic resistance of *L. plantarum* makes it a perfect microorganism to be used in the production of fermented foods with high salt concentrations **(Milesi et al., 2008; Gómez-Ruiz et al., 2008)**. A recent study shows the useful action of *L. plantarum* in making food safer and in increasing its shelf-life: in meat products *Lactobacillus plantarum*, as well as *Pediococcus acidilactici* cultures were able to decrease *Listeria monocytogenes* and *Salmonella typhimurium* until the day 3 of fermentation, compared to control samples, suggesting that the use of bioprotective starters can play a significant role in safety of meat foodstuffs and in the increase of their shelf-life as well as creating desired properties in meats **(Varnan et al., 2020)**. Yet, the growing interest in gluten-free products has intensified studies with the aim to use bacteria to improve the quality of products for people with celiac disease; in this context, *L. plantarum* has been found to reduce the anti-nutritional factors of cereals and to convert the toxic molecules responsible for the immune system reaction that occurs in celiac patients. In general, *L. plantarum* is useful to reach industrial objectives like the preservation, flavour and consistency of numerous foods and drinks (cheese,

kefir, sauerkraut, wine, fermented meat products and fermented vegetables) **(Al-Tawaha et al., 2018)**.

Recently, however, the focus on the use of *L. plantarum* has increased in particular due to its probiotic potential; it is recognized as a natural probiotic of the human gastrointestinal tract where this strain seems to be able to reduce the overall symptoms of infections and to inhibit the adhesion of any pathogens to enterocytes; it is also able to exert anti-oxidant and anti-mutagenic activity and to positively modulate immune system **(Behera et al., 2018)**.

Moreover, it has been reported a positive activity in obese patients; *Choi et al.*, for example, have recently evaluated the effect of long-term ingestion of *L. plantarum LMT1-48* on the expression of lipogenic genes in high-fat diet (HFD)-fed mice, observing that treatment of adipocytes with *L. plantarum LMT1-48* extract inhibited their differentiation and lipid accumulation by downregulating lipogenic genes (PPARγ, C/EBPα, FAS, and FABP4); the administration of *L. plantarum LMT1-48* was also able to reduce liver weight and liver triglycerides concurrently with the downregulation of the lipogenic genes PPARγ, HSL, SCD-1, and FAT/CD36 in the organ, resulting in the reduction of body weight and fat volume in HFD-fed obese mice. Moreover, they also observed that the administration of at least 10^6 CFU (colony-forming unit) of *L. plantarum LMT1-48* significantly lowered body weight and abdominal fat volume in modified diet-fed mouse models **(Choi et al., 2020)**.

1.4.1.4 *Lactobacillus casei*

Lactobacillus casei is a specie belonging to the *Lactobacillus casei* group (LCG), which is composed of three genotypically and phenotypically related facultatively heterofermentative species, *L. casei, L. paracasei,* and *L. rhamnosus* **(Hill et al., 2018)**.

The LCG is represented by bacillus bacteria, belonging to the *Lactobacillaceae* family, gram positive, anaerobic, microaerophilic and acidophilic (pH between 3.5 and 6.2). Mainly immobile, this group shows a typically fermentative metabolism with the production of lactic acid (homofermentative) **(Fanti, 2003)**. LCG members can be found in various habitats including decaying plant material, silage, the human reproductive and gastrointestinal tract (GIT), stool, and sewage, in many fermented foods of both plant (e.g., fermented vegetables, sourdough, wine) and animal (e.g., dairy products, cured meat and fermented sausages) origin. Members of this group are often used as nonstarter lactic acid bacteria in many ripened cheeses, contributing to development of cheese flavour. Selected strains from the *L. casei* group are sometimes added to cheese milk as adjuncts for outcompeting undesired microorganisms, and for improving and standardizing cheese quality. Some strains of this group have a probiotic potential and are included in functional cheeses and fermented milk beverages **(Minervini et al, 2015)**.

These microorganisms are largely studied as they can be used as probiotics supplements as well as in the food industry to confer particular properties like flavours and longer shelf life to the foodstuff. This is linkable to their capacity of endure many stresses during processing. They can survive bile salts **(Wang et al., 2011)**, oxidative stress, thanks to the high levels of magnesium and catalase **(van de Guchte et al., 2002; Ianniello et al., 2016)**, cold stress **(Beaufils et al., 2007)**, osmotic stress **(Palomino et al., 2013)**, acid stress and long-term storage **(Xu et al., 2016; Liao et al., 2017)**. Resistance to these stresses in LCG is strain

dependent, with some having a high resistance to multiple stressors while others confer little to no resistance **(Hill et al., 2018).**

As they are able to survive to the GI tract, they can be used as probiotics. Yet, they exert beneficial effects on human health, especially on the GI tract. For example, *L. casei* is reported to have positive effect in IBS patients. A study was conducted to evaluate the role of *Lactobacillus casei DG* (LC-DG) and its postbiotic (PB) in modulating the inflammatory/immune-response in post-infectious IBS in an ex-vivo organ culture model, reporting that LC-DG and its PB attenuate the inflammatory mucosal response by reducing inflammatory cytokines and increasing anti-inflammatory cytokines **(Compare et al., 2017)**. On the other hand, it is not perfectly clear the effect of this probiotic supplementation on the amelioration of quality life and symptoms in patients with IBS; some probiotics are an effective therapeutic option for IBS patients but the effects on each IBS symptom are likely species-specific **(Ortiz-Lucas et al., 2013)**.

Regarding diarrhoea, LCG strains have been associated with improving the symptoms and/or duration of diarrhoea in numerous studies, but specifically *Lactobacillus rhamnosus GG* (LGG) is a promising strain in this regard **(Hill et al., 2018)**. The ability of many *L. casei* strains to prevent antibiotic associated diarrhoea is related to its ability to maintain the diversity of the gut microbiome of individuals during antibiotic treatments **(Reunanen et al., 2012)**. Some studies reported also anti-tumoral properties linked to some strains of LCG **(Hill et al., 2018).** In an *in vitro* study on murine (CT26) and human (HT29) colon carcinoma cell lines, the administration of live *Lactobacillus casei* (as well as bacterial components thereof) was able to raise a significant concentration- and time-dependent anti-proliferative effect; this was confirmed in a mice experimental tumor model in which oral daily administration of 10^9 CFU live *L. casei* for 13 days significantly inhibited *in vivo* growth of colon carcinoma cells, resulting in approximately 80% reduction in tumor volume, accompanied by *L. casei*-driven

up-regulation of the TNF-related apoptosis-inducing ligand TRAIL and down-regulation of Survivin (a member of the inhibitor of apoptosis (IAP) protein family that inhibits caspases and blocks cell death) **(Tiptiri-Kourpeti et al., 2016)**.

Some *Lactobacillus casei* bacteria may have a role on obesity. A study conducted on mice fed with a fermented milk containing *L. casei CRL 431* shows that the strain positively affected some obesity biomarkers **(Núñez et al., 2014)**. In another recent study, obese induced rats were treated with different probiotics strains, including *L. casei IMVB-7280*; treatment with probiotics led not only to a significantly lower rates of obesity, but also to an improvement of the insulin resistance confirmed by a decrease in HOMA-IR (*homeostatic model assessment of insulin resistance*), a decrease in pro-inflammatory cytokines levels (IL-1β, IL-12) and elevation of adiponectin and TGF-β in comparison with MSG-obesity group **(Kobyliak et al., 2020)**. Regarding clinical trials, a study was conducted administering a multi-strain probiotic capsules (UB0316: *Lactobacillus salivarius UBLS-22, Lactobacillus casei UBLC-42, Lactobacillus plantarum, UBLP-40, Lactobacillus acidophilus UBLA-34, Bifidobacterium breve UBBr-01, Bacillus coagulans Unique IS2* 5×10^9 cfu each and fructo-oligosaccharide, 100 mg) on a group of subjects; in a total of 71 people who completed the scheduled study, a 12-week supplementation significantly reduced body mass index (BMI), body weight and waist-to-hip ratio from the baseline, compared to placebo **(Sudha et al., 2019)**.

Some strains of LCG group are also able to modulate the immune system by the upregulation of anti-inflammatory cytokines, such as IL-10 or the reduction of the secretion of pro-inflammatory cytokines (IL-12). Consequently, specific strains of the *L. casei* group may reverse dysfunctions of the intestinal epithelial barrier, thus potentially counteracting the onset of IBDs **(Minevrini et al., 2015)**.

1.4.1.5 Post-biotics and para-probiotics

Even if a large number of studies are conducted on viable bacteria used as probiotics, recent evidence suggests that bacterial viability is not necessary to attain the health-promoting effects, as not all mechanisms nor clinical benefits are directly related to viable microorganisms **(Aguilar-Toalá et al., 2018)**. This achievement gives raise to the new concept of post-biotics and para-probiotics. Para-probiotics, also known as "non-viable probiotics", "inactivated probiotics" or "ghost probiotics", is a term used to indicate inactivated (non-viable) microbial cells, which, when administered in sufficient amounts, confer benefits to consumers **(Tsilingiri et al., 2013)**. The bacteria can be inactivated through different strategies such as mechanical disruption, heat treatment, γ- or UV irradiation, high hydrostatic pressure, freeze-drying, sonication, acid deactivation; this makes bacteria become incapable of growing and therefore retain the beneficial health effects their viable form provides **(Aguilar-Toalá et al., 2018)**. Post-biotics, also known as *cell-free supernatants*, are represented by metabolic bacterial products released after bacterial lysis. Post-biotics and para-probiotics can include biosurfactants, exopolysaccharides, cell surface proteins, teichoic acids, peptidoglycans, as well as various metabolic products like short-chain fatty acids, organic acids, hydrocarbons, phenols, amino acids, benzoic acids, alcohol, sugars, peptides, etc **(Nataraj et al., 2020)**, depending on the strain and the inactivation method.

The goal of post-biotics and para-probiotic is that these molecules mime the beneficial and therapeutic effect of probiotics, eluding the living microorganism administration to a host with a compromised immune system **(Vernocchi et at., 2020)**. Indeed, post-biotics compared to the live bacteria have some advantages such as: no risk of bacterial translocation from the gut lumen to blood among vulnerable and immunocompromised subjects; no chances of acquisition and transfer of antibiotic resistance genes; more natural to extract, standardize, transport, and store; loss of viability by cell lysis can produce further beneficial effects;

enhanced interaction of every released molecule from the disrupted cells with the epithelial cells **(Piqué et al., 2019)**. On the other hand, it is known that probiotics may influence the congenital and acquired immunological system through metabolites, components of the cellular wall, and DNA, recognised by specialised cells of the host (e.g., those equipped with receptors) **(Oelschlaeger, 2010)**.

Numerous studies reported that post-biotic and para-probiotic are able to act on the immune system, in a way that sometimes is similar, sometimes higher than their former bacteria; anyway, the effect is dose and strain specific. For example, it is hypothesized that post-biotic compounds from Lactobacilli *spp.* can exert immunomodulation activity by increasing levels of Th1-associated cytokines and reducing Th2-associated cytokines **(de Almada et al., 2016)**. Lactobacilli can also act on the immune system through the stimulation of anti-inflammatory IL-12p40 (*L. plantarum L-137* inactivated by heat) **(Fujiki et al., 2012)** and through an improved expression of pro-inflammatory cytokines and the transcription of Toll-like receptors (TLR2, TLR3, TLR4, and TLR9), which increase the innate response of macrophages (*L. casei LcZ*) **(Wang et al., 2013)**. Several in vitro studies have shown that heat-treated *Bifidobacterium* cells induce cellular immune and anti-inflammatory responses by inhibiting IL-8 secretion in intestinal epithelial cells obtained from patients with UC. It was suggested that these effects in UC patients derived cells are induced by released microbial soluble anti-inflammatory factors that inhibit IL-8 secretion in intestinal epithelial cells **(Wegh et al., 2019)**. Another *in vivo* study on the immunomodulatory activity of both live and sonicated cells from a probiotic preparation (VSL#3) showed that both of them were able to modulate cytokines production by splenocytes in mice sensitised with Par j 1 (the predominant allergen protein from the pollen of *Parietaria judaica*) towards a Treg/Th0 profile characterised by increased IL-10 and IFN-γ production. Treatment of mice by intranasal administration of sonicated VSL#3 cells before immunisation with recombinant Par j induced

a significant reduction in serum antigen-specific IgG1, markedly reduced IL-13 and IL-4 mRNA and increased IL-10 expression in the lungs. These findings showed that inactivated VSL#3 preparations not only had the capacity to bias primary immune responses towards a Treg/Th0-type profile but also to modulate the development of Th2-biased responses **(Taverniti et al., 2011)**.

Regarding IBD and IBS, literature on the use of probiotics in order to ameliorate symptoms of UC and CD or IBS shows different results; depending on the strain, some probiotic resulted to have effects on IBS and IBD symptoms, some others were potentially able to worsen the clinical aspect of patients with one of these pathologies. In this context post-biotic can be helpful as they can alleviate symptoms without trigger the immune system, which is the prominent adverse effect of probiotics in these diseases; a potent post-biotic can protect against the inflammatory properties of invasive pathogens on healthy tissue and also downregulate ongoing inflammatory processes in IBD tissue **(Tsilingiri et al., 2012)**. Regarding IBDs in particular, SCFAs (*Short-Chain Fatty Acids*) production has been associated with a reduced risk of manifesting the pathology **(Machiels et al., 2014)**.

Post-biotics not only play a vital role in the maturation of the immune system, but they also affect barrier tightness and the intestinal ecosystem, and indirectly shape the structure of the microbiota; these make post-biotics an excellent prospective of use in treating or preventing many diseases, including those for which effective causal therapy has not yet been found (like Alzheimer's disease, IBD, or multiple sclerosis). Indeed, clinical trials aimed at modifying the microbiota of patients suffering from the abovementioned diseases are currently underway, and the first results are promising **(Żółkiewicz et al., 2020)**.

To date, the use of post-biotics to improve human health can be considered as a potent therapeutic option in order to prevent or to treat inflammatory diseases, also due to the

improving of gut microbiota, which is a crucial protagonist of the maintenance of an healthy gut environment.

1.5 The gut microbiota

1.5.1 General aspects

The human organism has been defined as a conglomerate of eukaryotic and prokaryotic cells, in which the latter are in a much greater number than the first, so much that the microbial genetic repertoire, better defined as microbiome, seems to be about 100 times greater than that of the human host **(Fujimura et al., 2010)**. The human body is inhabited by at least 10 times more bacteria than the number of human cells, and the majority of these, are found in the gastrointestinal tract **(Savage, 1977)**, forming the so-called gut microbiota. The microbiota and the human body create a mutual relationship in which the host provides a friendly environment and nutrients, and, in turn, the gut microbiota shapes immune system development and function, reinforces the gut barrier, metabolizes undigested nutrients and xenobiotics, modulates enteric and central nervous system activity, and protects against pathogens **(Karl et al., 2018)**.

The concept of the human microbiome is reported to be first suggested by *Joshua Lederberg*, who used the term "microbiome", to signify "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" **(Lederberg et al., 2001)**. Yet, the word microbiome is a basic microbiology term in common use for at least 50 years and is used to describe a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity **(Prescott, 2017)**. For example, the first report of living creatures in the human gastrointestinal tract dates from 1681 when *Antonie van Leeuwenhoek* reported a variety of 'little animals' in his stool samples and identified what is now thought to be a *Giardia spp.* when suffering from diarrhea **(Rajilić-Stojanović et al., 2014)**.

Gut microbiota is composed of different cell lineages with a capacity to communicate with one another and with the host; it consumes, stores, and redistributes energy; it mediates physiologically important chemical transformations; and it can maintain and repair itself through self-replication **(Bäckhed et al., 2005)**. Although bacteria predominate, archaea and eukarya are also represented. Acid, bile, and pancreatic secretions hinder the colonization of the stomach and proximal small intestine by most bacteria. Bacterial density increases in the distal small intestine, and in the large intestine rises to an estimated 10^{11}–10^{12} bacteria per gram of colonic content, which contributes to 60% of faecal mass **(O'Hara et al., 2006)**.

Most of colonic bacteria are members of a resident population that has a long-term association with the host. Other microorganisms found in the colon can inhabit other sections of the gastrointestinal tract (GIT), such as the mouth or the small intestine, shedding alive to the colon but being unable to replicate in this environment **(Xu et al., 2003)**. Furthermore, microorganisms ingested with food and water can transit through the upper intestine and reach alive the hindgut, after being challenged by the low pH of the stomach, the digestive enzymes, and the toxicity of bile salts **(Rossi et al., 2016)**.

A large number of studies focus on the genera and species that colonize the intestine; it is known that a prevalence of anaerobic bacteria can be found in the human gut, yet both anaerobic and aerobic genera concur to create the human gut bacterial population **(O'Hara et al., 2006)**. Most diverse and abundant microorganisms found in the adult GI tract belong to the phyla *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria*; the gastrointestinal microbiota also contains members of the less diverse, although in some cases still abundant, bacterial phyla, including the *Verrucomicrobia, Lentisphaerae, Synergistetes, Planctomycetes, Tenericutes* and the *Deinococcus-Thermus* group **(Rajilić-Stojanović et al., 2014)**.

Both endogenous (genetics, age) and environmental (diet, medication) factors **(Heeney et al., 2018)** influence the composition of the microbiota, emphasizing how challenging it is to

define and compare microbial community structures within and between specified intestinal niches of a given individual at a particular point in his or her life history and to compare the microbiota among groups of individuals living in a particular geographic area or among more broadly distributed populations **(Xu et al., 2003)**. For example, it has been shown that the intestinal microbiota undergoes dynamic changes in diversity and composition during the human lifespan and particularly for the period of development with the most substantial changes believed to occur throughout childhood **(Radjabzadeh et al., 2020)**.

The colonization of the new-born from microorganisms starts in utero by microbiota in the amniotic fluid and placenta; indeed, microbes have been detected in the placenta, amniotic fluid, foetal membrane, umbilical cord blood, and meconium **(Collado et al., 2016).** Lately the composition of the primary gut microbiota of the baby is massively influenced by the type of deliver with a prevalence of *Lactobacillus* and *Prevotella* (derived from the mother's vaginal microbiota) in new-borns delivered vaginally, and of *Streptococcus, Corynebacterium*, and *Propionibacterium* (derived from the skin) in those born via caesarean delivery **(Dominguez-Bello et al., 2010)**. It was also found that, especially in the first months of life, *Bifidobacterium* is more abundant in vaginal delivered children than in those born through caesarean delivery; *Bifidobacteria* are health-associated microbes, able to promote gut health and provide defence against pathogens **(Reyman et al., 2019)**. This underlines the importance of the early-life gut microbiota development for a balanced priming of the immune system; indeed birth by caesarean section (CS) has been associated with adverse effects on immune development, predisposing to infections, allergies, and inflammatory disorders **(Reyman et al., 2019).** Early-life assembly of the gut microbiota is also believed to play a role in subsequent emotional and cognitive development with impact on host health and leading or preventing disorders such as obesity, diabetes, inflammatory diseases, and even potentially neuropsychiatric illnesses, including anxiety and depression **(Wiley et al., 2017)**. After the birth, environmental factors

like diet (included the way of breast-feeding **(van den Elsen et al., 2019)**), the use of antibiotics and the environment, influence the modifications in the gut of the child **(Tamburini et al., 2016)**. For example, a meta-analysis conducted on seven studies about the effect of breast-feeding on gut microbiota of the infant, showed a consistent increase in the relative abundance of *Bacteroidetes* in non-exclusive breast-feeding (non-EBF) vs. exclusive breast-feeding (EBF) infants in all seven studies, and an overall significant increase in relative abundance of *Firmicutes*; the study showed a persistent increase in relative abundances of genera *Bacteroides, Eubacterium* (that are the most common bacterial genera in gastrointestinal tract of adults), and *Veillonella* in non-EBF vs. EBF infants **(Ho et al., 2018)**. *Lactobacillus, Staphylococcus, Enterococcus,* and *Bifidobacterium* are transferred through breastfeeding as they can be found both in breastmilk and in the mother's skin (e.g., *Staphylococcus*) **(Fernández et al., 2013)**. Non-EBF or shorter duration of EBF in the first 6 months of life has been associated with a bacterial composition more closely resembling the adult microbiota, higher relative abundance of bacterial functional pathways related to carbohydrate metabolism, and lower relative abundance of the ones related to lipid metabolism, detoxification, and cofactor and vitamin metabolism **(Ho et al., 2018)**. Another recent study observed a higher relative abundances of genus *Bacteroides* in children and higher relative abundances of genus *Blautia* in adults. Predicted functional metagenome analysis showed an overrepresentation of the glycan degradation pathways, riboflavin (vitamin B2), pyridoxine (vitamin B6) and folate (vitamin B9) biosynthesis pathways in children, in contrast with the gut microbiome of adults, which showed higher abundances of carbohydrate metabolism pathways, beta-lactam resistance, thiamine (vitamin B1) and pantothenic (vitamin B5) biosynthesis pathways, concluding that the predominance of catabolic pathways in children (valine, leucine and isoleucine degradation) as compared to biosynthetic pathways in adults (valine, leucine and

isoleucine biosynthesis) suggests a functional microbiome switch to the latter in adult individuals **(Radjabzadeh et al., 2020)**.

Nevertheless, the breastmilk microbiota evolves over the period of breastfeeding, since colostrum microbiota has a higher diversity than mature milk, influencing the shaping of the infant gut microbiota **(Cabrera-Rubio et al., 2012)**.

The changes and modelling of the gut microbiota in infants occur until the age of three, when the microbiota become to be more stable and similar to that of the adult **(Yatsunenko et al., 2012; Gensollen et al., 2016)**.

After this age, the microbiota is more stable and it can change in response to environmental stresses like toxic/inflammation products, antibiotics or dietary elements, that can alter the microbial population in a way that is often reversible **(Martens et al., 2018)**; moreover, microbial population can also be modified in a positive manner, with the administration of probiotics or prebiotics **(Hasan et al., 2019)**.

Anyway, differences in adult microbiota are more often found between different individuals, which have distinct diets and lifestyles, than the same adult during life. Children are significantly more diverse from one another than adults are in terms of their faecal bacterial community phylogenetic structure and of their repertoires of microbiome-encoded functions **(Yatsunenko et al., 2012).**

The influence of the environmental and geographical differences in gut microbiota of adults can be explained through the divergencies in diet composition within geographical areas; gut microbiota shape itself in the first years of life in order to adapt to the energy sources that arrive in the lumen. Studies have shown that host specificities or diet-related differences may explain variations observed in the microbiota composition **(Mueller et al., 2006).** For example, a study about the discrepancies of microbiota within age and geography in Malawian, Amerindian and USA population, conducted by *Yatsunenko*, showed that the most prominent

differences, in terms of ECs (enzyme classification, which is a way to understand the indispensable role played by microbe-encoded enzymes in the healthy functioning of human metabolic systems **(Mohammed et al., 2015)**), involved pathways related to vitamin biosynthesis and carbohydrate metabolism as long as their availability change between populations and during the growth. Malawian and Amerindian babies had higher representation of ECs that were components of the vitamin B2 (riboflavin) biosynthetic pathway compared to USA, while these differences were not evident in adults. Riboflavin is found in human milk and in meat and dairy products, so they speculate that the observed differences in baby microbiomes may represent an adaptive response to vitamin availability. Also compared to adults, baby microbiomes were enriched in ECs involved in foraging of glycans represented in mother's milk and the intestinal mucosa and a number of genes involved in utilizing these host glycans are significantly overrepresented in Amerindian and Malawian compared to USA baby microbiomes, while the glycoside hydrolases decreases as Malawian and Amerindian babies mature and transit to a diet dominated by maize-, cassava and other plant-derived polysaccharides **(Yatsunenko et al., 2012)**. In contrast, alpha-fucosidase gene representation increases as USA infants age and are exposed to diets rich in readily absorbed sugars **(Yatsunenko et al., 2012)**. Another example of how diet can interact with microbiota composition can be found in studies that reported a variation in bacteria population between vegetarian **(Walker et al., 2011)** and *Western diet* **(David et al., 2014)**. Vegetarian diets have been found to be associated with healthy diverse gut microbiota characterized by the domination of species that can metabolize insoluble carbohydrates, such as *Ruminococcus, Roseburia*, and *Eubacterium*, while a *Western diet* has been associated with a decreasing number of *Firmicutes* and an increase in *Bacteroides* **(Hasan et al., 2019)**.

The relationship between diet and microbiota is bidirectional as diet can shape microbiota and this latter can help the host to digest complex dietary macromolecules into

metabolites that vertebrates can then utilize nutritionally. For instance, the catabolism of fibers, large plant polysaccharides, requires enzymes that are expressed by the microbes and not the host; indeed, by using food fibers for their own metabolic benefit, microbes provide the host with end products such as simpler polysaccharides. The different metabolic pathways and requisites of distinct microbes provided in the host diet might be a key factor shaping the microbiome **(Ochoa-Repáraz et al., 2016)**.

1.5.2 Gut microbiota in human health

1.5.2.1 Early gut microbiota as factor that predisposes or protects from adult diseases

Human diseases have been increasingly correlated with faecal microbiota composition **(Heeney et al., 2018)**. Several studies have reported an association between alterations in the composition of the gut microbiome and various GI and non-GI disease conditions in both children and adults **(Radjabzadeh et al., 2020)**. Intestinal disorders include IBD, IBS, and coeliac disease, while extra-intestinal disorders include allergy, asthma, metabolic syndrome, cardiovascular disease, and obesity **(Carding et al., 2015)**. In general, with few exceptions, a more diverse gut microbiota, both in composition and genetic content, is considered a healthier microbiota **(Blaser et al., 2009)**.

The composition of the bacterial population, which is not identical in each human subject, with the prevalence of one specie against another, is a factor that may favour a health or a disease condition; the predisposition to a particular wellbeing condition is related to genes, but an association has been also found between particular species in infant gut microbiota and the development or prevention of diseases in the adulthood. The prove of this is the fact that developing a certain early microbiota, predispose the young child to be an adult with more or less prevalence of diseases like autoimmune problems, allergies, GI troubles. For instance, in their meta-analysis, *Ho et al.*, reported that the infant gut microbiota can exert a protective effect against diarrhoea, morbidity, and mortality and that it decreases long-term risk of diabetes and obesity, in breastfeeding infants compared to non-breastfeeding ones, which have a different relative abundance of the species composing their microbiota **(Ho et al., 2018)**. Moreover, the predisposition to infections, allergies, and inflammatory disorders have been associated with the hight relative abundance of *Bifidobacteria* in early microbiota **(Reyman et**

al., 2019). *Bisgaard et al.* demonstrated the inverse association between the early gut bacterial diversity and the risk of allergic sensitization, even if they did not report a relationship with asthma or atopic dermatitis **(Bisgaard et al., 2011)**. This association is also supported by another study that reported an increased abundance of *Lachnospiraceae* in infants with allergy to cow's milk compared to non-allergic ones **(Canani et al., 2016)**.

Regarding the adult gut microbiota, environmental stressor (like diet, lifestyle, toxin, drugs, pathogens, but also everyday stress) can cause an alteration in microbial population that leads to negative health conditions, with the increase or decrease in relative abundance and diversity of bacterial specie of GI and other body sites **(Belizário et al., 2018; Karl et al., 2018)**. When these changes, which can be temporary, occur, the result is a situation defined as dysbiosis. The term dysbiosis refers, in literature, to three main definitions: a general change in the microbiota composition (e.g., alteration, perturbation, abnormal composition, and loss of diversity), an imbalance in composition (almost always deemed to have negative effects), and a change to specific lineages in that composition (any named taxon change) **(Hooks et al., 2017)**. There is growing evidence that dysbiosis of the gut microbiota is associated with the pathogenesis of both intestinal and extra-intestinal disorders **(Carding et al., 2015)**.

An important association is that between gut microbiota and obesity; indeed even if bacterial population is different from healthy subjects, the functional gene profile associated with each microbiota tends to be similar: microbiota is reported to affect the intestinal genes expression related to energy balance, the regulation of intestinal barrier function, the intestinal satietogenic hormones release, the bile acids metabolic activity modulation, the digestion/absorption of nutrients by intestinal mucosa of the host, and the generation of short-chain fatty acids (SCFAs) via non-digestible carbohydrates bacterial fermentation **(Rivera-Piza et al., 2020)**. The most reported variation in gut microbiota from healthy subjects to obese is the reduction of abundance of *Bacteroidetes* and the increase of *Firmucutes* **(Kallus et al., 2012)**. More detailed, high-fat and/or high carbohydrate diet programmes the gut microbiota to one predominated by *Firmicutes, Prevotella* and *Methanobrevibacter* but deficient in beneficial genera/species such as *Bacteroides, Bifidobacterium, Lactobacillus* and *Akkermansia* **(Amabebe et al., 2020)**. Furthermore, when obese volunteers consumed a low-fat or low-carbohydrate diet for one year and lost as much as 25% of their body weight, the

proportion of *Firmicutes* in their colon decreased and that of the Bacteroidetes increased **(Ley et al., 2005)**. Obesity is strictly connected with metabolic syndrome, fatty liver disease, type 2 diabetes and cardiovascular disease. Since microbiota is related to obesity, it is also related to these conditions.

1.6 Gut microbiota metabolites

Functions of microbiota are related to the gut as well as to the systemic environment where it owns important and multiple functions. Gut microbiota is involved in protective, structural, and metabolic tasks. For example, microbes can exert protective roles against pathogens and dangerous products through the production of anti-microbial metabolites and the competition against pathogens for nutrients and receptors **(O'Hara et al., 2006)** and by metabolizing xenobiotics (such as the inactivation of heterocyclic amines formed in meat during cooking) **(Davis, 2016)**; they can also exert structural activities like acting on the barrier improvement (apical tightening of the TJs); they can control the IECs differentiation and proliferation, metabolize dietary carcinogens, synthesize vitamins, ferment non-digestible dietary residue and endogenous epithelial-derived mucus, act on the absorption of ions and produce short chain fatty acids. Gut bacterial population can also take action positively on the immune system and induce the production of IgA **(O'Hara et al., 2006)**.

The prominent way through which microbiota acts to help the host against possible pathologies and damages is by providing a large number of metabolites that are then absorbed by the gut. Indeed, the crosstalk between the host and its microbiome occurs in part through the secretion of metabolites, which have a profound effect on host physiology **(Levy et al., 2017)**. The microbiota has an important role in the regulation of the digestion, as they metabolize nutrients deriving from diet, releasing 'specialized metabolite' that may modulate immune cells through direct and indirect mechanisms in the context of health and disease **(Vernocchi et al., 2020)**.

Nevertheless, while several studies provided lots of information about gut microbiota composition and how it is influenced by the environment, nowadays, an increasing relevance has been given to the molecules that they are able to produce; metagenomic sequencing and other multi-omic technologies, such as metatranscriptomics, metaproteomics, and

metabolomics, are giving an increasing knowledge about in this field **(Martinez et al., 2017)**, important in order to achieve more therapeutical strategies leading to the shaping of the microbiota, most made through diet. Indeed, bacteria living in the gut are important for the processing and absorption of several nutrients and metabolites deriving from diet, including bile acids, lipids, amino acids (AAs), vitamins, and short-chain fatty acids **(Brestoff et al., 2013)**. As these molecules are linked to diet, is not surprisingly that it is the major exogenous factor able to shape the microbiota as well as the metabolic profile leading to the different species. Indeed, diet provides microorganisms with diverse types of macro and micro-nutrients which are able to select a population or another, as different microbial species own specific metabolic pathways; nevertheless, those nutrients are used by bacteria in order to produce metabolites that can influence the host in both a positive or a negative manner, or, in other words, to a healthy or a pathological status **(Vernocchi et al., 2020).**

For example, a study performed in 2010 on the relationship between diet and the gut microbiota, reported the differences in faecal microbiota between European children (EU), which diet was a *Western diet*, mostly based on fast food, and that of children from a rural African village of Burkina Faso (BF), where the diet was high in fiber; in finding different species abundances between EU and BF, and a higher production of SFCAs in the second group, De Filippo et al. hypothesized that gut microbiota coevolved with the polysaccharide-rich diet of BF individuals, allowing them to maximize energy intake from fibers while also protecting them from inflammations and non-infectious colonic diseases **(De Filippo et al., 2010)**. Similarly, De Filippis et al., in a study conducted on how Mediterranean diet beneficially impacts the gut microbiota and associated metabolome, detected significant associations between consumption of vegetable-based diets and increased levels of faecal short-chain fatty acids, *Prevotella* and some fibre-degrading *Firmicutes;* on the other hand omnivorous volunteers having low adherence to the Mediterranean diet (MD) had the highest

levels of detrimental microbial metabolites, such as phenolic and indole derivatives, and trimethylamine N-oxide (TMAO) **(De Filippis et al., 2016)**.

Gut microbes are able to metabolize indigested nutrients arriving in the colon, through anabolic pathways; this leads to the release of different types of metabolites, depending on the prevalent dietary pattern followed by the host, as microbiota can act on polysaccharides, mostly undigested fibers, protein and amino-acids, and lipids. While saccharolytic fermentation takes place mainly in the proximal colon, as most bacteria choose to utilize carbohydrates instead of proteins, proteolytic fermentation occurs in the distal colon, producing branched-chain fatty acids (BCFAs) and potentially detrimental metabolites such as ammonia (from amino acid deamination and urea hydrolysis), indoles, and phenols (from amino acid (AAs) carboxylation) **(Vernocchi et al., 2020)**.

1.6.1 Metabolites from carbohydrates: short-chain fatty acids (SCFAs)

The fermentation of complex carbohydrates, performed in the distal colon by gut bacteria, involves oligosaccharides and polysaccharides from the diet or mucins derived from host secretions. Especially relevant in the context of human health is the creation of metabolic end products known as short-chain fatty acids (SCFAs); SCFAs are carboxylic acids with aliphatic tails of 1–6 carbons of which acetate (C2), propionate (C3), and butyrate (C4) are the most abundant produced by anaerobic fermentation of dietary fibers in the intestine **(Parada Venegas et al., 2019**), but also from amino acids resulting from protein breakdown **(Louis et al., 2017)**. As a proof that SCFAs are formed in high amount in the intestine and that they exert their prevalent action in this site, it has been demonstrated that their levels are lower in other tissues such as liver or blood **(Cummings et al., 1987)**. The main substrates for bacterial fermentation and SCFA production are resistant starch (RS), inulin, oat bran, wheat bran, cellulose, Guar gum, and pectin **(Parada Venegas et al., 2019**).

While butyrate and propionate are especially involved in the regulation of the physiology of the intestine and in immunomodulation, acetate acts as a substrate for lipogenesis and gluconeogenesis **(Shahid et al., 2018**). SCFAs are also an energy substrate for enterocytes.

Butyrate is one of the most studied microbiota metabolites; its production starts from carbohydrates via glycolysis: two molecules of acetyl-CoA are combined to form acetoacetyl-CoA, which is then reduced to butyryl-CoA; the final formation of butyrate from butyryl-CoA, is operated either via butyryl-CoA-acetate CoA-transferase or via phosphotransbutyrylase and butyrate kinase **(Louis et al., 2009)**. Butyrate can also be produced from organic acids, glutamate and lysine **(Louis et al., 2017**).

Propionate can derive from carbohydrates, organic acids, and amino acids. With a focus on carbohydrates, in order to form propionate, hexose and pentose sugars are processed through

the succinate pathway whereas the deoxy sugars fucose and rhamnose are metabolized by the propanediol pathway **(Louis et al., 2017)**.

Lastly, acetate can occur from fermentation of fibers, as well as the ones of peptides and fats. Acetate is mainly generated from fibers via acetogenesis or via the carbon fixation pathway. Acetogenesis is mediated by homoacetogenic bacteria or acetogens (found in the digestive tract of humans and ruminants), which are capable to produce acetate from H_2 and carbon dioxide (CO_2); on the other hand, the carbon fixation pathway (also known as *Wood-Ljungdahl* pathway) produces acetate from CO_2 as a precursor **(González Hernández et al., 2019)**.

Clostridium clusters such as *Eubacterium*, *Roseburia*, *Faecalibacterium*, as well as *Lactobacillus spp*, *Bifidobacterium spp*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii* and *Ruminococcus bromii* are the bacteria known to be most implicated in SCFAs metabolism from polysaccharide derived from plants (leading to the formation of SCFAs and gases), undigested oligosaccharide and water-soluble fiber (leading to the formation of SCFAs and lactate) **(Vernocchi et al., 2020)**.

SCFAs have been largely studied because of their multiple beneficial effects on the host health, especially through their activity on the immune system. For example, a recent study showed that SCFAs promote IL-22 (which is critical for intestinal immunity) production by CD4+ T cells and ILCs through G-protein receptor 41 (GPR41) and inhibit histone deacetylase (HDAC) **(Yang et al., 2020)**. In general, as reviewed by Vernocchi et al., important functions of SCFAs are triggering of Foxp3+ T regulatory (Treg) cells and tolerance, induction of IgA secretion from B cells, “competitive exclusion”, due to high-fiber diet that spreads commensal bacteria and limits the access of pathogenic bacteria to the gut epithelium, promotion of mucus secretion by gut epithelial cells, contribution to the intestinal barrier integrity, in particular by stimulating the formation of the proteins of TJs, such as claudin, occludin, and zonulin as well

as promotion of tissue repair and wound healing, and inhibition of the proinflammatory transcription factor (NF-κB) and decreasing of oxidative stress **(Vernocchi et al., 2020)**.

An important role of SCFAs is that regarding the improvement of the intestinal membrane integrity, as it leads to both intestinal and systemic pathologies and to an increased level of inflammation and oxidative stress; in particular, butyrate maintains and/or increases transepithelial electrical resistance (TEER) in *in vitro* studies on multiple types of cell lines. In an *in vitro* system simulating the mucus- and lumen-associated microbiota where butyrate-producing bacteria (three mono-species and one multispecies mix) were supplemented to the faecal microbial communities of ten Crohn's disease patients, researchers found an increase in epithelial barrier integrity **(Geirnaert et al., 2017)**. The same effect has been reported for rat small intestine cdx2-IEC monolayers, where the supplementation with sodium butyrate enhanced intestinal barrier function through increasing Claudin-1 transcription via facilitating the association between SP1 and Claudin-1 promoter **(Wang et al., 2012),** and for small intestine porcine IPEC-J2 cells exposed to LPS, through a selective upregulation of TJ proteins and activation of the Akt signaling pathway **(Yan et al., 2017)**. In Caco-2 cell monolayers, the treatment with butyrate is able to enhances the intestinal barrier by facilitating TJs assembly via activation of AMP-activated protein kinase **(Peng et al., 2009),** while on T84 cells, the same result was obtained after administration of microbial-derived butyrate acting through IL-10 receptor-dependent repression of Claudin-2 **(Zheng et al., 2017)**.

The reduction of the intestinal membrane permeability and the following inflammation is strictly connected with pathologies characterized by a low-grade inflammation such as IBDs. SCFAs are reported to be able to ameliorate both intestinal membrane permeability (as described above) and inflammation, for example acting as ligands for G-protein coupled receptors (GPCRs), including GPR109A, GPR43, and GPR41, activating anti-inflammatory signalling cascades **(Parada-Venegas et al., 2019)**. Several studies have been conducted in

order to find the correlation between IBD and gut microbiota or its metabolites. An anti-inflammatory role for one of the SCFAs, butyrate, is nowadays strongly sustained and we know that low levels of this metabolite may be involved in the pathogenesis of IBDs. For example, different gut microbiota metabolites, in particular SCFAs and secondary bile acids, were found in patients who achieved remission, in a double-blind trial where 81 individuals with active ulcerative colitis were randomly assigned to groups that received an initial colonoscopic infusion and then intensive multidonor faecal microbiota transplantation or placebo enemas, 5 days a week for 8 weeks **(Paramsothy et al., 2019)**. A recent study presented the molecular mechanisms involved in the anti-inflammatory effect of butyrate against IBDs as conducted through histone deacetylase inhibitor activity, activation of PPARγ and of GPR109A, GPR41 and GPR43 receptors; the study highlights that the histone deacetylase inhibitor activity of butyrate depends on its absorption by colonocytes, suggesting that butyrate transporters are also important for the anti-inflammatory effect at colonic level **(Couto et al., 2020)**. GPR43 are central in SCFA-induced neutrophil chemotaxis36 and for the expansion and suppressive function of T-reg cells44; GPR109A prevented colitis and colon carcinogenesis through increased expression of anti-inflammatory effector molecules by monocytes and induced differentiation of T-reg cells and interleukin-10 (IL-10)-producing T cells **(Rooks et al., 2016)**.

Free SCFAs can also cross the blood-brain barrier, thus acting on central nervous system; quantitative analysis of cerebrospinal fluid by high-resolution nuclear magnetic resonance spectroscopy (HR-NMR) has revealed the existence of approximately 30 – 40 μM SCFAs in CSF, and in particular, acetate serves as an important energy source for astrocytes in brain **(Hu et al., 2018)**. The capacity of SCFAs to pass the blood-brain barrier suggest their role in neurological functionalities **(Martinez et al., 2017)**. The action of SCFAs in the brain is also reported as connection between the gut and the brain; animal and human data demonstrated that, in particular, acetate beneficially affects host energy and substrate

metabolism via secretion of the gut hormones as glucagon-like peptide-1 and peptide YY, which, thereby, affects appetite, via a reduction in whole-body lipolysis, systemic pro-inflammatory cytokine levels, and via an increase in energy expenditure and fat oxidation **(González Hernández et al., 2019)**.

At systemic level, SCFAs have been shown to have effect on blood pressure, in particular via the sensory receptors Olfr78 (*Olfactory receptor 78*) and GPR41 (*G protein-coupled receptor 41*), acting in opposition to one another **(Pluznick, 2014)**; in hypertension patients compared to a control group, was evidenced a decrease in relative abundance of several SCFAs (mainly butyrate) producers, including members of the *Lachnospiraceae* and *Ruminococcaceae* families, such as *Roseburia* and *Faecalibacterium* **(Silveira-Nunes et al., 2020)**.

2. Aim of the study

The human gut represents a key structure in the selection of molecules and nutrients that the body demands to absorb, through digestion, and what it needs to block. It establishes a connection between the internal and external environment and has a selective control on what arrives in its lumen and passes through it **(Salvo Romero et al., 2015)**.

These functions are allowed by the intestinal membrane, a barrier that includes physical, chemical/biochemical and immunological components, and represents the first line of host defense against the external environment and against both commensal bacteria (in order to block them in the lumen where they absolve their roles) and invading enteric pathogens **(Martens et al., 2018)**. Indeed, the intestinal mucosa owns important functions like nutrient absorption, excretion and secretion of different products, barrier against pathogens **(Shimizu et al., 2007)**.

The mucosal barrier is indispensable for the maintenance of the gut environment and to prevent intestinal inflammation; when the production of mucosal barrier components such as mucus and antimicrobial peptides is altered, this situation leads to an increase in membrane permeability and a malfunctioning of the physical barrier itself, that may be easily passed by undesirable elements causing inflammation **(Okumura et al., 2018)**. On the other hand, inflammation has been demonstrated to impact intestinal integrity, determining the loss of epithelial barrier function, a crucial factor in the initiation and progression of pathological intestinal disorders such as IBDs and cancer. This was observed for example in the intestine of some IBD patients; some evidence reported that IBD arises when the mucosal barrier is compromised in its defense against challenges from the intestinal microbiota and, in particular in ileal disease, a strong association can be found between diminished expression or defective function of defensins and the advent of intestinal inflammation **(Jäger et al., 2013)**.

Despite all the protective mechanisms that allow the gut membrane to work properly (mucus, GALT, microbiota, TJs) it is possible that both external and internal stimuli cause an

alteration in its functionality. In this context, diet represents a central factor, as through it we introduce both dangerous and protective compounds. To the first group belong for example oxysterols, while in the second resides probiotics. Oxysterols have been shown, in *in vitro* studies, to alter intestinal barrier integrity, through a complex pro-oxidant and pro-inflammatory action not fully elucidated yet **(Vejux et al., 2009).** Indeed, a high intake of oxysterols has been associated to the onset of IBDs and cancer **(Poli et al., 2013; Kloudova et al., 2017; Willinger, 2019)**.

However, it appears that oxysterol-dependent intestinal damage might be prevented by antioxidant and anti-inflammatory agents. In the last decade, several studies highlighted the ability of probiotic Lactobacilli to exert antioxidant and anti-inflammatory action in the gut lumen, through a direct effect on the intestinal mucosa or by the modulation of the bacterial population that resides in the gut **(Ayyanna et al., 2018; Ghyselinck et al., 2020)**.

Nevertheless, diet is the prominent factor that influences the composition of the gut microbiota and, more important, its metabolism; indeed, resident gut bacteria are known to use macro- and micro-nutrients arriving from food to produce small molecules, like SCFAs, which are the key point of the crosstalk between gut microbiota and the host **(Vernocchi et al., 2020)**, and accountable for most of the beneficial effects ascribed to a healthy microbiota **(Paramsothy et al., 2019; Yang et al., 2020)**.

In this context, the aim of the present research project was to evaluate the possible protection exerted by probiotics against oxysterols-induced alteration of the intestinal permeability and to investigate the mechanism of action in relation to cellular signaling. A preliminary study was also conducted to investigate any modifications induced by oxysterols or probiotics on the metabolic activity of the microbiota.

We choose two different strains of probiotics, *Lactobacillus plantarum 299v®* (DMS 9843) and *Lactobacillus casei DG®* (CNCMI1572), already commercialized for their

properties. As administration of probiotics leads to an action exerted by bacteria arriving alive in the gut, but also by the cellular content they release after death, we decided to study both the bacterial extract obtained from the two strains, and the bacteria as pure cultures. This because a major knowledge about the post-biotic compounds, including the cellular content of probiotic bacteria, is a goal to achieve in order to ameliorate and personalize therapeutic strategies; indeed, the use of post-biotics instead of the probiotic itself could avoid the living microorganisms' administration to a host with a compromised immune system **(Vieira et al., 2016)**.

The oxysterols were tested as a mixture containing a representative percentage and concentration of the most widely oxysterols present in cholesterol-rich foods: 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol **(Plat et al. 2005; Biasi et al., 2009)**. The concentration used is reported to cause oxidative stress and inflammation on intestinal cell models in vitro **(Vejux et al., 2009)**. The research project was focused on three objectives:

- The first was to evaluate the alteration of the intestinal membrane permeability induced by oxysterols and to investigate the potential protective role played by Lactobacilli bacterial extract. To achieve this objective, we used an *in vitro* intestinal barrier model represented by monolayers of differentiated Caco-2 cells, human adenocarcinoma-derived cells that, once differentiated, assume phenotypical characteristics of human enterocytes. The value of the transepithelial electrical resistance (TEER) was measured to monitor the alteration of permeability of the cell monolayers, treated with an oxysterol mixture alone or together with the bacterial extract. To explore the mechanism underlying the alteration of the permeability, the modulation of tight junctions, occludin, zonulin and JAM-A, was investigated in relation to MAPKs, p38 and ERK1/2 activation.

- The second objective was to evaluate the effect of the bacteria as pure culture against oxysterols induced intestinal damage. Live Lactobacilli were co-cultured with Caco-2 cells and adherent bacteria were tested for their protective ability in the same experimental conditions used for the bacterial extract.
- The third objective was to evaluate the interaction between oxysterols and/or probiotics (bacterial extract or pure culture) and the microbiota metabolites production. For this aim, in a preliminary study, batch cultures were prepared by inoculating a human fecal sample from a healthy donor into vessels containing a basal medium and treating it with oxysterols alone or with the bacterial extract or the pure culture of *L. plantarum 299v* or *L. casei DG*; samples were collected and analyzed through NMR, to determine the relative abundance of the major biologically relevant SCFAs.

3. Materials and methods

3.1 Reagents and chemicals

5-cholesten-3β,7α-diolo (7α-hydroxycholesterol) was purchased from Avant Polar Lipids (Alabaster, Alabama, USA). 5-cholesten-3β-ol-7-one (7-ketocholesterol), 5-cholesten-3β,7β-diolo (7β-hydroxycholesterol), cholesterol 5α,6α-epoxide (5α,6α-epoxicholesterol), cholesterol 5β,6β-epoxide (5β,6β-epoxicholesterol), 2,5-Diphenyltetrazolium Bromide (MTT), dimetyl-sulfoxide (DMSO), Bradford reagent, CellLytic-M, NaCl, K_2HPO_4, KH_2PO_4, $MgSO_4x7H_2O$, $CaCl_2x6H_20$, $NaHCO_3$, tween 80, vitamin K, L-Cysteine, resazaurin and all solvents of analytical grade were purchased from Sigma Aldrich (Milano, Italy). Nitrocellulose membranes, gels and all material for electrophoresis and immunoblotting, yeast extract, peptone water and bile salts were purchased from ThermoFisher (Massachusetts, United States). Vessels and all component of the batch cultures system were purchased from Soham Scientific (UK).

3.2 Caco-2 cell cultures

Caco-2 cell line was purchased from ECACC (Salisbury, UK). Caco-2 are cells from human colorectal adenocarcinoma which, once reached confluence, spontaneously differentiate into intestinal epithelial cells, thus expressing all the characteristics of enterocytes.

3.2.1 Materials for cell cultures

Phosphate-buffered saline (PBS) with and without $MgCl_2$ and $CaCl_2$, Dulbecco's modified eagle medium (DMEM) with low glucose and with L-Arginin, bovine foetal serum (FBS) and penicillin/streptomycin 1X were purchased from Euroclone (Milano, Italy). Transwell inserts were obtained from Corning Costar Corp. (New York, N.Y., USA). EZBlockTM Phosphatase Inhibitor Cocktail II, and EZBlockTM Protease Inhibitor Cocktail were purchased from ThermoFisher (Massachusetts, United States).

3.2.2 Maintenance of cell cultures

Caco-2 cells were maintained in T75 flasks until their confluence reached the 80%, in D-MEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin) at 37°C in a 5% CO_2 humidified atmosphere. At passage 45-60, cells were removed from flasks by adding a trypsin solution at 1% and incubating at 37°C for 5-10 minutes; Caco-2 were collected, centrifuged, counted in a *Burker chamber* and then seeded into 6 or 24 well plates at a concentration of 5×10^4/mL for different experiments. Cells were incubated for 14-21 days, replacing the medium twice weekly, to allow their spontaneous differentiation.

3.3 Oxysterol mixture

3.3.1 Preparation of the oxysterol mixture

A mixture of the most widely represented dietary oxysterols was prepared using pure standard compounds diluted in ethanol. The oxysterols and their relative percentage used in the mixture were: 7α-hydroxycholesterol (4.26%), 7β-hydroxycholesterol (14.71%), 7-ketocholesterol (42.96%), 5α,6α-epoxycholesterol (32.3%), 5β,6β-epoxycholesterol (5.76%) **(Plat et al., 2005)**. The concentration of the oxysterol mixture was calculated using an average molecular weight of 403 g/mol **(Biasi et al., 2009)**.

3.4 Bacterial strains

All the experiments were conducted using two bacterial strains known to exert probiotic activities (*Lactobacillus plantarum* 299V® and *Lactobacillus casei DG*, LP-DG® - *Lactobacillus paracasei* CNCM I-1572) isolated from the commercial probiotic preparations, respectively *Probi Mage®* **(Johansson et al., 1993)** and *Enterolactis* **(Radicioni et al., 2019)**, in collaboration with the Laboratory of Applied Hygiene (Department of Medical Science and Public Health), University of Cagliari. The probiotic strains were tested as bacterial extracts obtained as described in the next paragraph and used at a concentration of 70 µL/mL or as pure culture of live bacteria, at a concentration of 10^8 CFU/mL.

Bacterial strains were isolated from commercial probiotic preparations. Briefly, one gram or mL of sample was suspended in 9 mL of *De Man Rogosa Sharpe agar* (MRS, Microbiol, Cagliari Italy) broth and incubated at 30 °C in 5% CO_2 for 1 h. Then 0.1 mL was cultivated in MRS agar in microaerophilic conditions at 30 °C for 48 h. After taxonomic identification, the probiotic isolates were maintained at -20°C in MRS broth with 15% (v/v) glycerol (Microbiol, Cagliari, Italy) and propagated three times in MRS broth for activation prior to experimental use.

3.4.1 Bacterial extracts (preparation of cell free-supernatant)

Bacterial extracts, from *L. plantarum 299v* and from *L. casei DG*, were prepared in collaboration with the Laboratory of Applied Hygiene. Briefly, the overnight bacterial cultures were harvested by centrifugation (6000 rpm for 15 minutes at 4°C) washed three times and resuspended in 5 mL of PBS. The bacterial count in the suspension was in the range of 1-9 x 10^9 CFU/mL. After treatment with 1 mg/mL lysozyme at 37°C for 1 h, the cells were subjected to ultrasonic disruption. Sonication was carried out at 4°C for 5 minutes followed by a break of 20 seconds, for 10 times in order to break cell wall and cell membrane and collect cellular

content. Cell debris were then removed by centrifugation at 6000 rpm for 20 minutes at 4°C and the resulting supernatant was filtered through a 0.45 µm pore-size cellulose acetate filter (Millipore, Bedford MA, USA) prior to use as intracellular cell free extracts of the two probiotic *Lactobacillus* strains.

3.4.2 Bacterial pure culture

Bacteria from commercial probiotic preparations were revitalized and grown in MRS broth with the same procedure used to prepare the bacterial extract: bacteria were added into MRS broth in a concentration of 1:10 and incubated at 30°C, 5% of CO_2 for 48 h and then incubated in new MRS until they were revitalized. In order to remove MRS, bacterial solutions were centrifuged at 6000 rpm for 15 minutes and suspended in PBS for three times. Dilutions were prepared in order to evaluate the optical density (OD_{600}) of the culture. The OD was used to determine the volume of solution to be added in DMEM to have a final concentration of 10^8 CFU/mL. The DMEM solution containing bacteria was used for treatment of Caco-2 cell monolayers.

3.5 Bacterial adhesion to Caco-2 cell monolayers

Adhesion tests were conducted in order to evaluate if and how the two strains of probiotics were able to adhere to Caco-2 cell monolayers, a preliminary step to exert their protection against oxysterols-caused damage on intestinal membrane permeability. The overnight cultures of the two *Lactobacillus* strains were centrifuged at 6000 rpm for 15 minutes and the pellet washed twice with PBS and diluted to the concentration of 2 x 10^8 CFU/mL in DMEM (serum and antibiotic free). For the adhesion assay cells were seeded in 6-well plates and cultured for 14 days as described above; cell culture medium was changed every other day and replaced by fresh DMEM supplemented with 2% (w/v) FBS and without antibiotic at least

1 h before the adhesion assay. The cells were pre-treated with 1 mL aliquot of the probiotic *Lactobacillus* strains at a concentration of 2 x 10^8 CFU/mL for 1 hour before adding the oxysterol mixture at a concentration of 60 µM and incubated at 37°C, 5% of CO_2. After 3- and 24-hours, cells with adherent bacteria were washed three times with 1mL of PBS in order to remove nonadherent bacteria and lysed by addition of Triton X 100 (0.05% solution) for 10min; then appropriate dilutions were plated on MRS agar. Adhesion was expressed as the percentage of bacteria adhered to Caco-2 cells compared to the initial number of bacteria.

3.6 Transepithelial electrical resistance

Caco-2 cells were plated in transwell plates (polycarbonate membrane with 0.4 µm diameters pores, Sigma-Aldrich) in a concentration of 5x10^4 cells/mL in 500 µL of growth media and used to measure the transepithelial electrical resistance, TEER **(Serreli et al., 2017)**. Briefly, cells differentiated on the inserts showing TEER values higher than 300 Ω/cm^2 were pre-treated with 70 µL/mL of bacterial extract for 1 hour or 10^8 CFU/mL of bacterial pure culture (*L. plantarum 299v* o *L. casei DG*) for 1 hour before adding a proper volume of oxysterol mix to reach a concentration of 60 µM inside the insert. TEER values were monitored at different times and values reported as TEER percentage referred to t_0.

3.7 Western blotting

Caco-2 cells in 6-well plates (5x10^4 cells/mL in 2 mL of growth media), were pre-treated with bacterial extracts (70 µL/mL) for 2 hours or bacterial pure culture for 1 hour (10^8 CFU/mL) before adding the oxysterol mixture (60 µM) or treated with oxysterol mixture (60 µM), bacterial extracts (70 µL/mL)/bacterial pure culture (10^8 CFU/mL) or media (control) for different times (2h, 4h, 6h, 18h, 24h, 48h, 72h). Samples were collected using cell lysis buffer added with protease and phosphatase inhibitors and stored at -20°C until use. Protein

concentration was determined through Bradford protocol **(Bradford, 1976)**. Denatured proteins (20–50 µg) were separated using 10% polyacrylamide gel, then transferred into nitrocellulose membrane where they were blocked with 25 mL of a TBS (Tris/HCl, pH 7.5, 100 mM NaCl) and 4% milk solution for 30 minutes. After washing with TBS solution, membranes where incubated over-night, at 4°C, with primary polyclonal antibodies, anti-Occludin and anti-Junctional Adhesion molecule A (JAM-A), anti-Zonulin 1 (ZO-1) and then washed two times with TTBS (TBS with Tween 20 0.5%) before adding the secondary antibody IgG peroxidase-conjugated. Both primary and secondary antibody were prepare adding an aliquot of the original solution in 10 mL of TTBS solution with 1% of milk. Membranes were washed twice with TTBS and one time with TBS, exposed to Clarity™Western-ECL (Bio-Rad) reagents (4-5 minutes) and observed through ChemiDoc™MT System. Analysis of the imagine obtain from ChemiDoc where analysed using Quantity One (*Biorad, Hemel Hempstead UK*) software in order to determine the molecular weight of the protein bands, through the comparison with bands obtained by separation of a marker run together with proteins.

3.8 Faecal samples

Faecal samples were obtained from healthy donors who had not taken antibiotics in the previous six months and who were not taking any probiotic or prebiotic supplementation. Samples were kept frozen until the experiment and then diluted 1/10 w/w in PBS (0.1 mol/L phosphate buffer, pH 7.4) and homogenised.

3.9 Batch cultures experiments

The preliminary study of the possible interactions between probiotics and oxysterols with microbiota was performed using batch culture in vitro fermentation systems, as previously

described by *Gibson et al.* **(Gibson et al., 2000)**. These are *in vitro* models, consisting of multiple vessels, each one representing a specific combination or substrate, able to simulate the distal colon of the human large intestine and all fermentation processes occurring in it. Temperature was maintained at 37°C through a water flux, while pH value, set between 6.7-6.9 was constantly checked with a pH controller and adjusted with 0.5 M NaOH or HCl solutions if necessary. Anaerobic conditions were created through the injection of filtered N_2 gas flux inside the media (15 ml/min). Vessels were sterilized and filled with a total volume of 80 mL of growth basal media and inoculated with 10% w/v faecal slurry (diluted with PBS at a final concentration of 1%), before adding a mixture of oxysterols (60 µM), the bacterial extract (1%) or the live bacteria (10^8 UFC/mL) or a combination of both oxysterols and bacterial extract/live bacteria. Two controls were prepared in two separated vessels, each one containing only the basal media and the feacal slurry as a negative control and maintained at the same conditions as the others, whereas a positive one was prepared by adding a well validated prebiotic compound, inulin HP. Batch cultures were run up to 24 hours and 5 mL samples were collected at time 0 and after 4, 6 and 24 hours and processed. Briefly, each sample had been separated in two Eppendorf in order to have a duplicate and centrifuged for 10 minutes, 13.500 rpm, 4 C. Each fermentation supernatant was collected in order to perform metabolite' leading to characterize the SCFAs production.

3.10 Batch cultures basal media

Basal media were prepared using the following component for 1 litre of water: peptone water (2 g), yeast extract (2 g), NaCl (0.1 g), K_2HPO_4 (0.04 g), KH_2PO_4 (0.04 g), $MgSO_4x7H_2O$ (0.01 g), $CaCl_2x6H_20$ (0.01 g), $NaHCO_3$ (2 g), tween 80 (2 mL), hemin dissolved in a few drops of NaOH 1M (0.05 g), vitamin K (10 µl), L-Cysteine HCl (0.5 g), bile salts (0.5 g), resazaurin 0.025g/100 mL solution (4 mL).

3.11 Lactobacilli growth curve in presence of oxysterols

In order to verify if oxysterols were able to induce the death of *L. plantarum 299v* and *L. casei DG* or alter their growth curve, experiments were conducted with batch cultures system as previously described, with the only difference that no stool sample were used. Briefly, vessels were filled with media and inoculate with probiotics (10^8 CFU/mL) or probiotics and oxysterols (60 µM) together. Samples were collected at 2, 4, 6 and 24 hours, diluted and plated in MRS agar. After 48 hours, colonies were counted, and numbers of bacteria were calculated through the following formula:

$$\text{Log}_{10}\ \text{CFU/mL} = n \times 50 \times d$$

n = number of colonies; d = dilution factor

3.12 Short-chain fatty acids analysis by NMR

3.12.1 Sample Preparation for 1H-NMR experiments

1 mL of faecal broth was aliquoted in Eppendorf tubes and centrifuged at 5500 g, 10 min, 4 °C to remove any particulate. Then, 1.2 mL of cold methanol/water (80:20) was added to the supernatant and the extraction was combined with 10 min of ultrasonic treatment at a controlled temperature (4 °C). Finally, the solution was centrifuged at 5500 g for 30 min at 4°C and 700 µL of supernatant was aliquoted and dried in an Eppendorf™ Concentrator Plus overnight.

For ^{1}H-NMR analysis, dried hydrophilic faecal extracts were re-dissolved with 690 µL 100 mM KH_2PO_4/D_2O buffer pH 7.2 (99,8%, Cambridge Isotope Laboratories Inc, Andover, USA) and added with 10 µL of internal standard solution 5 mM (sodium 3- trimethylsilyl-propionate-2,2,3,3,-d4, TSP, 98 atom % D, Sigma-Aldrich, Milan, Italy). An aliquot of 650 µL was transferred to 5-mm NMR tube.

3.12.2 ^{1}H-NMR spectroscopy analysis

^{1}H-NMR experiments were carried out using a Varian UNITY INOVA 500 spectrometer operating at 499.839 MHz for proton and equipped with a 5 mm double resonance probe (Agilent Technologies, CA, USA). ^{1}H-NMR spectra were acquired at 300K with a spectral width of 6000 Hz, a 90° pulse, an acquisition time of 2 s, a relaxation delay of 2 s, and 256 scans. The residual water signal was suppressed by applying a presaturation technique with low power radiofrequency irradiation for 2 s. ^{1}H-NMR spectra were imported in ACDlab Processor Academic Edition (Advanced Chemistry Development, 12.01, 2010) and pre-processed with line broadening of 0.1 Hz, zero-filled to 64K, and Fourier transformed. Each spectrum was manually phased and baseline corrected. Chemical shifts were referred to the TSP single resonance at 0.00 ppm.

3.12.3 Identification and quantification of ^{1}H-NMR spectra

Metabolites were identified on the basis of literature information and by using a dedicated library, such as the Human Metabolome Database (HMDB, http://www.hmdb.ca) and the 500 MHz library from Chenomx NMR suite 7.1.

The concentration of the different metabolites was obtained using Chenomx NMR suite 7.1 (Chenomx Inc., Edmonton, Alberta, Canada). Chenomx NMR Suite is an integrated set of tools for identifying and quantifying metabolites in NMR spectra. It is equipped with reference libraries that contain numerous pH-sensitive compound models that are identical to the spectra of pure compounds obtained under similar experimental condition.

3.13 Statistical analysis

The statistical analysis was performed using the average ± standard deviations for each of the groups in all the experiments (each experiment was performed at least 3 times);

significative differences were found through the software GraphPad Prism 5 (GraphPad software, San Diego, CA, USA), using the analysis of variance “one-way ANOVA” and post-hoc Tukey’s test.

4. Results

Part I - Oxysterols-induced alteration of Caco-2 cell monolayer permeability and protective effect exerted by Lactobacilli bacterial extracts

4.1 Oxysterols-induced alteration of Caco-2 cell monolayer permeability

The damage caused by oxysterols on the intestinal epithelial membrane was evaluated *in vitro* in Caco-2 cell monolayers, as alteration of permeability, measured as transepithelial electrical resistance (TEER).

Cells were treated with a mixture of oxysterols (60 μM), reported in literature as representative in concentration and composition of what can be introduced from a high cholesterol diet, and incubated for 72 hours. TEER value was detected at time 0, 2, 6, 18, 24, 48, 72 hours.

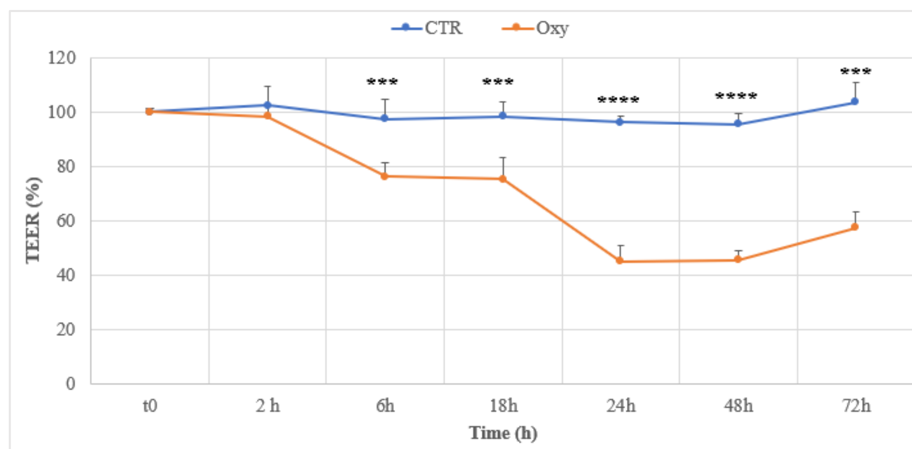

Figure 3. Changes in Caco-2 cell monolayer permeability after treatment with oxysterols. TEER values were measured with time (hours), in Caco-2 cells without any treatment (CTR) or treated with the oxysterol mixture 60 μM (Oxy) and incubated for 72 hours. Data are reported as percentage of TEER value compared with time 0 (t0) for each sample. **** = $p < 0.0001$, *** = $p < 0.001$ Oxy compared to CTR (n=6).

Figure 3 shows the comparison between untreated Caco-2 cells (CTR) and cells treated with the oxysterol mixture. Treatment with oxysterols led to a significant decrease in TEER value, starting from 6 hours of incubation (about 25% less than time 0) and showing a major

decrease, ranging from 45 to 55%, at 24 and 48 hours. After 48 hours TEER value started to increase again.

4.2 Oxysterols-induced alteration of TJs level, occludin, zonulin and JAM A, in Caco-2 cell monolayers

In order to investigate the mechanism underlying the alteration of permeability induced by oxysterols on Caco-2 cell monolayers, we evaluated the modulation of tight junctions, occludin, zonulin-1 (ZO-1) and junctional adhesion molecule (JAM-A) with time. Obtained results are shown in the figures below.

4.2.1 Determination of TJs (occludin, JAM-A, ZO-1) levels in Caco-2 cells treated with oxysterols

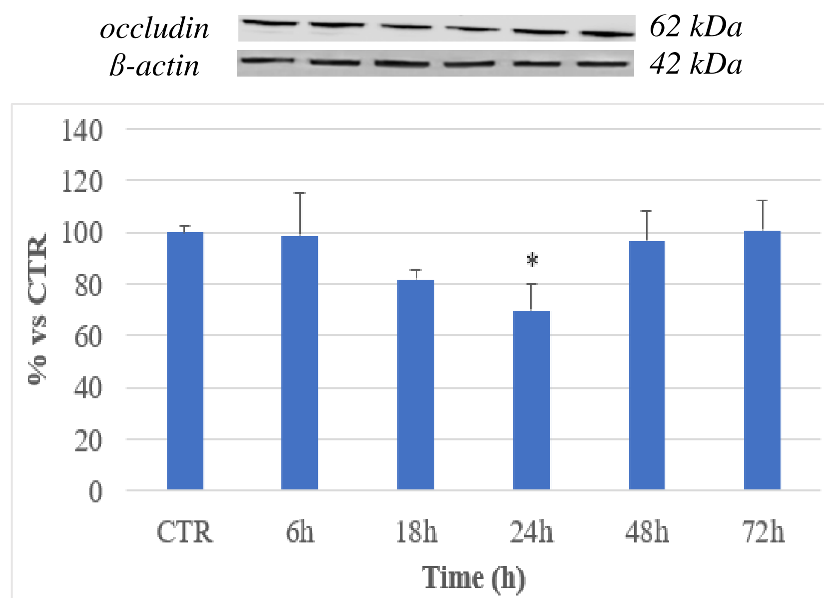

Figure 4. Percentage of occludin/β-actin ratio measured in Caco-2 cells not treated (CTR) or treated with a mixture of oxysterols 60 μM for 6, 18, 24, 48 and 72 hours. Data are reported as percentage compared to CTR for each time. Representative WB images of the experiment are shown. * = $p < 0.05$ Oxy compared to CTR (n=3).

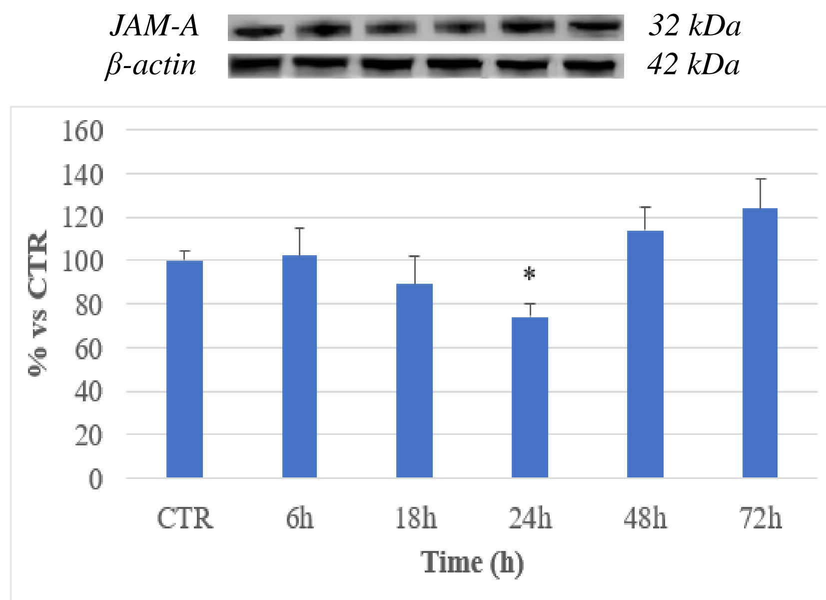

Figure 5. Percentage of JAM-A/β-actin ratio measured in Caco-2 cells not treated (CTR) or treated with a mixture of oxysterols 60 μM for 6, 18, 24, 48 and 72 hours. Data are reported as percentage compared to CTR for each time. Representative WB images of the experiment are shown. * = $p < 0.05$ Oxy compared to CTR (n=3).

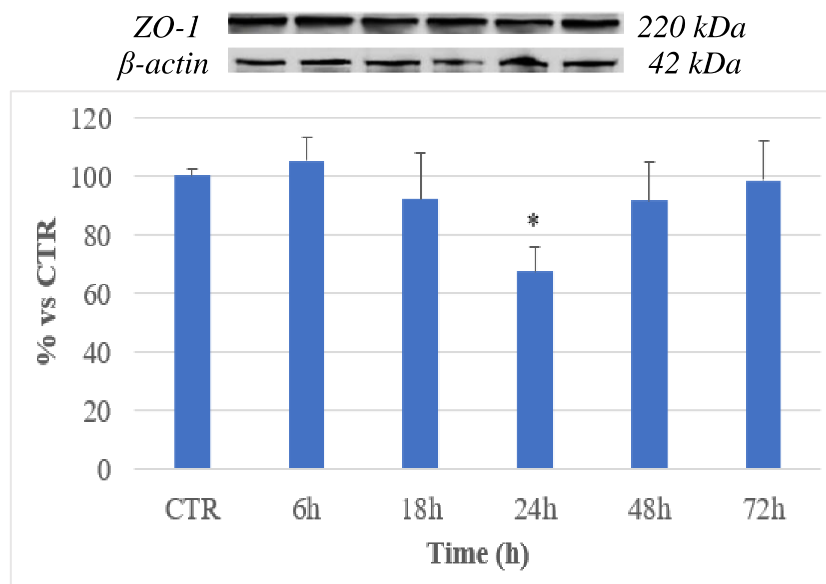

Figure 6. Percentage of ZO-1/β-actin ratio measured in Caco-2 cells not treated (CTR) or treated with a mixture of oxysterols 60 μM for 6, 18, 24, 48 and 72 hours. Data are reported as percentage compared to CTR for each time. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR (n=3).

Treatment with the oxysterol mixture led to a decrease in all three TJs level compared to CTR (100%), as shown in figures 4, 5, 6, where the amount of occludin, JAM-A and ZO-1 measured in Caco-2 cells not treated (CTR) or treated with oxysterols (60 μM) for 6, 18, 24, 48, 72 hours is reported. The level of each protein showed a not-significant decrement at 18 hours, that became significant at 24 hours, with a percentage of about 25-30% lower than CTR. The levels of occludin, JAM-A and ZO-1 started to increase again at 48 hours, reaching a value similar to the CTR, after 72 hours.

4.3 Modulation of MAPKs activation, p38 and ERK, by oxysterols

4.3.1 Measure of p38 level in Caco-2 cells treated with oxysterols

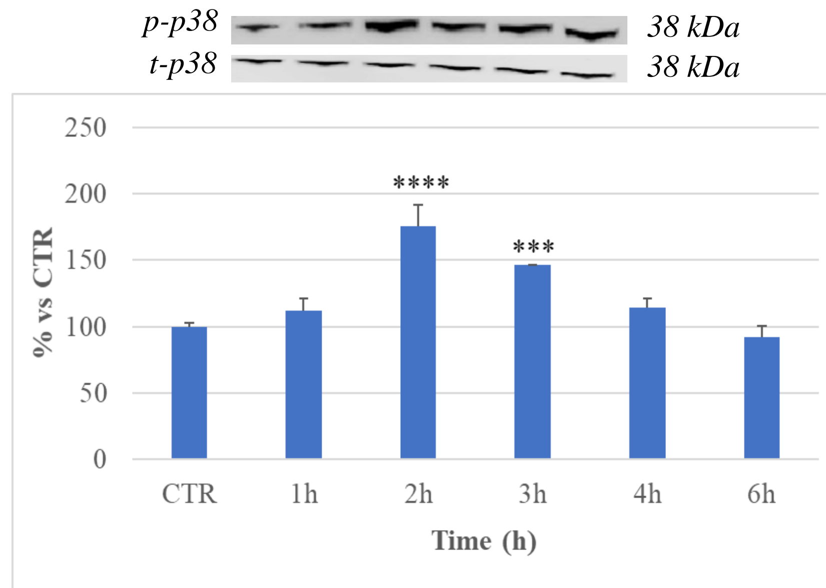

Figure 7. Percentage of p-p38/t-p38 ratio in Caco-2 cells not treated (CTR) or treated with a mixture of oxysterols 60 µM for different times. Data are reported as p-p38/t-p38 ratio percentage compared to CTR p-p38/t-p38 ratio for each sample. Representative WB images of the experiment are shown. **** = $p < 0.0001$, *** = $p < 0.001$ Oxy compared to CTR (n=3).

Figure 7 shows the differences in p38 phosphorylation between samples untreated (CTR) and the ones treated with oxysterols for 1, 2, 3, 4, and 6 hours. Oxysterols treatment stimulated Caco-2 cells to activate p-38 protein through its phosphorylation, which occurred mostly at 2 and 3 hours when the level of p-p38 was significantly higher in samples with oxysterols compared to CTR (100%). A minor activation was still visible at 4 hours, while after 6 hours the level of p-p38 was similar to the control.

4.3.2 Measure of ERK levels in Caco-2 cells treated with oxysterols

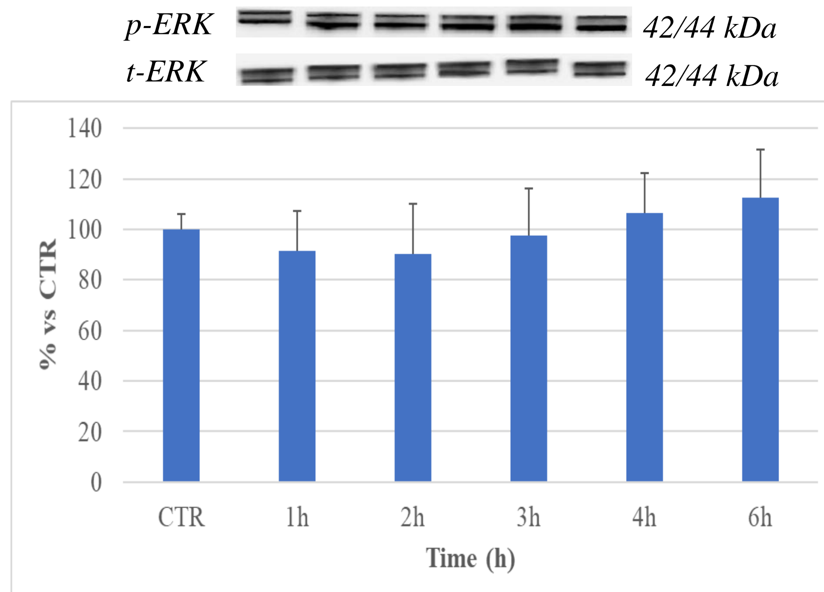

Figure 8. Percentage of p-ERK/t-ERK ratio measured in Caco-2 cells not treated (CTR) or treated with a mixture of oxysterols 60 µM for different times. Data are reported as p-ERK/t-ERK ratio percentage compared to CTR p-ERK/t-ERK ratio for each sample. Representative WB images of the experiment are shown (n=3).

Figure 8 shows the level of ERK phosphorylation determined in samples untreated (CTR) and the ones treated with oxysterols for 1, 2, 3, 4, and 6 hours. Contrary to what observed for p38 protein, no significant activation was evidenced in cells treated with the oxysterol mixture compared to CTR, as levels of p-ERK were similar between the two type of samples, for each incubation time.

4.4 Protective effect exerted by Lactobacilli bacterial extracts against alteration of Caco-2 cell monolayer permeability

In order to evaluate the possible protective effect exerted by the two bacterial extracts, from *L. plantarum 299v* and from *L. casei DG*, against oxysterols-induced alteration of Caco-2 cell monolayers permeability, cells were pre-treated with the bacterial extract for 2 hours, before treatment with oxysterols. TEER values were determined at time 0, 2, 6, 18, 24 hours.

4.4.1 The effect of the bacterial extract from *L. plantarum 299v*

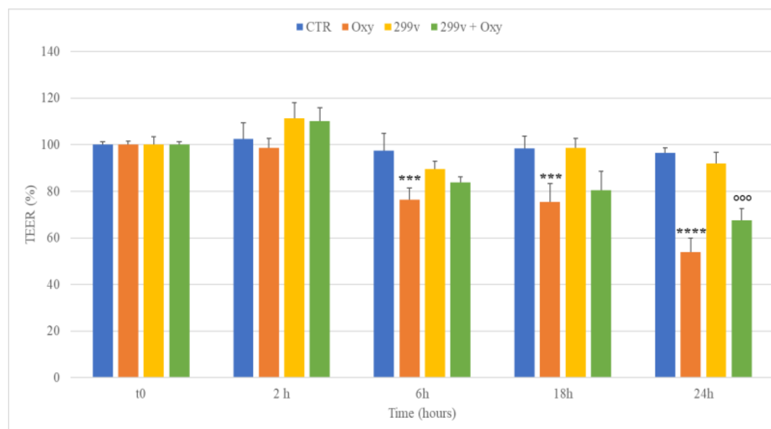

Figure 9. Changes in Caco-2 cell monolayer permeability after treatment with oxysterols, bacterial extract from *L. plantarum 299v*, or both. TEER values were measured after 2, 6, 18 and 24 hours, compared to t0 (100%), in Caco-2 cells not treated (CTR), treated with the oxysterol mixture 60 µM (Oxy), with the bacterial extract (299v) or with both of them. Data are reported as percentage of TEER value compared with time 0 (t0) for each sample. **** = $p < 0.0001$, *** = $p < 0.001$ Oxy compared to CTR; °°° = $p < 0.001$ 299v + Oxy compared to Oxy (n=6).

Figure 9 shows TEER values in Caco-2 cells not treated (CTR), treated with the oxysterol mixture, with the bacterial extract from *L. plantarum 299v* or with both of them. While treatment with oxysterols caused a decrease in TEER value, most at 24 hours, treatment with the bacterial extract did not alter membrane permeability; pre-treatment with the extract, before adding oxysterols, slowed TEER decrease with time, with a significant effect at 24

hours, when TEER value appeared to be about 25% higher than that detected in the samples treated with oxysterols only.

4.4.2 The effect of the bacterial extract from *L. casei DG*

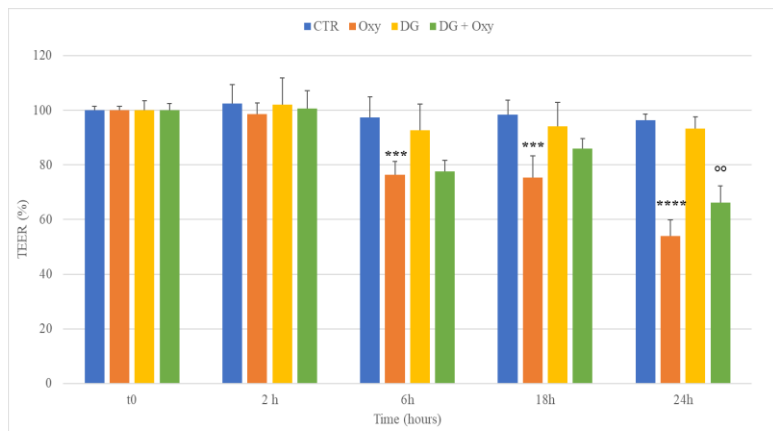

Figure 10. Changes in Caco-2 cell monolayer permeability after treatment with oxysterols, bacterial extract from *L. casei DG*, or both. TEER values were measured after 2, 6, 18 and 24 hours, compared to t0 (100%), measured in Caco-2 cells not treated (CTR), treated with the oxysterol mixture 60 µM (Oxy), with the bacterial extract or with both of them. Data are reported as percentage of TEER value compared with time 0 (t0) for each sample. **** = $p < 0.0001$, *** = $p < 0.001$ Oxy compared to CTR; °° = $p < 0.01$ DG + Oxy compared to Oxy (n=6).

TEER values measured in Caco-2 cells not treated (CTR), treated with the oxysterol mixture, with the bacterial extract from *L. casei DG* or with both of them are reported in Figure 10. As previously observed for *L. plantarum 299v*, the addition of the bacterial extract from *L. casei DG* to Caco-2 cells did not cause any alteration in monolayer permeability compared to control. Moreover, similar to *L. plantarum 299v*, the effect of *L. casei DG* on Caco-2 cell monolayers, when administered before treatment with the oxysterol mixture, slowed TEER decrease compared to the samples treated with oxysterols alone; this effect was significant at 24 hours, when TEER value was about 10-12% higher for cells pre-treated with the bacterial extract then that detected in the samples treated with oxysterols alone.

4.5 Protective effect exerted by Lactobacilli bacterial extracts against alteration of TJs level

4.5.1 Measure of TJs level (occludin, JAM-A, ZO-1) in Caco-2 cells treated with oxysterols and/or bacterial extract (*L. plantarum 299v*) for 24 hours

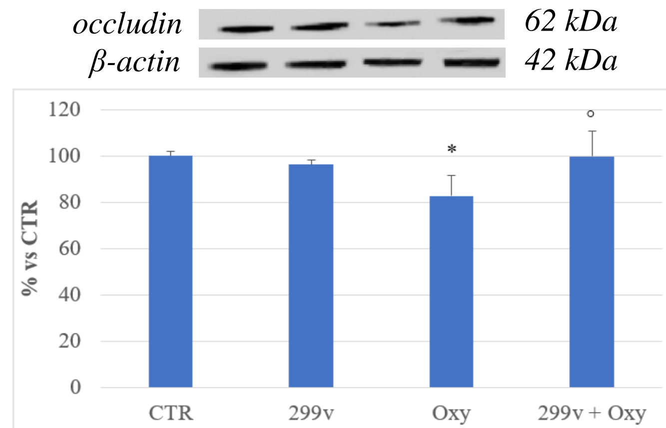

Figure 11. Percentage of occludin/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial extract (*L. plantarum 299v*), with a mixture of oxysterols 60 µM or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.05$, 299v + Oxy compared to Oxy (n=3).

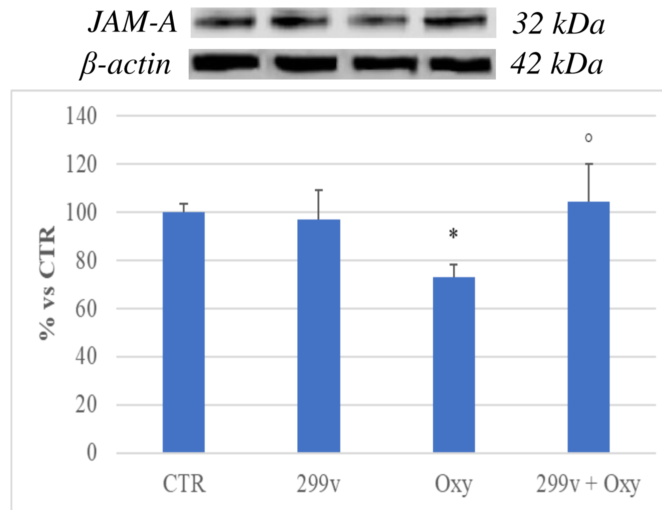

Figure 12. Percentage of JAM-A/β-actin ratio in Caco-2 cells not treated (CTR), treated with the bacterial extract (*L. plantarum 299v*), with a mixture of oxysterols 60 µM or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.05$, 299v + Oxy compared to Oxy (n=3).

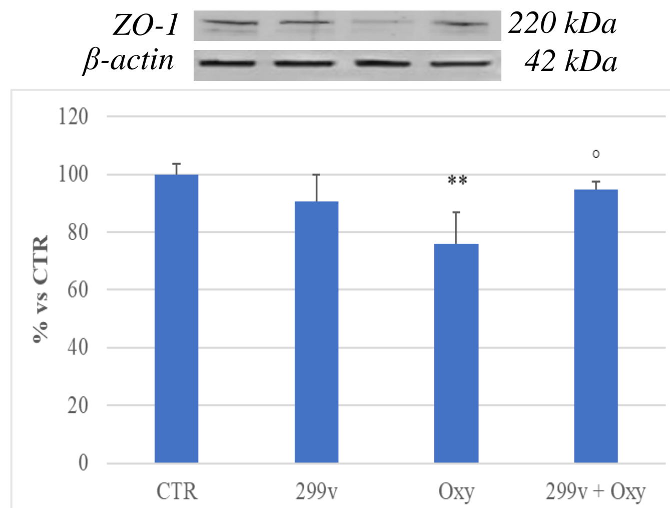

Figure 13. Percentage of ZO-1/β-actin ratio in Caco-2 cells not treated (CTR), treated with the bacterial extract (*L. plantarum 299v*), with a mixture of oxysterols 60 µM or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$, Oxy compared to CTR; ° = $p < 0.05$, 299v + Oxy compared to Oxy (n=3).

Figures 11, 12 and 13 show the protection against oxysterols-induced decrease in occludin, JAM-A and ZO-1 level, exerted by the extract from *L. plantarum 299v* on Caco-2 cells pre-treated before the addition of the oxysterol mixture (60 μM) and incubated for 24 hours. As previously reported, oxysterols were able to cause a significant decrease in occludin level that was about 20% lower compared to CTR (100%). JAM-A and ZO-1 levels were reduced of about 23% and 20% compared to CTR (100%) respectively. When cells were treated with the bacterial extract alone, no significant modifications were detected in TJs level, compared to CTR. The bacterial extract showed a significant protective effect; occludin, JAM-A and ZO-1 levels were similar to the CTR for sample 299v + Oxy, demonstrating that bacterial extract was able to preserve protein's level.

4.5.2 Measure of TJs level (occludin, JAM-A, ZO-1) in Caco-2 cells treated with oxysterols and/or bacterial extract (*L. casei DG*) for 24 hours

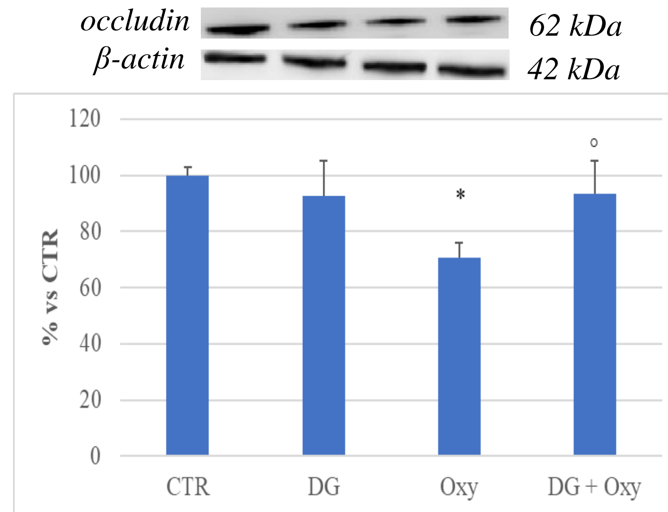

Figure 14. Percentage of occludin/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial extract (*L. casei DG*), with a mixture of oxysterols 60 μM or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.05$, DG + Oxy compared to Oxy (n=3).

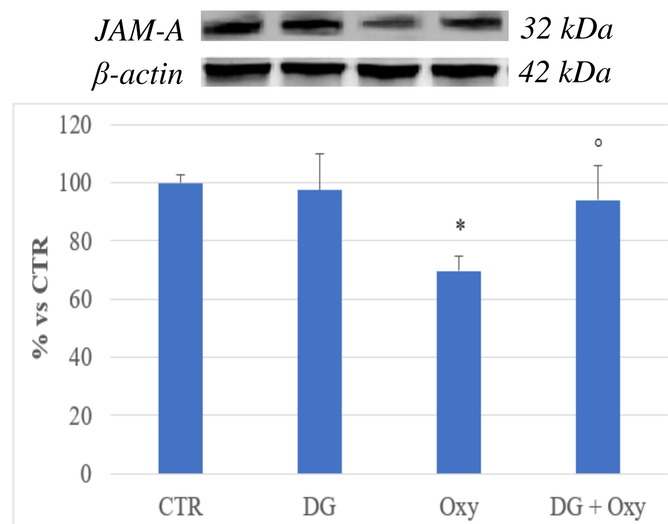

Figure 15. Percentage of JAM-A/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial extract (*L. casei DG*), with a mixture of oxysterols 60 μM or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.05$, DG + Oxy compared to Oxy (n=3).

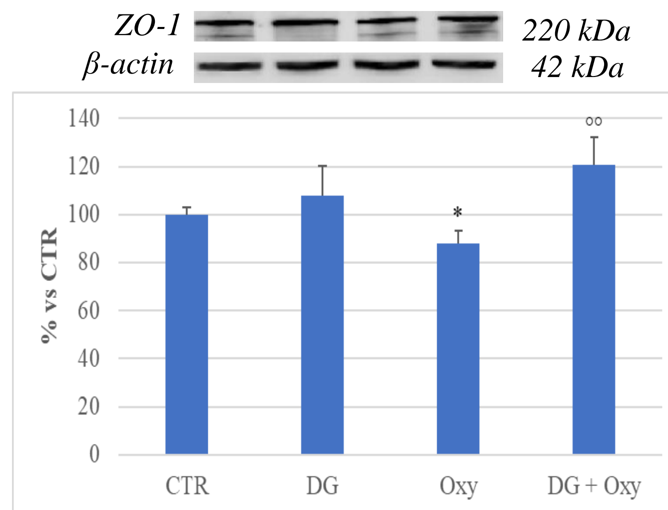

Figure 16. Percentage of ZO-1/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial extract (*L. casei DG*), with a mixture of oxysterols 60 μM or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; °° = $p < 0.01$, DG + Oxy compared to Oxy (n=3).

Oxysterols-induced decrease of TJs level after 24 hours incubation and the effect exerted by the bacterial extract from *L. casei DG* on Caco-2 cells are shown in Figures 14, 15, 16. Results were similar for all three TJs. The bacterial extract showed a significant ability to inhibit the decrease in occludin, JAM-A and ZO-1 levels caused by oxysterols: in each of the three set of experiments, when cells were treated with the oxysterol mixture, the level of TJs decreased of about 20-25% compared to the control, but no significant decrement was detected when cells were treated with the bacterial extract alone or when monolayers were pre-treated with the bacterial extract before adding oxysterols. The bacterial extract exerted a significant protection as TJs level in the samples DG + Oxy were similar to CTR for each experiment.

4.6 Modulation of p38 MAPK activation by Lactobacilli bacterial extracts

4.6.1 Measure of p38 levels in Caco-2 cells treated with oxysterols and/or bacterial extract (*L. plantarum 299v*) for 2 hours

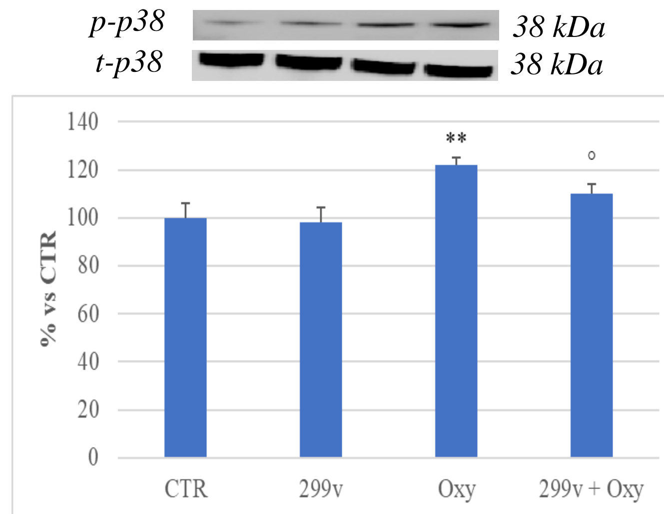

Figure 17. Percentage of p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTR) or treated with the bacterial extract from *L. plantarum 299v* (299v), a mixture of oxysterols 60 µM (Oxy) or both (299v + Oxy). Data are reported as p-p38/t-p38 ratio percentage compared to CTR p-p38/t-p38 ratio for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$ Oxy compared to CTR; ° = $p < 0.05$ 299v + Oxy compared to Oxy (n=3).

Figure 17 shows the rate of phosphorylation of p-38 MAPK detected in Caco-2 cells treated with the oxysterol mixture, the bacterial extract from *L. plantarum 299v*, or both. After 2 hours of incubation, oxysterols induced a significant phosphorylation of the protein, with respect to the control, as also reported in Figure 7. Pre-treatment of Caco-2 cells with the bacterial extract inhibited p-38 MAPK activation, keeping phosphorylation at control level.

4.6.2 Measure of p38 levels in Caco-2 cells treated with oxysterols and/or bacterial extract (*L. casei DG*) for 2 h

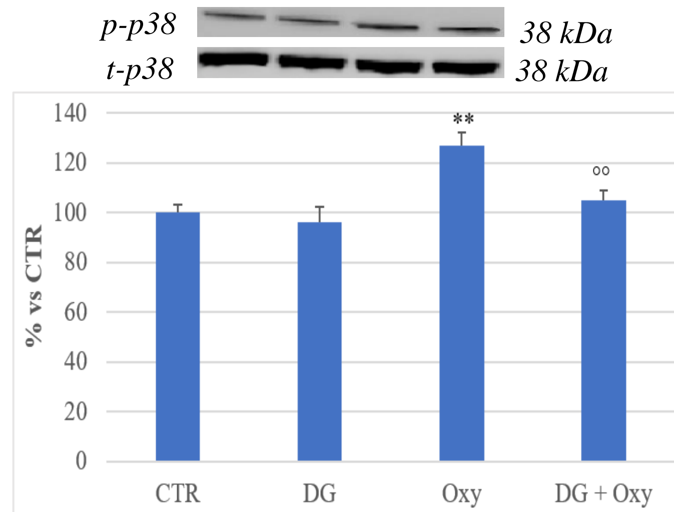

Figure 18. Percentage of p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTR) or treated with the bacterial extract from *L. casei DG* (DG), a mixture of oxysterol 60 μM (Oxy) or both (DG + Oxy). Data are reported as p-p38/t-p38 ratio percentage compared to CTR p-p38/t-p38 ratio for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$ Oxy compared to CTR; °° = $p < 0.01$ DG + Oxy compared to Oxy (n=3).

Phosphorylation level of p-38 MAPK detected in Caco-2 cells treated with the oxysterol mixture, the bacterial extract from *L. casei DG* or both at 2 hours incubation is reported in Figure 18. Oxysterols induced a significant phosphorylation of the protein (about 28%), with respect to the control, which was significantly inhibited in presence of the bacterial extract from *L. casei DG*.

Part II: - Protective effect exerted by Lactobacilli pure cultures against oxysterols-induced damage in Caco-2 cell monolayers

4.7 Adhesion of live bacteria to Caco-2 cell monolayers

In order to establish a system in which Caco-2 cell monolayers and live bacteria could be cultivated together, we conduced preliminary tests on the adherence of *L. plantarum 299v* and *L. casei DG* to cell monolayers. Results are reported below.

4.7.1 Adhesion of live *L. plantarum 299v* to Caco-2 cell monolayers

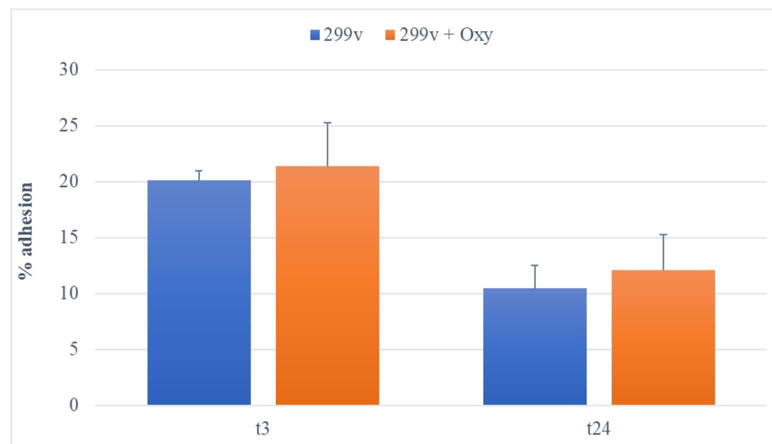

Figure 19. Influence of oxysterols on the adhesion capacity of the probiotic *L. plantarum* 299v to the Caco2-cells. Values are means (±SEM) of two independent experiments.

Figure 19 reports the percentage of adhesion of *L. plantarum 299v* pure culture to Caco-2 cells after 3 and 24 hours of incubation, in presence or absence of the oxysterol mixture. As reported, no significant differences have been detected between samples treated with the pure culture only and those treated with the probiotic and the oxysterols, indicating that the adhesion of *L. plantarum 299v* to Caco-2 cells is not influenced by the presence of the oxysterol mixture.

4.7.2 Adhesion of live *L. casei DG* to Caco-2 cell monolayers

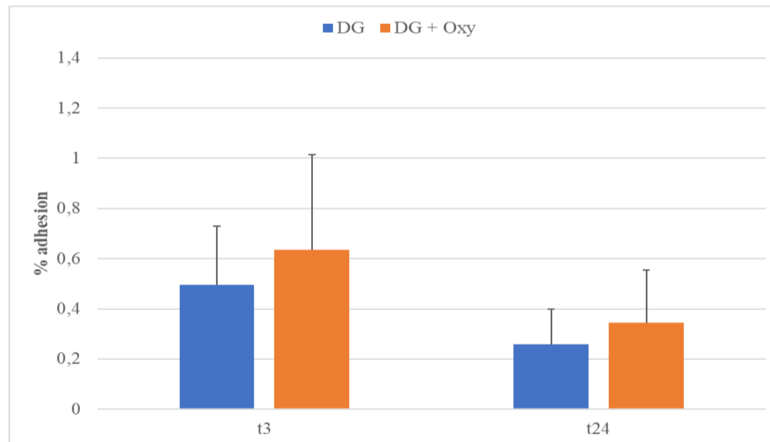

Figure 20. Influence of oxysterols on the adhesion capacity of the probiotic *L. casei* DG to the Caco-2 cells. Values are means (±SEM) of two independent experiments.

Figure 20 reports the percentage of adhesion of *L. casei DG* pure culture to Caco-2 cells after 3 and 24 hours, when it was added alone to the monolayer and in presence of the oxysterol mixture. Results were similar to that reported for *L. plantarum 299v*; indeed, no significant differences have been detected between samples treated with the pure culture only and those treated with the probiotic and the oxysterols, indicating that, even in the case of *L. casei DG*, adhesion to Caco-2 cells was not influenced by the presence of the oxysterol mixture. In general, adhesion of *L. casei DG* to the monolayer was lower than that of *L. plantarum 299v*.

4.8 Protective effect exerted by Lactobacilli pure cultures against alteration of Caco-2 cell monolayers permeability

4.8.1 The effect of *L. plantarum 299v* pure culture

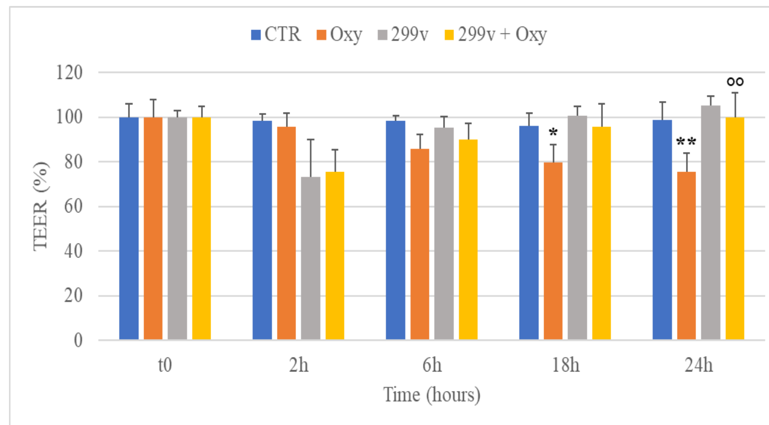

Figure 21. Changes in Caco-2 cell monolayers permeability after treatment with oxysterols, pure culture from *L. plantarum 299v*, or both. The figure shows changes in TEER values after 2, 6, 18 and 24 hours, compared to t0 (100%), measured in Caco-2 cells not treated (CTR), treated with the oxysterol-mixture 60 µM (Oxy), with the pure culture or with both of them. Data are reported as percentage of TEER value compared with time 0 (t0) for each sample. ** = $p < 0.01$, * = $p < 0.05$, Oxy compared to CTR; °° = $p < 0.01$ 299v + Oxy compared to Oxy (n=6).

Figure 21 shows the trend of TEER value over time in Caco-2 cells not treated (CTR), treated with the oxysterol mixture, with the pure culture of *L. plantarum 299v* or with both of them; time 0 (t0) represents, for each sample, the time immediately before the treatment (100% of TEER value). While untreated samples showed similar TEER values at t0 and after 6, 18 and 24 hours, treatment with the oxysterol mixture caused a significant decrease in TEER value after 18 and, mostly, 24 hours compared to CTR. Samples treated with the pure culture of *L. plantarum 299v* only showed a TEER value similar, and even higher although not significant, compared to CTR. Pre-treatment with the pure culture, before adding oxysterols, kept TEER value at control level.

4.8.2 The effect of *L. casei DG* pure culture

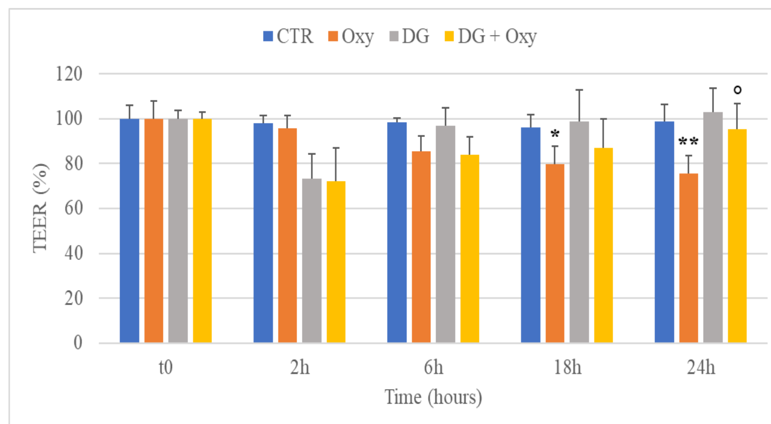

Figure 22. Changes in Caco-2 cell monolayers permeability after treatment with oxysterols, pure culture from *L. casei DG*, or both. The figure shows changes in TEER values after 2, 6, 18 and 24 hours, compared to t0 (100%), measured in Caco-2 cells not treated (CTR), treated with the oxysterol mixture 60 μM (Oxy), with the pure culture or with both of them. Data are reported as percentage of TEER value compared with time 0 (t0) for each sample. ** = $p < 0.01$, * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.05$, 299v + Oxy compared to Oxy (n=6).

Figure 22 shows TEER values measured over time in Caco-2 cells not treated (CTR), treated with the oxysterol mixture, with the pure culture of *L. casei DG* or with both of them; time 0 (t0) represents, for each sample, the time immediately before the treatment (100% of TEER value). As reported above, no significant differences were detected at t0 and after 6, 18 and 24h in control samples and in those treated with the pure culture, whose value was similar to that of the CTR. As already observed for *L. plantarum 299v*, while treatment with the oxysterol mixture caused a significant decrease of TEER values after 18 and 24 hours compared to CTR, pre-treatment with the pure culture of *L. casei DG* maintained TEER value similar to the CTR.

4.9 Protective effect exerted by Lactobacilli pure cultures against alteration of TJs level

4.9.1 Measure of TJs level (occludin, JAM-A, ZO-1) in Caco-2 cells treated with oxysterols and/or bacterial pure culture (*L. plantarum 299v*) for 24h

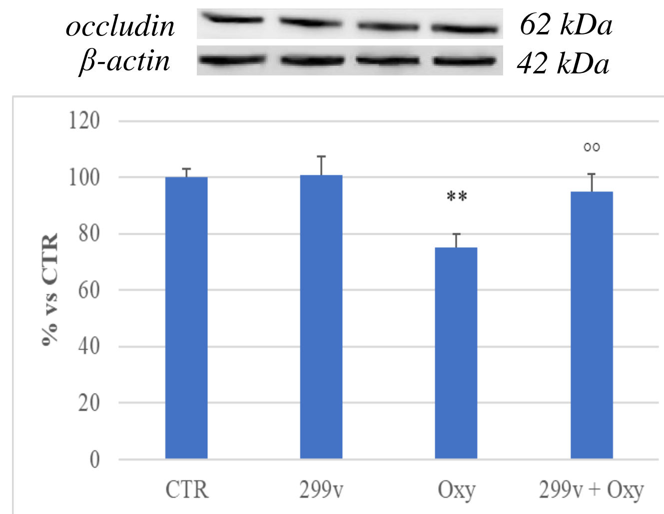

Figure 23. Percentage of occludin/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial pure culture (*L. plantarum 299v*), with a mixture of oxysterols 60 µM or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$, Oxy compared to CTR; °° = $p < 0.01$, 299v + Oxy compared to Oxy (n=3).

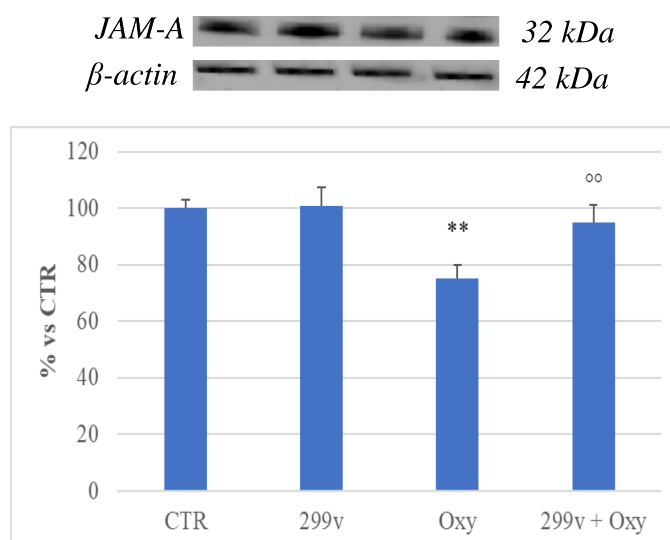

Figure 24. Percentage of JAM-A/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial pure culture (*L. plantarum 299v*), with a mixture of oxysterols 60 µM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$, Oxy compared to CTR; °° = $p < 0.05$, 299v + Oxy compared to Oxy (n=3).

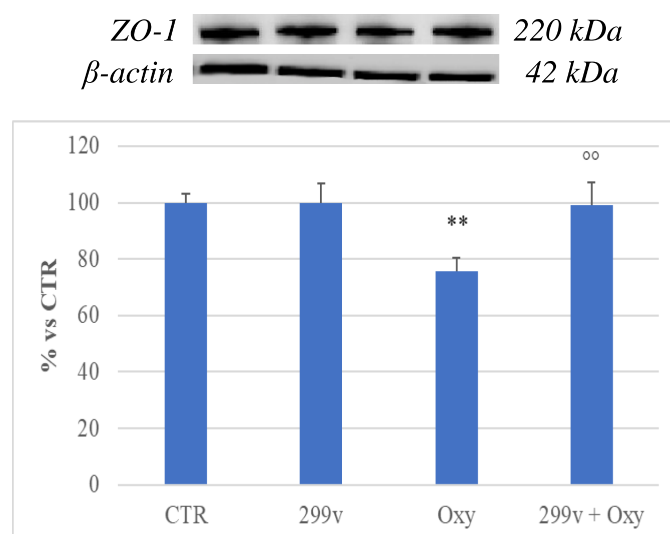

Figure 25. Percentage of ZO-1/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial pure culture (*L. plantarum 299v*), with a mixture of oxysterols 60 µM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$, Oxy compared to CTR; °° = $p < 0.01$, 299v + Oxy compared to Oxy (n=3).

Data regarding the protective action exerted by the pure culture from *L. plantarum 299v* against oxysterols-caused decrease of TJs level in Caco-2 are reported in figures 23, 24 and 25. Results are similar for all the three TJs. While the oxysterol mixture was able to cause a decrease in TJs level of about 20-25% compared to CTR (100%) after 24 hours, occludin, JAM-A and ZO-1 levels were similar to the CTR for sample 299v + Oxy, and significative higher compared to Oxy, showing that bacterial pure culture was able to counteract the decrease of protein level caused by oxysterols. No significant modifications compared to CTR, were detected in TJs level, when cells were treated with the bacterial pure culture alone.

4.9.2 Measure of TJs level (occludin, JAM-A, ZO-1) in Caco-2 cells treated with oxysterols and/or bacterial pure culture (*L. casei DG*) for 24h

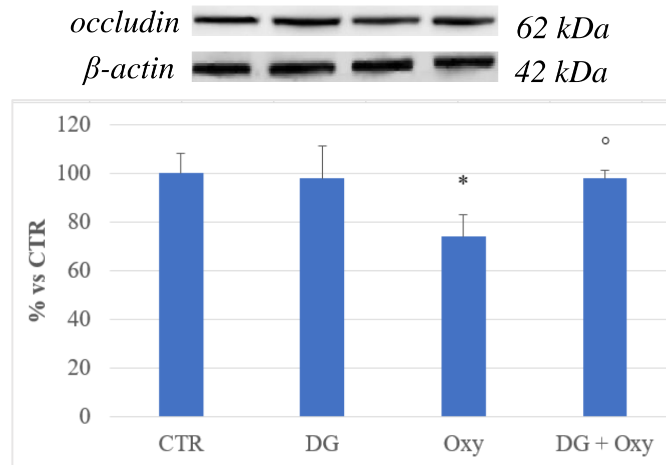

Figure 26. Percentage of occludin/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial pure culture (*L. casei DG*), with a mixture of oxysterols 60 μM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.05$, DG + Oxy compared to Oxy (n=3).

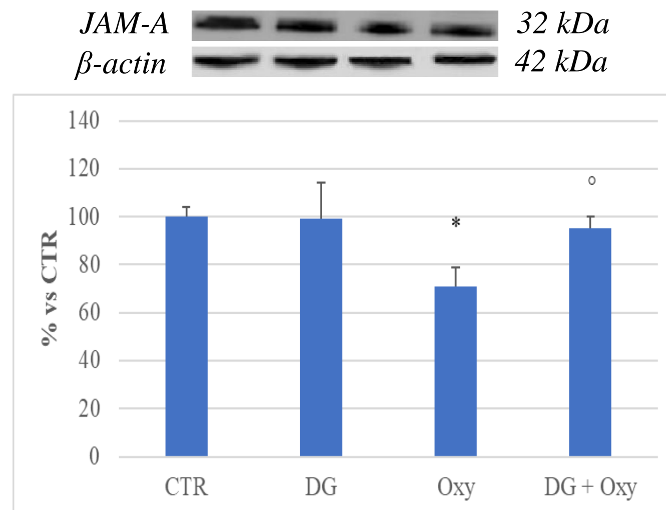

Figure 27. Percentage of JAM-A/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial pure culture (*L. casei DG*), with a mixture of oxysterols 60 μM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.05$, DG + Oxy compared to Oxy (n=3).

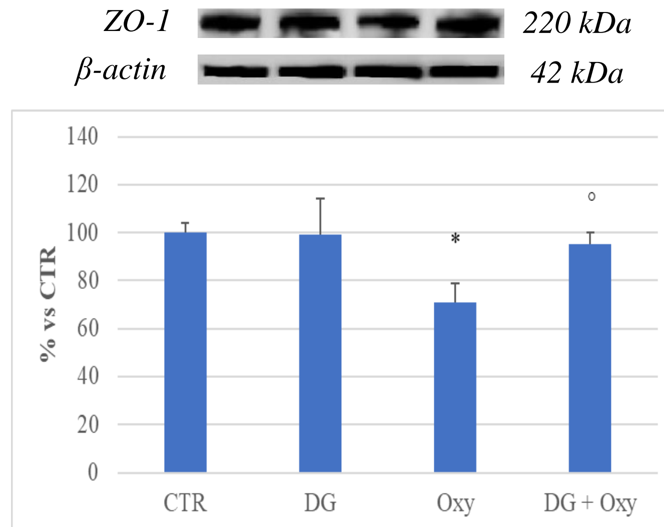

Figure 28. Percentage of ZO-1/β-actin ratio measured in Caco-2 cells not treated (CTR), treated with the bacterial pure culture (*L. casei DG*), with a mixture of oxysterols 60 μM, or with both for 24 hours. Data are reported as percentage compared to CTR for each sample. Representative WB images of the experiment are shown. * = $p < 0.05$, Oxy compared to CTR; ° = $p < 0.01$, DG + Oxy compared to Oxy (n=3).

Figures 26, 27 and 28 show the protection against oxysterols-induced decrease in occludin, JAM-A and ZO-1 levels, exerted by the pre-treatment of Caco-2 cells with the pure culture from *L. casei DG* before incubation with the oxysterol mixture (60 μM) for 24 hours. The oxysterol mixture led to decrease of about 20-25% in TJs level compared to CTR (100%), while no significant decrease was detected when cells were treated with the bacterial pure culture alone or pre-treated with the bacterial extract before adding oxysterols. Indeed, a significative protection was exerted by the bacterial extract as proteins levels were about 20% higher for sample DG + Oxy (similar to CTR level) compared to the samples incubated with the oxysterol mixture alone.

4.10 Modulation of p38 MAPK activation by Lactobacilli pure cultures

4.10.1 Measure of p38 level in Caco-2 cells treated with oxysterols and/or pure culture (*L. plantarum 299v*) for 2 hours

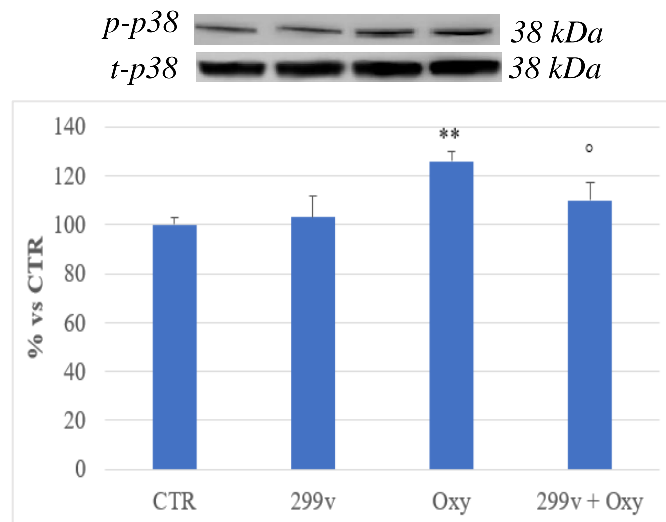

Figure 29. Percentage of p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTR) or treated with *L. plantarum 299v* pure culture 10^8 UFC/mL (299v), a mixture of oxysterols 60 µM (Oxy) or both (299v + Oxy). Data are reported as p-p38/t-p38 ratio percentage compared to CTR p-p38/t-p38 ratio for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$ Oxy compared to CTR; ° = $p < 0.05$ 299v + Oxy compared to Oxy (n=3).

Figure 29 shows the phosphorylation of p-38 MAPK induced by oxysterols and the effect exerted by the pure culture from *L. plantarum 299v* on Caco-2 cells. Oxysterol mixture was able to significantly enhance the levels of p-p38 compared to not treated samples (CTR) after 2 hours of incubation, when the ratio p-p38/t-p38 was about 25% higher in Oxy samples, than in CTR (100%). As observed for the bacterial extract from *L. plantarum 299v*, the pure culture (299v) did not cause changes in p-p38 levels, compared to CTR, and significantly inhibited oxysterols-induced phosphorylation, when present in the incubation mixture.

4.10.2 Measure of p38 level in Caco-2 cells treated with oxysterols and/or pure culture (*L. casei DG*) for 2 hours

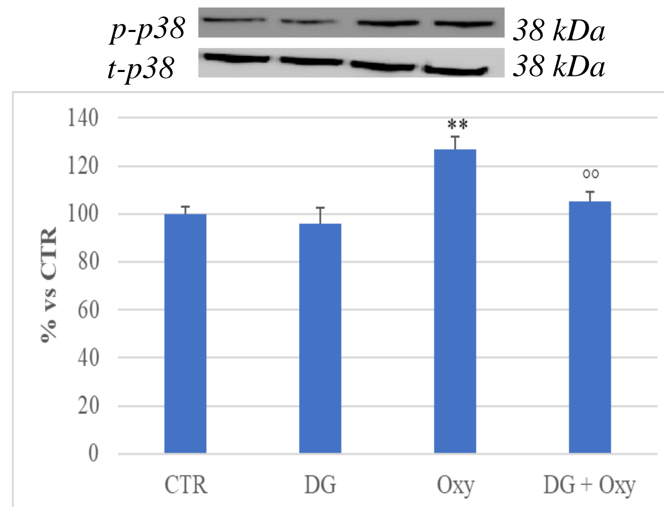

Figure 30. Percentage of p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTR) or treated with *L. casei DG* pure culture 10^8 UFC/mL (DG), a mixture of oxysterols 60 µM (Oxy) or both (DG + Oxy). Data are reported as p-p38/t-p38 ratio percentage compared to CTR p-p38/t-p38 ratio for each sample. Representative WB images of the experiment are shown. ** = $p < 0.01$ Oxy compared to CTR; °° = $p < 0.01$ DG + Oxy compared to Oxy (n=3).

Figure 30 shows the oxysterols-induced phosphorylation of p-38 MAPK, and the effect exerted by the pure culture from *L. casei DG* on Caco-2 cells after 2 hours of incubation. Results are similar to what reported for *L. plantarum 299v*. Indeed, even in this case, while the pure culture (DG) did not cause changes in p-p38 levels compared to CTR, the oxysterol mixture enhanced the p-p38/t-p38 ratio compared to not treated samples (CTR), in a significative manner (about 25%). In samples pre-treated with the probiotic before treatment with the oxysterol mixture, p-p38 level was significantly lower than in those treated with the oxysterol mixture alone.

Part III: Modulation of microbiota metabolites production exerted by oxysterols and/or probiotics (bacterial extract or pure culture) in batch cultures.

In order to study the possible effect of Lactobacilli and/or the oxysterol mixture on the gut microbiota, a preliminary study was conducted using in vitro batch cultures system. We focused on the production of three SCFAs (acetate, butyrate and propionate). Levels of the three SCFAs are reported as the average of relative abundances of each one compared to the total; moreover, taking into consideration the high inter-individual variability usually reported in these kind of studies, to facilitate the interpretation of the data, the results of each experiment report the comparison between time 0 and time 24 for the control and for each sample in the same experiment.

4.11 Modulation of bacteria growth by oxysterols

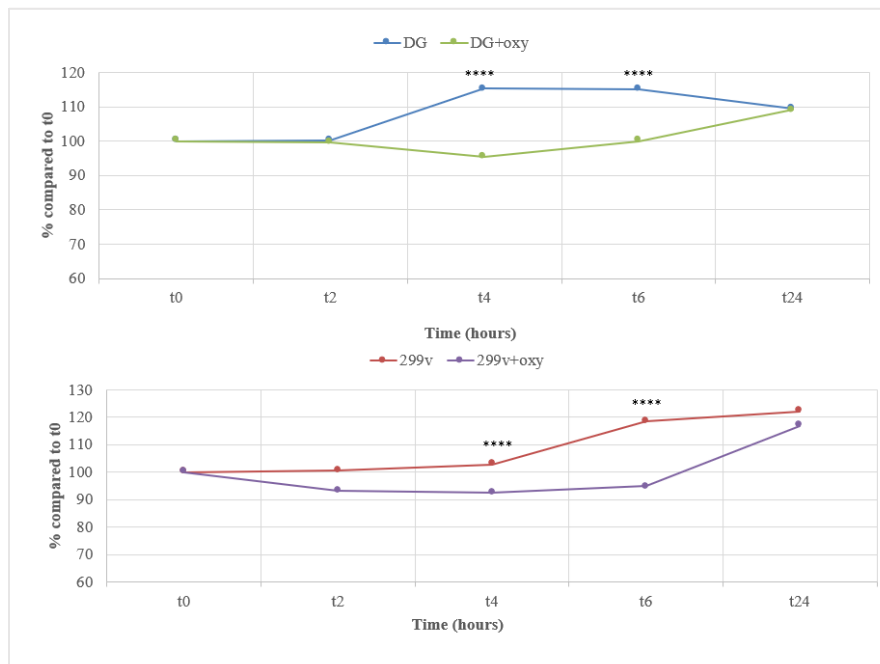

Figure 31. Bacteria pure culture and bacteria pure culture + oxy detected in culture batch broths by viable plate count. The first graph reports the results for *L. casei DG* (DG/DG + Oxy), while the second refers to *L. plantarum 299v* (299v/299v + Oxy). Results are reported as average % compared to the CTR (100%) of the data (n=6). *L. plantarum 299v* and *L. casei DG* were inoculated at the final concentration of 10^8 CFU/80ml of batch culture medium (final volume vessel) and challenged with the mixture of oxysterols (60 µM). Standard deviation was between 5 and 10%. **** = $p < 0.0001$.

In order to evaluate any impact on probiotics' growth curve of the oxysterol mixture, we performed a preliminary set of experiments, by preparing 4 vessels with basal media, without faecal sample, inoculated with the probiotic or with both probiotic and oxysterol mixture. Samples at different times were collected and plated in MRS agar. Figure 31 shows the growth curve of *L. casei DG* or *L. plantarum 299v* alone and of the same strain in presence of oxysterols. As can be observed in the upper graph, in presence of oxysterols *L. casei DG* growth curve was different from the one obtained with its inoculation alone; indeed, the growth curve reached significant lower values between 4 and 6 hours.

A similar trend was observed for *L. plantarum* 299v, where the strain seemed to have a decrease in its growth between 2 and 6 hours in presence of oxysterols. These data indicated that the

oxysterol mixture was able to interact with both probiotic strains, decreasing their growth, without causing death. On the other hand, however, while *Lactobacillus* growth was influenced by oxysterols between 2 and 6 hours, after 24 hours the bacteria growth level in presence of oxysterols was the same as in the controls.

4.12 Changes in metabolic profile of microbiota after treatment with inulin

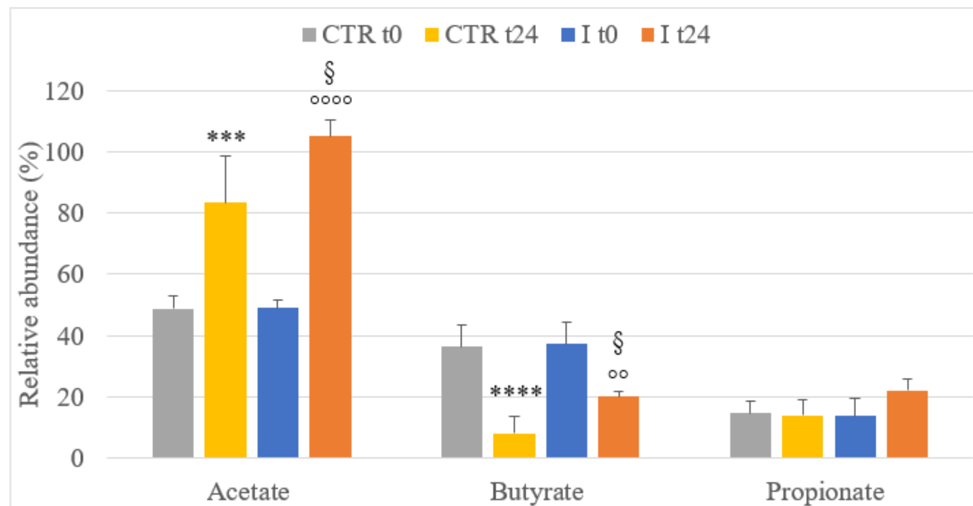

Figure 32. Changes in SCFAs production by microbiota population after 24 hours, in samples untreated (CTR) and treated with inulin (I). The same faecal sample was used in batch cultures system composed of different vessels with basal media, each one representing the CTR or the I. Samples were collected at time 0 and t24. Data are reported for acetate, butyrate, and propionate as the average of relative percentage compared to the total amount of SCFAs. **** = $p < 0.0001$, *** = $p < 0.001$ CTR t0 compared to CTR t24; °°°° = $p < 0.0001$, °° = $p < 0.001$ I t0 compared to I t24; § = $p < 0.05$ I t24 compared to CTR t24 (n=3).

Figure 32 shows the relative abundance of three SCFAs such as acetate, butyrate and propionate, detected in vessels containing a faecal sample and a basal media, and added or not with inulin, in order to observe possible changes in SCFAs profile after 24 hours. As reported, significant changes were detected between t0 and t24 in both CTR and I for acetate and butyrate; indeed, in CTR and I, level of acetate were respectively about 35% and 57% higher at 24 hours than at t0, while butyrate level was about 17% (I) and 28% (CTR) lower after 24 hours with respect to the initial value. Regarding propionate, no significant differences were reported between t0 and t24 for CTR and I, although when inulin was added, propionate level increased after 24 hours. To summarize, while both CTR and I samples showed an increase in acetate and a decrease in butyrate relative abundance, in I t24, levels of SCFAs are significantly higher for acetate and butyrate and higher, even if not in a significant manner, in the case of

propionate compared to the control at the same time, thus underlying the prebiotic action of inulin.

4.13 Changes in metabolic profile of microbiota after treatment with oxysterols

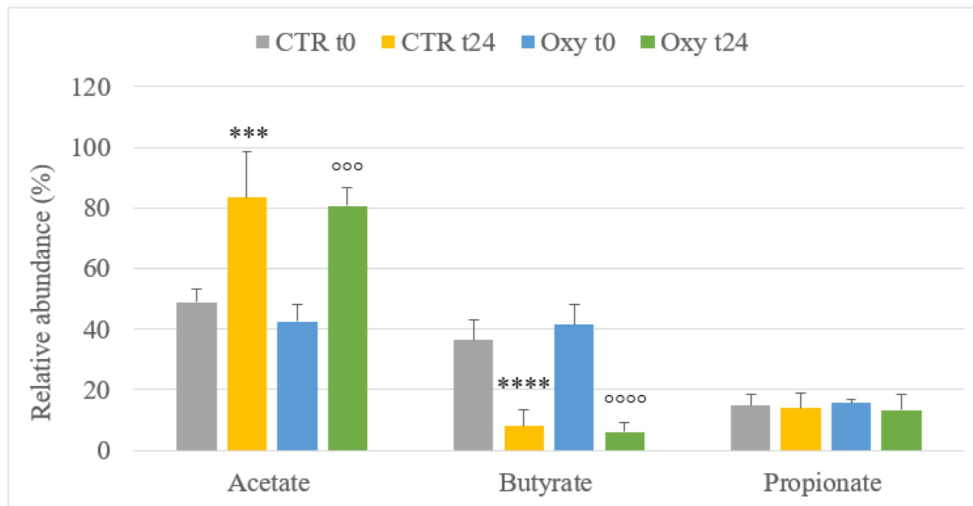

Figure 33. Changes in SCFAs production by microbiota population after 24 hours, in samples untreated (CTR) and treated with oxysterol 60 µM (Oxy). The same faecal sample was used in batch cultures system composed of different vessels with basal media, each one representing the CTR or the Oxy sample. Samples were collected at time 0 and t24. Data are reported for acetate, butyrate, and propionate as the average of relative percentage compared to the total amount of SCFAs. **** = $p < 0.0001$, *** = $p < 0.001$ CTR t0 compared to CTR t24; °°°° = $p < 0.0001$, °°° = $p < 0.001$ Oxy t0 compared to Oxy t24 (n=3).

Figure 33 shows the relative amount of three SCFAs, acetate, butyrate and propionate detected in vessels containing a faecal sample and a basal media, and added or not with oxysterols, in order to observe possible changes after 24 hours. Significant changes in acetate and butyrate levels were detected between t0 and t24 in both CTR (about 35% higher in t24 compared to t0 for acetate and about 30% lower in t24 compared to t0 for butyrate) and Oxy (about 40% higher in t24 compared to t0 for acetate and about 35% lower in t24 compared to t0 for butyrate). No significant differences were observed instead between CTR and Oxy samples after 24 hours, compared to t0 in the level of propionate. In these experimental conditions, oxysterols determined the same changes observed with time in the CTR, as the common tendence was towards an increase in acetate, a decrease in butyrate and a substantial similar value of propionate relative abundance.

4.14 Changes in metabolic profile of microbiota after treatment with bacterial extract from *L. plantarum 299v*

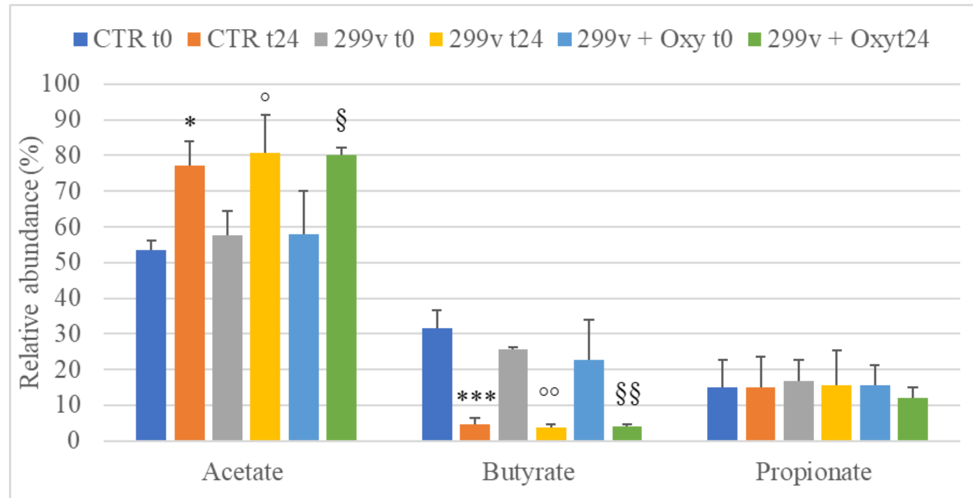

Figure 34. Changes in SCFAs production by microbiota population after 24 hours, in samples untreated (CTR), treated with bacterial extract from *L. plantarum 299v* (299v) or treated with 299v and oxysterols (299v + Oxy). The same faecal sample was used in batch cultures system composed of different vessels with basal media, each one representing the CTR, 299v or 299v + Oxy sample. Samples were collected at time 0 and t24. Data are reported for acetate, butyrate, and propionate as the average of relative percentage compared to the total amount of SCFAs. *** = $p < 0.001$, * = $p < 0.05$, CTR t0 compared to CTR t24; °° = $p < 0.01$, ° = $p < 0.05$, 299v t0 compared to 299v t24; §§ = $p < 0.01$, § = $p < 0.05$, 299v + Oxy t0 compared to 299v + Oxy t24 (n=3).

Figure 34 shows the relative amount of three SCFAs, acetate, butyrate and propionate detected in vessels containing a faecal sample and a basal media, untreated or added with bacterial extract from *L. plantarum 299v* alone or together with the oxysterol mixture at a concentration of 60 µM, in order to observe possible changes after 24 hours. Data showed a change in SCFAs relative abundance in all samples at t24 comparing to t0; significant differences were reported for the increase in acetate (t24 was 24% for CTR, 23% for 299v 22% for 299v + Oxy higher) and for the decrease in butyrate relative abundance in all t24 samples (t24 was 27% for CTR, 22% for 299v 18% for 299v + Oxy lower), compared to t0. Propionate amount was not changed after 24 hours in none of the samples. Thus, all treatments caused similar shifts in SCFAs profile, as that reported for CTR.

4.15 Changes in metabolic profile of microbiota after treatment with bacterial extract from *L. casei DG*

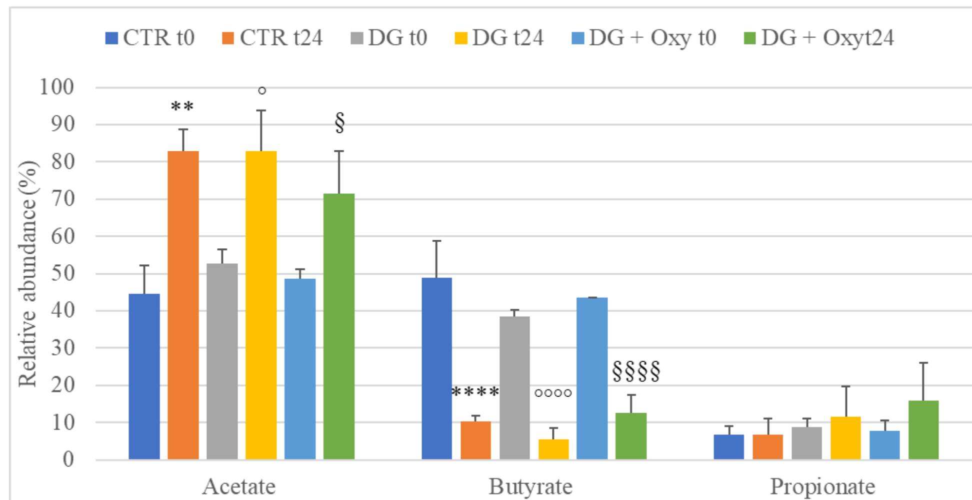

Figure 35. Changes in SCFAs production by microbiota population after 24 hours, in samples untreated (CTR), treated with bacterial extract from *L. casei DG* (DG) or treated with DG and oxysterols (DG + Oxy). The same faecal sample was used in batch cultures system composed of different vessels with basal media, each one representing the CTR, DG or DG + Oxy sample. Samples were collected at time 0 and t24. Data are reported for acetate, butyrate, and propionate as the average of relative percentage compared to the total amount of SCFAs. **** = $p < 0.0001$, ** = $p < 0.01$, CTR t0 compared to CTR t24; °°°° = $p < 0.0001$, ° = $p < 0.05$, DG t0 compared to DG t24; §§§§ = $p < 0.0001$, § = $p < 0.05$, DG + Oxy t0 compared to DG + Oxy t24 (n=3).

Figure 35 shows the relative amount of three SCFAs, acetate, butyrate and propionate detected in vessels containing a faecal sample and a basal media, untreated or added with bacterial extract from *L. casei DG* alone or together with the oxysterol mixture at a concentration of 60 µM, in order to observe possible changes after 24 hours. While propionate relative abundance did not show significant changes after 24 hours compared to t0, acetate and butyrate profiles significatively changed in all samples after 24 hours. In particular, acetate relative abundance was higher (about 38% in CTR ($p < 0.01$), about 30% in DG ($p < 0.05$) and about 23% in DG + Oxy ($p < 0.05$)) at t24 compared to each t0. Butyrate relative abundance was about 38% in CTR t24, 32% in DG t24 and 31% in DG + Oxy t24 lower compared to each sample at t0 ($p < 0.001$).

4.16 Changes in metabolic profile of microbiota after treatment with bacterial pure culture from *L. plantarum 299v*

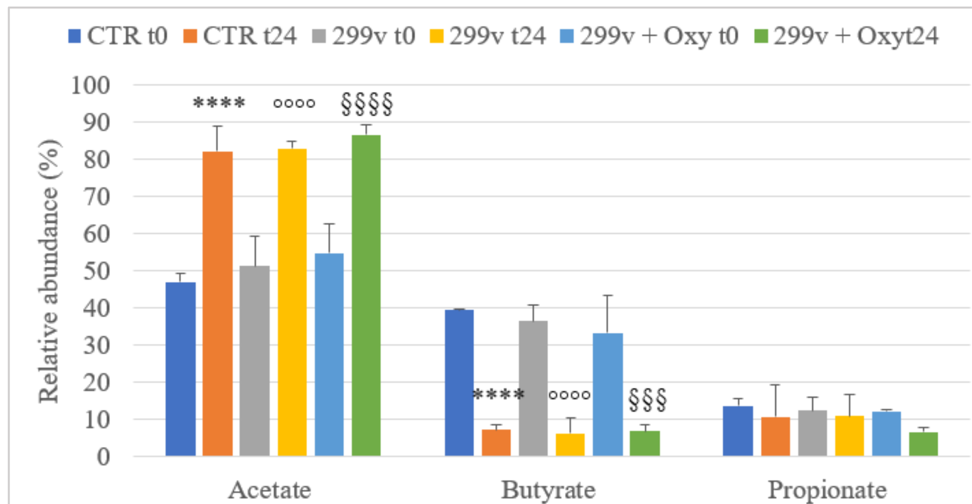

Figure 36. Changes in SCFAs production by microbiota population after 24 hours, in samples untreated (CTR), treated with bacterial pure culture from *L. plantarum 299v* (299v) or treated with 299v and oxysterols (299v + Oxy). The same faecal sample was used in batch cultures system composed of different vessels with basal media, each one representing the CTR, 299v or 299v + Oxy sample. Samples were collected at time 0 and t24. Data are reported for acetate, butyrate, and propionate as the average of relative percentage compared to the total amount of SCFAs. **** = $p < 0.0001$, CTR t0 compared to CTR t24; °°°° = $p < 0.0001$ 299v t0 compared to 299v t24; §§§§ = $p < 0.0001$, §§§ = $p < 0.001$ 299v + Oxy t0 compared to 299v + Oxy t24 (n=3).

Figure 36 shows the relative amount of three SCFAs, acetate, butyrate and propionate detected at t0 and t24 in vessels containing a faecal sample and a basal media, untreated or added with bacterial pure culture from *L. plantarum 299v* alone or together with the oxysterol mixture at a concentration of 60 µM. Propionate relative abundance was similar between t0 and t24 in all samples, while acetate and butyrate profiles significantly changed after 24 hours in all samples and followed the same trend: acetate increased at t24 compared to t0 in all samples (indeed it was 35% in CTR, 31% in 299v and 32% in 299v + Oxy higher at t0 compared to t24), while butyrate was lower at t24 compared to t0 in all samples (of about 32% in CTR, 30% in 299v and 27% in 299v + Oxy).

4.17 Changes in metabolic profile of microbiota after treatment with bacterial pure culture from *L. casei DG*

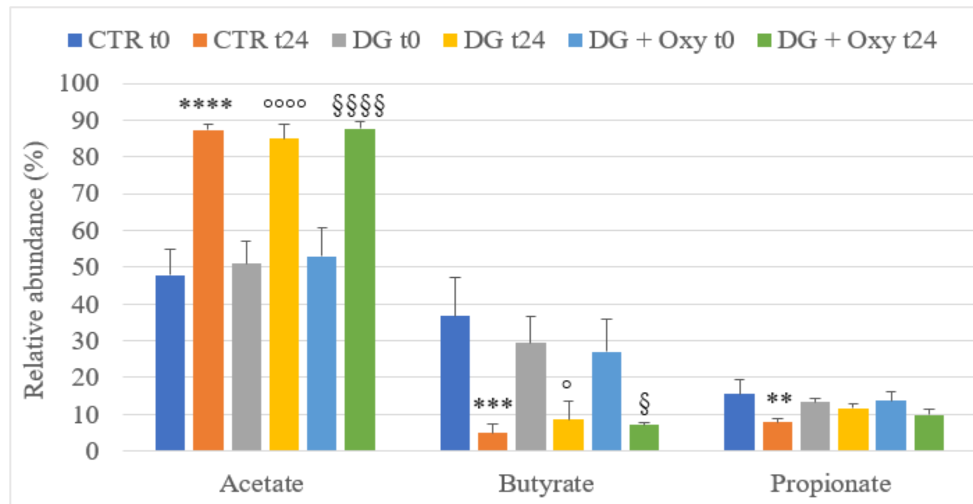

Figure 37. Changes in SCFAs production by microbiota population after 24 hours, in samples untreated (CTR), treated with bacterial pure culture from *L. casei DG* (DG) or treated with DG and oxysterols (DG + Oxy). The same faecal sample was used in batch cultures system composed of different vessels with basal media, each one representing the CTR, DG or DG + Oxy sample. Samples were collected at time 0 and t24. Data are reported for acetate, butyrate, and propionate as the average of relative percentage compared to the total amount of SCFAs. **** = $p < 0.0001$; *** = $p < 0.001$, CTR t0 compared to CTR t24, ** = $p < 0.01$; °°°° = $p < 0.0001$, ° = $p < 0.05$, DG t0 compared to DG t24; §§§§ = $p < 0.0001$, § = $p < 0.05$ DG + Oxy t0 compared to DG + Oxy t24 (n=3).

Figure 37 shows the relative amount of three SCFAs, acetate, butyrate and propionate detected at t0 and t24 in vessels containing a faecal sample and a basal media, untreated or added with bacterial pure culture from *L. casei DG* alone or together with the oxysterol mixture at a concentration of 60 µM. Significant differences were observed between t0 and t24 for all three SCFAs profiles. In particular, acetate significantly increased in all samples at t24 compared to t0 (40% in CTR, 35% in DG and 35% in DG + Oxy), while butyrate significantly decreased at t24 compared to t0, more in the CTR ($p < 0.005$) than in DG ($p < 0.05$) and DG + Oxy ($p < 0.05$) samples (32% in CTR, 21% in DG and 20% in DG + Oxy). Considering propionate relative abundances, there was a significant decrease between t0 and t24 only in CTR samples, where it was 8% lower at t24 compared to t0, while in DG and DG + Oxy propionate relative

abundances were slightly decreased, in a not significant way, indicating a possible major production of the SCFA when the pure culture is added to the system, however not significant compared to that of the CTR at 24 h.

5. Discussion

The intestinal epithelial mucosa is an important structure for the maintenance of a good homeostasis of the intestine itself and of the human body at systemic level; indeed, the mucosal surface is lined by epithelial cells and provide a barrier to the flux of antigens from the outside **(Glover et al., 2016)**. This barrier is composed of various elements, cells like intestinal enterocyte (IECs), goblet cells, and immune cells, cellular junctions (AJ, TJ, GJ), an overlaying layer of mucus, (AMPs) and the microbiota **(Martens et al., 2018)**. Each of these components absolve a crucial role that leads to the maintenance of a healthy and functional mucosa. Despite that, antigens, pathogens or toxic compounds can induce a damage on the intestinal wall, mostly acting as triggers for the immune system **(Okumura et al., 2018)** and thus leading to the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 **(Reimund et al., 1996)**; the chronical dysregulated homeostasis of the epithelium is the key point in a number of intestinal and systemic pathologies, such as metabolic and autoimmune disorders, as well as IBS **(González-Castro et al., 2017)** and IBDs in which the predominant characteristic is the presence of a low grade inflammation **(Hill et al., 2009)**.

The activation of the immune system towards the development of an inflammatory environment has been linked to an increase of the barrier permeability; when this increase occurs, the function as a barrier is lost **(Arrieta et al., 2006)**. This creates leaks in the membrane, a situation known as *Leaky gut*, which in turns allows pathogens and antigens to pass through it towards the circulating system, concurring to the initiation and the progression of a number of disorders, where the common characteristic is the presence of an inflammatory status. This condition can also evolve as a real syndrome, called *Leaky gut syndrome* **(Citi, 2018; Ghosh et al., 2020)**.

Different factors can concur to the impairment or the loss of activity of the epithelial membrane as a barrier, which are both intrinsic and extrinsic and which act directly on the IECs

or indirectly challenge the stability of the membrane, for example through an action on gut microbiota.

In fact, the homeostasis and the balance of the species composing the gut microbiota is a key factor to keep a good operation of the intestinal mucosa **(Takiishi et al., 2017)**. The gut microbiota is a concert of resident bacteria which protect the wellbeing of the intestine and improve the good status of the IECs and the functioning of the immune system, mostly through the production of several metabolites **(Parada Venegas et al., 2019)**. Among microbial metabolites, SCFAs, derived from the fermentation of undigested fibers, are particularly relevant as they have been demonstrated to act as nutrients for IECs and to positively stimulate the immune system **(Rivera-Piza et al., 2020)**.

Exogenous bacteria introduced with diet, as probiotic preparations, have been reported for their multiple positive effects on human health, especially in the intestinal tract. In this site they may improve the health of microbiota and the production of specific metabolites and exert direct anti-inflammatory effects, which make them useful in the prevention of diseases and, in some cases, in the amelioration of symptoms connected to them **(Sanders et al., 2019)**. Thus, diet is a source of agents that at systemic level, but above all at intestinal level, may exert a protective role, inhibiting the onset and progression of oxidative and inflammatory processes, maintaining intestinal homeostasis.

On the other hand, dietary factors may also affect the gut membrane in a negative manner. Different diets have been reported to have divergent effects on human health as the predomination of a macro or a micro-nutrient compared to another can provide the host with a preventive effect or with a trigger for the inflammation process **(Suzuki, 2020; Vernocchi et al., 2020)**. Among dietary compounds that have been proved to trigger a prooxidant and pro-inflammatory environment at intestinal level, there are oxidized lipids, as oxysterols. These cholesterol oxidation products are present at relevant concentration in highly processed

cholesterol rich foods **(Lizard, 2016)**, as meat and animal products, typical of the so-called *Western diet*, strongly associated with a higher prevalence of an inflammatory condition inside the gut **(Christ et al., 2019)**. Indeed, oxysterols have been reported to cause oxidative stress and inflammation on intestinal cell models in vitro **(Vejux et al., 2009)**.

In this context, the aim of the present research project was to evaluate the possible protection exerted by probiotics against oxysterols-induced alteration of the intestinal permeability and to investigate the mechanism of action, in relation to cellular signalling and to any modifications induced by oxysterols or probiotics/post-biotics on the metabolic activity of the microbiota.

Evaluation of membrane permeability alteration induced by oxysterols and potential protective role exerted by Lactobacilli bacterial extract and pure culture.

In previous studies we demonstrated that a mixture of oxysterols at a concentration that is representative of that introduced through diet, 60 µM, was able to cause a damage on Caco-2 cell monolayers, leading to the induction of a proinflammatory response **(Deiana et al., 2017)**. This concentration has been reported to increase in the same experimental model the expression of IL-6 and IL-8 mRNA, thus assuming a central role in chronic intestinal inflammation and in sustaining intestinal barrier disruption, by furthering release of pro-inflammatory mediators **(Biasi et al., 2013)**.

In the present study, we evaluated the alteration induced by a mixture of oxysterols (7α-hydroxycholesterol (4.26%), 7β-hydroxycholesterol (14.71%), 7-ketocholesterol (42.96%), 5α,6α-epoxycholesterol (32.3%), 5β,6β-epoxycholesterol (5.76%) **(Plat et al. 2005)**) at a concentration of 60 µM in monolayers of differentiated Caco-2 cells, recognized to be a model

of enterocyte-like cells which, once reached confluence, show absorptive properties similar to those of small intestine mucosa **(Sun et al., 2008)**.

After 72 h of incubation, oxysterols caused a temporarily alteration of the monolayer permeability, measured as transepithelial electrical resistance (TEER), which started to be strongly and significatively decreased at 24 h. After 48 h, TEER value began to increase again, indicating a possible capacity of cells to reorganize and restructure the monolayer **(Biasi et al., 2008)**.

As the alteration of the intestinal permeability is mostly connected to modifications at the tight junctions (TJ) level **(Chelakkot et al. 2018)**, we investigated any modulation induced by oxysterols on Caco-2 TJ in relation to the alteration of the monolayer permeability. In particular, we focused our attention on occludin and JAM-A, as "intercellular bridge proteins" **(Furuse et al., 1993; Martìn-Padura et al., 1998)** and on zonulin, the intracellular anchor between transmembrane proteins and the peri-junctional actomyosin ring **(Ulluwishewa et al., 2011)**.

TEER decrease in Caco-2 cell monolayers treated with oxysterols was indeed paralleled by a decrease of occludin, JAM-A and zonulin level, has shown by western blot quantification. The decrease, in our system, began at 18 h and was no longer present at 48 h in the case of occludin and JAM-A, while was still evident, but not significant, at 48 h when zonulin was examined. However, the major significant decrease was observed at 24 h for all the proteins.

The addition of the bacterial extracts to the monolayers, before the treatment with oxysterols, determined a minor alteration of TEER values, significant at 24 e 48 hours.

The two probiotics used, *Lactobacillus plantarum 299v* and *Lactobacillus casei DG*, are already commercialized and recognized for their protective role against inflammation **(Compare et al., 2017; Le et al., 2018)**, even though their mechanism of action is still not completely understood.

The observed effect was similar for both bacterial extracts *L. plantarum 299v* and *L. casei DG*, with minor differences between them in the fact that, while TEER values were higher in samples treated with bacterial extract than in those treated with oxysterols alone at each time for *L. plantarum 299v*, they began to be higher than that of oxysterols-treated samples after 18 h when *L. casei DG* was used. These experiments underlined the ability of the tested strains of probiotics, as bacterial extracts, to counteract the alteration of membrane permeability induced by oxysterols in the system used.

State that the oxysterols-induced damage on intestinal membrane permeability started to be significant after 24 h, we studied in the same experimental system the protective effect exerted by live bacteria used as pure culture. We observed not only a protective action of the live bacteria against oxysterols induced damage, but also an increasing in TEER level when the bacterium was administered alone, although not statistically significant. Indeed, it is known that bioactive factors released by probiotics trigger the activation of various cell signalling pathways that lead to strengthening of tight junctions and the barrier function (**Krishna et al., 2013**). Both *L. plantarum* and *L. casei* have been reported to exert this protective effect on different type of cells, such as Caco-2 and T84 monolayers, animal models of colitis and in human trials (**Parassol et al., 2005; Karczewski et al., 2010; Wang et al., 2019**).

When we evaluated the modulation of TJs related to the increase of monolayer permeability, we observed that pre-treatment of Caco-2 cell monolayers with the bacterial extract or the pure culture, before adding the oxysterol mixture, was able to counteract the level decrease of occludin, JAM-A and zonulin for both *L. plantarum 299v* and *L. casei DG*. Data are supported by literature; indeed, a number of studies reported an action of Lactobacilli group and of *L. plantarum* e *L. casei*, on the amelioration of the intestinal permeability and the defence of the gut mucosa against noxious stimuli, mostly by acting on the organization of TJs and on the transcription of gene coding for these proteins (**Compare et al., 2017; Behera et**

al., 2018). This ability is strictly strain-specific; the present study shows this effect in particular for two different strains of Lactobacilli against a specific stimulus, represented by oxysterols, and highlighted that *Lactobacillus plantarum 299v* and *Lactobacillus casei DG* were able to increase the tightness of the TJ even in the presence of oxysterols.

The bacterial extract showed a significant effect against oxysterols-induced alterations of Caco-2 cell monolayers permeability, but lower than that exerted by the live bacteria. Live bacteria as pure cultures show good characteristics to think about a preventive effect on healthy individuals, as they seem to be able to improve intestinal membrane permeability in the absence of a damaging stimulus. However, the use of bacterial extracts, still biologically active, may be a good compromise for patients with intestinal inflammation, such as IBD patients, as literature reports some cases of the activation of the immune system when live bacteria are administered to these subjects **(Vernocchi et al., 2020).**

A contributing mechanism that is involved in the transcriptional regulation of tight junction proteins, is the stimulation of MAPKs; these are factors able to respond to a multitude of stimuli arriving from inside and from outside the cell, through the triggering of a cascade of phosphorylation, which in turns, determine the activation (phosphorylation) or inactivation (dephosphorylation) of proteins involved in different cellular mechanisms such as growth, proliferation, differentiation, motility, stress response, survival and apoptosis **(Owens et al., 2007; Plotnikov et al., 2011)**. In this context, it has been supposed that intracellular oxysterols could act as secondary messengers in cell signal transduction pathways, of which ERK1/2, p38 MAPK, and JNK play important roles **(Guina et al., 2015)**. Among studied MAPKs, the earlier activation induced by oxysterols in Caco-2 cells at a concentration of 60 µM seems to involve MAPK/p38 pathway **(Guina et al., 2015)**. Thus, we investigated if the protective mechanism exerted by bacterial extract or bacterial pure culture on oxysterols-induced alteration of TJs resided in a minor activation of the MAPK pathways, focusing our attention on the modulation

of MAPKs p38 and ERK. The results showed that p-p38 levels changed during time, with a major significant activation at 2 h after oxysterols treatment and a less but still significant activation at 3 h compared to control; on the other hand, levels of p-ERK were not significantly different during time. The results confirmed that oxysterols are able to activate MAPK/p38 pathway, but they do not determine any changes in the phosphorylation of ERK. The activation of p-p38 can be a key point in the modification of tight junction levels. Literature reports an involvement of p-p38 protein in the increase of pro-inflammatory cytokine such as IL-6 and IL-8 through NF-kB activation **(Garat et al., 2003)**; on their side, IL-6 and IL-8 have been indicated to be involved in the down regulated expression of mRNA and production of TJs **(Desai et al., 2002; Yu et al., 2013)**. Moreover, increase in Caco-2 permeability was accompanied by a rapid activation of NF-κB; this increase was not reported if inhibitors of NF-κB were used **(Al-Sadi et al., 2007)**.

To investigate if probiotics mediated protection on the loss of membrane permeability induced by oxysterols and linked to TJs was connected with MAPKs, Caco-2 cell monolayers were pre-treated with the bacterial extract or the bacterial pure culture before adding the oxysterol mixture. We observed that when Caco-2 cells were pre-treated with the bacterial extract or the pure culture obtained from both *L. plantarum 299* and *L. casei DG*, levels of p-p38 were increased compared to the control, but they were also lower than that reported for samples treated with the only oxysterols. These data indicate a possible mechanism of protection against oxysterols-induced alteration at TJs level exerted by both *L. plantarum 299v* and *L. casei DG* as bacterial extracts or pure cultures, residing in a minor activation of the MAPK/p38 pathway, thus leading to an anti-inflammatory effect.

Indeed, these probiotics are reported to have anti-inflammatory properties at intestinal level. For instance *L. plantarum* modulates the ratio of Th1 and Th2 cells by stimulating the production of different inflammatory cytokines such as tumor necrosis factor-alpha, interleukin

(IL)-1β, IL-6, IL-10, IL-12, and interferon-gamma **(Le et al., 2018)**; *L. casei* has been reported to down-regulate the transcription of a number of genes encoding pro-inflammatory effectors such as cytokines and chemokines, for example after the infection with dangerous bacteria in the intestine, resulting in an anti-inflammatory effect that appeared mediated by the inhibition of the NF-κB pathway **(Tien et al., 2006)**.

Modulation of microbiota metabolites production by oxysterols and/or probiotics

Intestinal microbiota does not just reside inertly in the gut; rather, it confers vital benefits to the host by digesting dietary fibers to short-chain fatty acids (SCFAs) that can be used as an energy source, synthesizing vitamin B and vitamin K, and metabolizing bile acids. Evidence indicated that intestinal microbiota also influences host immunity by directly interacting with host cells or producing several metabolites, including SCFAs **(Okumura et al., 2017; Furusawa et al., 2013; Atarashi et al., 2008)**. SCFAs have been reported to be implied in anti-inflammatory processes, as well as in the improvement of symptoms related to intestinal disfunctions; they have been also linked to an amelioration of the intestinal barrier integrity, through the stimulation of the formation of TJs and the promotion of tissue repair and wound healing. Furthermore, they are implicated in the inhibition of the proinflammatory transcription factor (NF-κB) and decreasing of oxidative stress **(Vernocchi et al., 2020)**.

Diet may cause a shift in the relative abundance of species forming the gut microbiota, as well as in their metabolic profile, stimulating the host in a positive or negative manner. For these reasons we decided to perform a preliminary study to evaluate the possible changes induced on the metabolism of the intestinal microbiota, obtained from faecal samples from healthy donors, induced by two kind of dietary external stimuli: a potentially damaging one, represented by oxysterols, and a potentially protective one represented by probiotic

Lactobacillus strains, used as bacterial extracts or bacterial pure culture. For this aim we employed a batch culture system, which have been largely used as model of *in vitro* colonic fermentation, where conditions of temperature, CO_2 and pH are maintained, in order to mimic the distal region of the human large intestine **(Gibson et al., 2000)**. This *in vitro* system has already been used for the analysis of the composition and of the metabolic production of the gut microbiota after fermentation with media added with different type of compounds **(Tzounis et al., 2008; Grimaldi et al., 2016; Yousi et al., 2019)** such us prebiotics, probiotics and antioxidants. For example, this approach has been employed by other authors for the selection of probiotics and prebiotics **(Liu et al., 2016; Takagi et al., 2016; Valdés et al., 2017; Gaisawat et al., 2020)**. On the other hand, so far, no studies have been conducted on possible effects induced by oxysterols on gut microbiota itself or on its metabolic profile.

In particular, we focused on most studied SCFAs, acetate, butyrate and propionate, which were detected through ^{1}H-NMR spectroscopy analysis, one of the methods commonly used for SCFAs evaluation in fecal samples (**Monleon et al., 2009; Ndagijimana et al., 2009; Le Gall et al., 2011; Primec et al., 2017**). Indeed, those are the most abundant (≥95%) SCFAs in the human colon and stool and are present in an approximate molar ratio of 60:20:20 **(Den Besten et al., 2013)**. An imbalance of the intestinal microbiome and a decrease in the number of bacteria producing metabolites such as SCFAs (acetic, propionic and butyric acid) often occur in patients with IBDs, IBS, type 2 diabetes (T2D), obesity, autoimmune disorders or in cancer patients **(Markowiak-Kopeć et al., 2020)**.

Acetate is a molecule that can be produced by gut resident bacteria from undigested fibers, peptides, fat and H_2 and CO_2; butyrate can derive from the degradation of undigestible carbohydrates, organic acids, glutamate and lysin; propionate can be formed from carbohydrates, organic acids and amino acids **(Louis et al., 2017; González Hernández et al., 2019)**. Acetate, butyrate and propionate have been related to the reduction of the intestinal

inflammation and the improvement of the intestinal permeability (**Tedelind et al., 2007; Laffin et al., 2019; Zheng et al., 2020**). *Lactobacillus spp*. are reported, among others, as producers of SCFAs (**Vernocchi et al., 2020).**

In batch culture experiments, the faecal sample without any addition was considered as negative control to which all different combinations used were referred to, as literature reports a large inter-individual variability among relative abundances of species shaping the microbiota, and consequently on their metabolites production. This variability needs to be considered when responses to external stimuli exerted by microbiota are taken into consideration. The most predominant factor able to influence microbiota composition is diet: for example, a diet full of fibers (fruit, vegetables, whole grain cereals) is linked to a major production of SCFAs, while a nutrition poor of these elements is connected to a minor production of SCFAs, even when fibers are administered, as it contributes to select microorganism that are not able to digest non-fermentable carbohydrates (**Vernocchi et al., 2020**). These differences can greatly influence the response of the microbiota to both protective and damaging exogenous factors (**Healey et al., 2018**).

We also prepared a positive control using inulin to treat faecal microbiota; indeed, it is a well-known prebiotic compound, that can be fermented by bacteria as a substrate with the final production of SCFAs (**Kolida et al., 2002**). Due to the β-(2,1) bonds in its chain, the inulin molecules are not digestible by human enzymes. On the other hand, healthy species in the gut microbiota, such as Lactobacilli and *Bifidobacteria* are able to hydrolase inulin polymers leading to the production of SCFAs, mostly butyrate and propionate (**Barclay et al., 2016**).

In accordance with this, in our experiments, the addition of inulin to the basal media, was related to a higher amount of SCFAs acetate, butyrate and propionate, after 24 hours, compared to the negative control, confirming the good operation of the system carried out.

Regarding experiments conducted with the oxysterol mixture, significant changes were detected at t24 compared to t0, in the enhancement of acetate relative abundance and a reduction of that of butyrate; propionate levels were not affected by any significant changes. To note, the same trend in SCFAs profile was reported for negative controls, suggesting that oxysterols did not exert any effect on SCFAs production. Oxysterols pro-inflammatory effect, also observed in our *in vitro* intestinal cells model, seems to be not linked to metabolic variation of microbiota; however, the experimental conditions, such as an insufficient exposure time (24h) may have affected obtained results. Thus, an interference with the metabolic profile of the microbiota cannot be excluded. Indeed, the characterization of microbiota population would be of great relevance in order to verify if changes occur on species relative abundance and so if the metabolic shift could follow this modification.

As regard to bacterial extracts, we found that after 24 hours, they were able to increase acetate and decrease butyrate relative abundances, while propionate levels were basically similar to the t0 or slightly enhanced in the case of *L. plantarum 299v* bacterial extract. Yet it is important to note that the same trend was observed in the negative control average of each experiment.

Similar results were observed for the strains as bacterial pure culture, alone or used together with the oxysterol mixture; the trend, after 24 hours compared to t0, was an increase in acetate production, a decrease in butyrate and low no-significant changes in propionate.

In each of these cases, at 24 hours, the proportion of SFCAs shows a prevalence of acetate, followed by propionate and butyrate; previous studies reported the same trend after 24 hours (**Takagi et al., 2016**).

Regarding the effect of probiotic on SCFAs production, literature reports a variety of papers where different species of probiotics have been tested in batch culture system obtaining results that are divergent in relation to the experimental condition used, reporting cases of

increase in all three SCFAs production as well as no changes in the metabolic profile. Different microbial fermentation patterns can be obtained depending on physic-chemical characteristics of the substrates and fermentation media, speed of fermentation and the microbial populations involved in the process **(Ríos-Covián et al., 2016).** For example, in a number of studies, different substrate, such as prebiotic have been added to the media together with probiotic, obtaining positive results in terms of SCFAs increase **(Yang et al., 2013; Bergillos-Meca et al., 2015; Ríos-Covián et al., 2016)**, thus demonstrating the massive influence of the diet on the positive modulation of the microbiota, its metabolic activity and its response to probiotics. Moreover, in studying the response of gut microbiota to probiotics and the capacity of these last to modulate the microbiota and its metabolic production, it is fundamental to consider the basal microbiota composition. As reported by *Valdés et al.* **(Valdés et al., 2017)** human probiotics have often shown only limited microbiota modulation ability, which seems to depend on the basal microbiota composition, which may determine the responder or non-responder phenotype towards probiotic intervention **(Valdés et al., 2017)**.

In summary, each experiment performed for all samples including oxysterol mixture (Oxy), *L. plantarum* 299v, *L. plantarum* 299v + Oxy, *L. casei DG* and *L. casei DG* + Oxy (both extracts and pure culture) reported an increase in acetate, a decrease in butyrate and similar levels of propionate, a trend totally similar to that of the negative control. This suggests that in our experimental system, after 24 hours, the addition of any agents or a combination of them was able to cause the same changes reported for the control and this could be related to the initial microbiota composition, the prevalent diet of the healthy donor and the time. However, changes in microbiota population cannot be excluded.

5.1 Conclusions and further studies

In conclusion, our data clearly show that intestinal epithelial cells represent a direct target of the pro-inflammatory action of dietary oxysterols, giving further evidence of their possible implication in IBD development. Our results also provide, for the first time, evidence of the ability of *Lactobacillus spp.* probiotics to protect intestinal cells against the pro-inflammatory effect of oxysterols, *in vitro*. The two strains of tested probiotics (*L. plantarum 299v* and *L. casei DG*) exerted their inhibitory effect acting on one key inflammatory mechanism such as the alteration of the intestinal permeability, caused by the oxysterols-induced TJs disruption. Probiotics effect seems to be due, at least in part, to the modulation of MAPK/p38 pathway and the bacterial extracts and the pure cultures showed a comparable efficacy, suggesting a promising effect of both probiotic strains as bacterial extracts in the treatment of intestinal inflammation, avoiding the stimulation of the immune system, which is a side effect of the use of the commercialised pure cultures.

Batch cultures did not highlight any significant interaction among oxysterols, probiotics and intestinal microbiota metabolic activity in the experimental condition used. However, these are preliminary results of an on-going study, which is being expanded with a higher number of samples. Further studies are going to proceed with the identification of microbiota population of each sample, through DNA sequencing, in order to fully evaluate the possible effects of oxysterols and probiotics on the different species shaping the microbiota. Nevertheless, the fact that Lactobacilli growth curve was not affected by oxysterols after 24 hours, when added together to a basal media without microbiota, is a promising point for major investigations on the possible use of probiotic as a defence against a damage caused by oxysterols in the gut.

Taken together our data strengthen the link between diet and IBD pathogenesis and progression and encourages us to continue studying probiotics as a useful tool in the prevention and management of such pathologies.

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