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Role of organic acids and phytonutrients as natural alternatives to antibiotics in supporting intestinal functionality

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List of abbreviations

2-AG: 2-Arachidonoylglycerol

5-HT: serotonin

5-HT1aR: serotonin 1A receptor

ABF: antibiotic-free

Ach: acetylcholine

AEA: anandamide

Caco-2: Cancer coli-2

CB1: cannabinoid receptor 1

CB2: cannabinoid receptor 2

CBD: cannabidiol

CCK: cholecystokinin

CNS: central nervous system

COX: cyclooxygenases

DAGL: diacylglycerol lipase

ECS: endocannabinoid system

EGCs: enteric glial cells

ENS: enteric nervous system

EO: essential oil

FAAH: fatty acid amino hydrolase

FD4: fluorescein isothiocyanate–dextran

GEO: ginger essential oil

GI: gastrointestinal

GIT: gastrointestinal tract

List of abbreviations

GPCRs: G protein-coupled receptors

GSE: grape seed extract

GSH: glutathione

IBD: inflammatory bowel disease

IL: interleukin

IFN-γ: interferon-gamma

JAMs: junctional adhesion molecules

LPS: lipopolysaccharide

MAGL: monoacyl glycerol lipase

MCFA: medium-chain fatty acids

MP: myenteric plexus

NAE: no-antibiotic ever

NAPE-PLD: N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D

NIC: nature-identical compound

NOS: nitric oxide synthase

OA: organic acid

OR1G1: olfactory receptor 1G1

PCP: paracellular permeability

ROS: reactive oxygen species

SCFA: short-chain fatty acids

TCA: tricarboxylic acid

TEER: transepithelial electrical resistance

THC: Δ^9-tetrahydrocannabinol

TJ: tight junction

TNF-α: tumor necrosis factor-alpha

TRP: transient receptors potential channel

List of abbreviations

TRPA1: transient receptor potential ankyrin 1

TRPV1: transient receptor potential vanilloid 1

TRPV3: transient receptor potential vanilloid 3

TTO: tea tree oil

ZO: zonula occludens

ABSTRACT

The gastrointestinal tract (GIT) represents the major portion of the body that interfaces with the external environment, with the double function of food processing and line of defense of the body. In fact, besides absorptive function, the GIT constitutes a physical barrier against harmful agents, reducing their contact with the epithelium, due to its semipermeable characteristics. Numerous components support and regulate the barrier function of the GIT, such as tight junctions (TJs), cytokines, commensal and pathogenic microorganisms, and other systems of the organism, as the endocannabinoid system (ECS). In fact, the ECS can control several gastrointestinal functions, as well as the regulation of intestinal inflammation. Also, external factors can influence the barrier equilibrium, as the composition of the diets, the use of feed additives, but also toxins, antigens, and inflammatory stimuli. Failure of the intestinal barrier function triggers an increase of the concentration of pro-inflammatory cytokines and leads to a reduction in intestinal functionality, with a consequent enhancement of the translocation of pathogens, resulting in pathological conditions of livestock animals. This thesis aimed to explore the potential of natural compounds as a new alternative approach to antibiotics not only as antimicrobial, but also supporting intestinal maturation and integrity, and as immune-boosting agents. Different experiments were performed to evaluate the potential of nature-identical compounds (NICs), organic acids (OAs), and essential oils (EOs) to support and fight various stressful stimuli.

In vitro, a well characterized blend of NICs and OAs were able to improve TJs and transepithelial electrical resistance (TEER) in an intestinal cell line, exerting an anti-inflammatory potential. EOs enhanced TEER and TJs mRNA levels, with a reduction of paracellular permeability (PCP), showing also antioxidant and antimicrobial properties. *In vivo*, thymol modulates the gene expression of ECS and gut chemosensing in the GIT of piglets,

where the precise localization of the cannabinoid receptors was immunohistochemically confirmed, suggesting an anti-inflammatory potential. In conclusion, natural alternative molecules represent an important and effective alternative to support or replace the classical pharmacological prophylaxis. These alternative molecules act not only as antimicrobial agents, but also exerted a crucial role in supporting the intestinal barrier function, preventing oxidative stress, and reducing inflammation. Moreover, the NIC thymol seems able to modulate the ECS, representing a novel frontier to support animal health and productivity.

INTRODUCTION

INTESTINAL BARRIER

The gastrointestinal tract (GIT) represents the largest fraction of the body that interfaces with the external environment. It achieves the function of food processing and allows the survival of commensal symbiotic microbes, while eliminating pathogens. Therefore, the GIT is one of the major lines of defense of the body, in which epithelial cells offer a physical barrier and work together with immune and stromal cells to fight harmful agents and reduce their direct contact with the epithelium. For these reasons, the gastrointestinal (GI) epithelium acts as a semipermeable barrier, allowing the absorption of nutrients and immune sensing, while limiting the transport of potentially harmful antigens and microorganisms (Farhadi et al., 2003; Wijtten et al., 2011).

Numerous components aid the GI barrier in its function as an immunological and physical defense barrier (Figure 1). These main components of the intestinal barrier include:

- the mucus layer, together with the commensal gut microbiota mainly located in the outer mucus layer, and antimicrobial proteins (AMPs), mucins, and secretory immunoglobulin A (sIgA) molecules located in the inner mucus layer (Schroeder, 2019);
- the central polarized epithelial cells monolayer, with specialized epithelial cells interconnected by protein complexes (Salvo Romero et al., 2015);
- the inner lamina propria where innate and adaptive immune cells reside, like macrophages, lymphocytes, plasma cells, dendritic cells, together with a structured lymphoid tissue made up of Peyer's patches, containing M cells, dendritic cells, and lymphocytes (Salvo Romero et al., 2015).

Due to the components of the intestinal barrier, the defenses performed by the GIT include both immunogenic mechanisms (immunoglobins and mucosal lymphocytes) and selective intestinal permeability, which represents a highly regulated dynamic process mainly controlled by the

protein complexes that connect the monolayer of epithelial cells. In fact, the intestinal barrier not only protects the deeper layers of the intestinal structure, but also strictly regulates the passage of pro-inflammatory molecules, microorganisms, toxins, and antigens thanks to the role of the epithelium in barrier permeability (Farhadi et al., 2003).

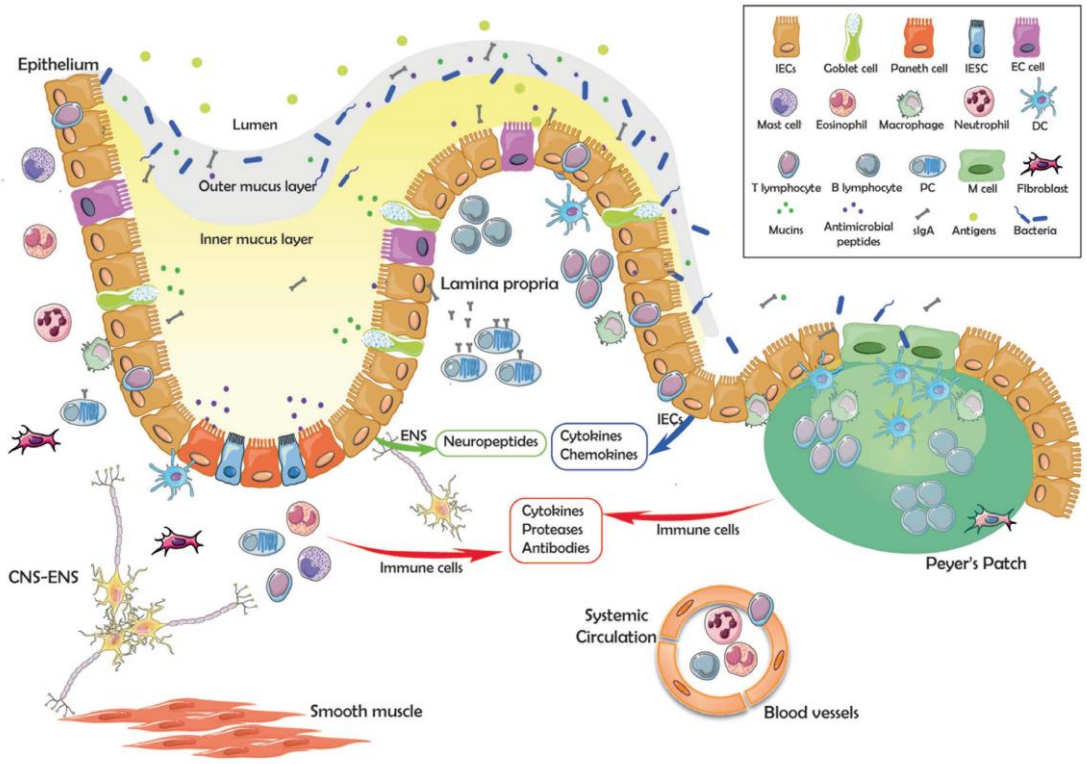

Figure 1. Anatomy of the intestinal barrier (Salvo Romero et al., 2015).
The intestinal barrier comprises 3 layers: mucus layer, intestinal epithelium, and lamina propria. The mucus layer is divided in outer, which contains microbiota, and inner, or non-stirred mucus layer. The intestinal epithelium includes a monolayer of polarized epithelial cells and a subepithelial region that contains the lamina propria, enteric nervous system (ENS), connective tissue, and muscular layers.

Structure of the intestinal epithelium

The intestinal epithelial barrier is constituted of a single layer of different types of epithelial cells, such as enterocytes, Paneth cells, goblet cells, enterochromaffin cells, and enteroendocrine cells, strictly interconnected among each other (Salvo Romero et al., 2015). The most abundant type of cells composing the intestinal epithelium are the enterocytes, for which the major function is the maintenance of epithelial barrier integrity. Enterocytes have a

key role in digestion by ensuring the uptake of ions, water, nutrients, vitamins, and absorption of unconjugated bile salts (Salvo Romero et al., 2015).

Epithelial cells are interconnected with each other due to the presence of specific protein complexes able to control the permeability of the intestinal epithelium. The intestinal epithelium mediates selective permeability via two major routes: transepithelial/transcellular and paracellular pathways (Figure 2) (Groschwitz and Hogan, 2009). Transepithelial or transcellular permeability is generally related to solute transport through the epithelial cells, mediated by the presence of selective transporters for amino acids, electrolytes, short-chain fatty acids (SCFA), and sugars. On the other hand, paracellular permeability (PCP) is associated with transport in the space between epithelial cells, and the paracellular route is regulated by intercellular complexes between cells. The interconnection between intestinal epithelial cells includes three components: desmosomes, adherens junctions, and tight junctions (TJs), aligned in this order from the basal end of the junction up to the apical part (Groschwitz and Hogan, 2009). TJs regulate the paracellular pathway for the movement of ions and solutes in-between cells. These junctions consist of the transmembrane proteins occludin and claudins, and the cytoplasmic scaffolding proteins belonging to the *zonula occludens* protein (ZOs) family. The adherens junctions and TJs complexes are also important in the regulation of cellular proliferation, polarization, and differentiation (Suzuki, 2020).

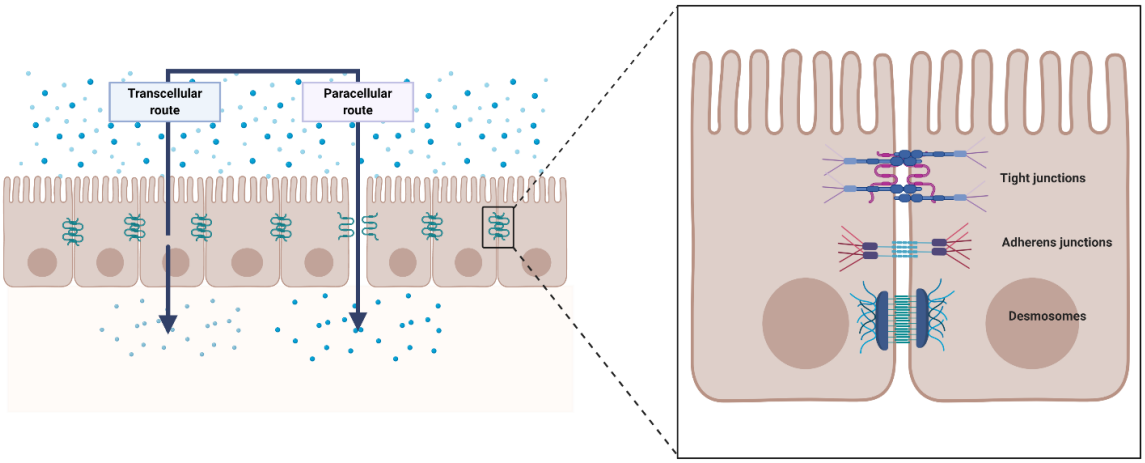

Figure 2. Pathways of epithelial permeability and epithelial cell junctional complexes (created with Biorender.com by Vetagro Marketing Dept.). The transcellular route is related to the movement of solute or water through intestinal epithelial cells. The paracellular route is represented by the movement of molecules through the intercellular space between epithelial cells. The interconnection between intestinal epithelial cells comprises TJs, adherens junctions, and desmosomes, aligned in this order from the apical to the basolateral part. The paracellular route is mainly regulated by tight junctions localized at the junction of the apical-lateral membranes.

TJs are crucial for the maintenance of the barrier integrity since they represent an essential component of the intestinal barrier machine, able to create a seal between neighboring intestinal epithelial cells, located close at the apical portion of the lateral membrane of epithelial cells. At the transmission electron microscopy, the TJs appear to eliminate the intracellular space at the so-called “kissing points” (Farquhar and Palade, 1963). Among their roles, the TJs count the regulation of the PCP, that facilitates the passage of ions and solutes through the intercellular space, while preventing the translocation of luminal antigens, pathogens, and their toxins (Suzuki, 2020). TJs are constituted by different transmembrane proteins, like claudins, occludin, junctional adhesion molecules (JAMs), and intracellular proteins, such as ZOs. The intracellular domains of transmembrane proteins, such as claudins and occludin, are associated with cytosolic plaque proteins, as ZOs, which provide a structural scaffold to the TJs (Suzuki, 2020).

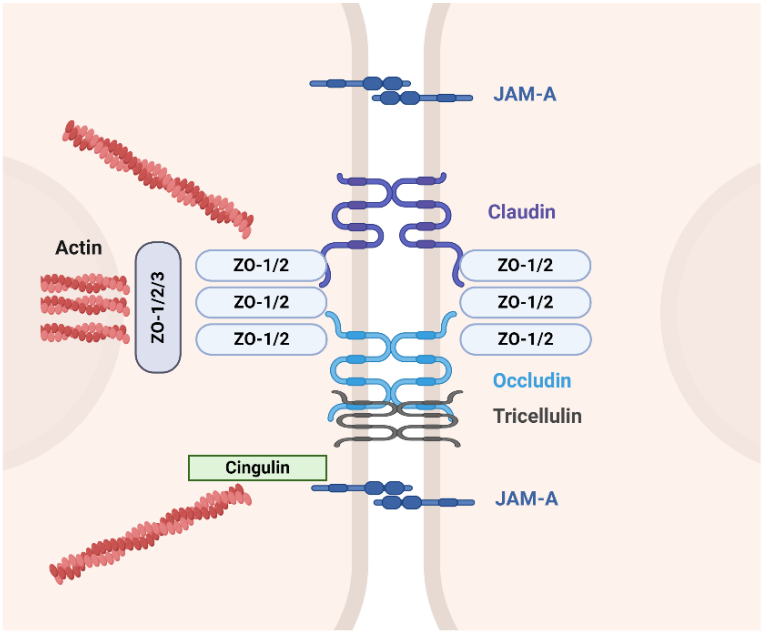

Figure 3. Schematic representation of the structural transmembrane components of TJs (created with Biorender.com by Vetagro Marketing Dept.). ZO-1 and -2 are important for assembling claudins and occludin, allowing the formation of TJ strands. The ZOs and cingulin can provide a direct link to the actin cytoskeleton. Occludin plays a role in the maintenance and assembly of TJs, while claudins contribute to the tightness of paracellular barriers. The ZOs are multi-domain proteins that provide an intracellular scaffold in the TJs (Niessen, 2007).

TJs are in a dynamic balance, regulated by both intracellular and extracellular factors. Several pieces of evidence suggest their involvement in the pathogenesis of inflammatory and non-inflammatory intestinal diseases. In fact, TJs disruption leads to an inadequate epithelial barrier, followed by intense water and electrolyte loss. Moreover, TJs may be disrupted during intestinal diseases, leading to the additional worsening of the pathogenesis, as bacteria and endotoxins can cross the intestinal barrier and activate an intestinal inflammatory response (Senthil et al., 2006). Last but not least, they are also the target of several enteric pathogens that exert their pathogenesis through alteration of TJs mediated by their toxins (Guttman and Finlay, 2009). Changes of TJs are associated with several disease in humans such as inflammatory bowel diseases (IBD), Crohn's disease, ulcerative colitis, and celiac disease, as well as in livestock animals. For example, after porcine epidemic diarrhea virus (PEDV) infection a loss of tight junction proteins, including ZO-1, ZO-2, occludin, and some claudins, were reported (Chen et

al., 2021; Jung et al., 2015) indicating impaired intestinal integrity, that promotes secondary bacterial infections. Increasing investigations also reported the influence of enterotoxigenic *Escherichia coli* (ETEC) on intestinal TJ functionality, suggesting its involvement in the reduction of TJ strand numbers, delocalization of ZO-1, reduction of occludin amounts, and dephosphorylation of occludin, allowing an increase in the intestinal permeability (Johnson et al., 2010).

Evaluation of the intestinal epithelium integrity – *in vitro* models

Considering that the intestinal single layer of epithelial cells represents a critical barrier protecting the host against diverse luminal noxious agents, as well as preventing the uncontrolled uptake of bacteria, it is important to develop and use *in vitro* model of intestinal cell culture that could allow to determine the effect of bioactive compounds in enhancing said barrier properties. The intestinal barrier functionality could be expressed as transepithelial electrical resistance (TEER) and PCP, two electrophysiological parameters able to evaluate the passage of ions, waters, and molecules through the intestinal epithelium (Srinivasan et al., 2015). TEER is the measurement of the electrical resistance of the ionic conductance across the cellular monolayer, used as a reliable method to confirm the integrity and permeability of the monolayer. On the other hand, the permeability of the intestinal epithelium can be studied by measuring the passive transport of small hydrophilic molecules across the monolayer. Such molecules mostly pass the monolayer via the paracellular route through the TJs. In fact, PCP indicates the measure of the paracellular passage of marker molecules, as well as the pore size selectivity of the tight junctions (Srinivasan et al., 2015; Verhoeckx et al., 2015).

One of the most used *in vitro* models of the intestinal epithelial barrier is the human epithelial cell line Caco-2 (***Ca***ncer ***co***li-2). This cell line is originally established from a human colorectal carcinoma from a 72 years-old Caucasian man in 1977 (Fogh et al., 1977). Caco-2 cells are successfully used as a model of intestinal epithelial barrier thanks to their properties, including

the most advantageous one: the ability to spontaneously differentiate into a monolayer of cells able to express various properties typical of absorptive enterocytes. Towards confluency, once seeded on specific supports, Caco-2 cells start to polarize, developing a typical apical brush border with microvilli. TJs form between adjacent cells, and they express enzyme activities typical of enterocytes (Lea, 2015).

The cultivation of Caco-2 cells on filter supports improves their morphological and functional differentiation into absorptive enterocytes, allowed by their ability to spontaneously specialize their structure, in order to better meet all their properties (Figure 4). During the differentiation on filter supports, Caco-2 cells seem to follow a specific time schedule in the development of their biochemical and morphological properties. When polarized and confluent, the cell layer forms a continuous barrier between the upper and lower compartments (apical/mucosal and basolateral/serosal, respectively) (Lea, 2015).

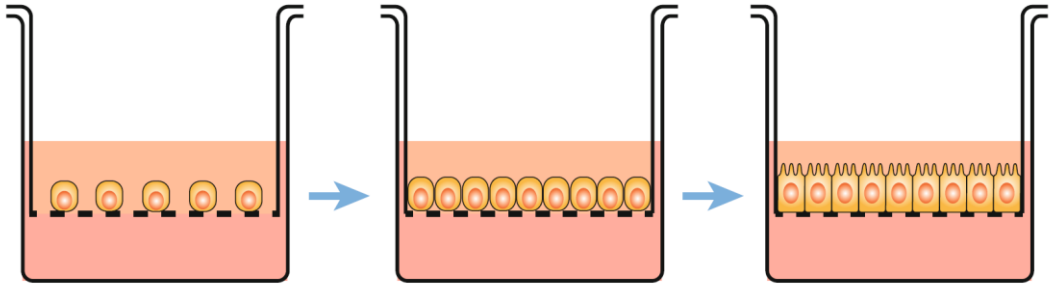

Figure 4. Differentiation of Caco-2 cells on a filter support insert. When reaching the confluence (middle) Caco-2 cells start to differentiate spontaneously, and after a total culture period of around 21 days they will appear with dense microvilli (Lea, 2015).

It is possible to evaluate electrophysiological parameters (TEER and PCP) with Caco-2 cells and the classical setup for the measurement of TEER consists of a cellular monolayer cultured on a semipermeable filter insert. TEER is measured with two electrodes, one electrode placed in the upper compartment (apical part) and the other in the lower compartment (basolateral part); the electrodes are separated by the cellular monolayer (Figure 5). The ohmic resistance is calculated based on Ohm's law, such as the ratio of the voltage and current. The simplified

model of the resulting electrical circuit for the TEER measurement contains electrical resistances for both the transcellular and paracellular pathways in parallel (Figure 3) (Hickman, 2016). Typically, TEER values > 300 Ω cm^2 indicate adequate monolayer integrity (Chen et al., 2015).

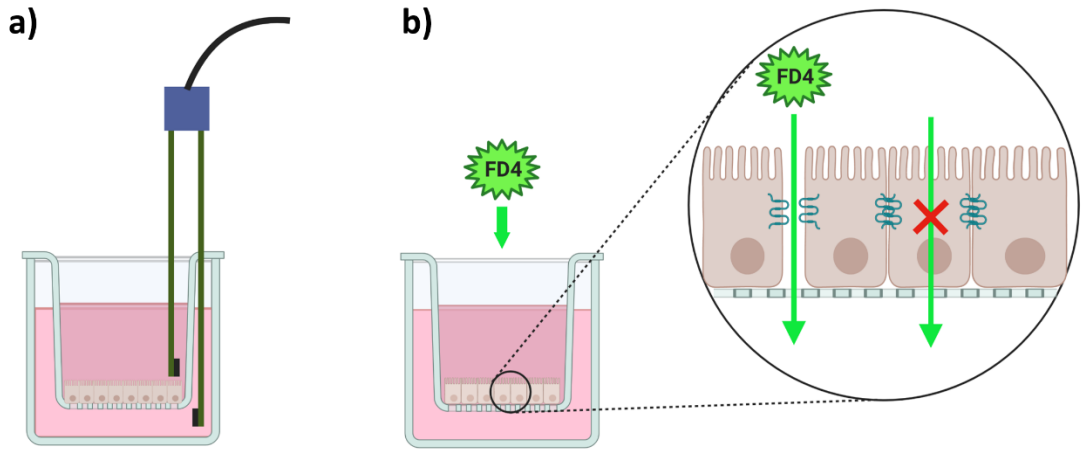

Figure 5. Evaluation of electrophysiological parameters in Caco-2 cells on porous insert (created with Biorender.com by Vetagro Marketing Dept.). (**a**) Schematic representation of the measure of TEER with chopstick-like electrodes for use on standard transwell inserts. (**b**) Schematic representation of the measure of PCP with a fluorescent tracer (fluorescein isothiocyanate–dextran, FD4); the dextran tracers can pass through the epithelium using the paracellular way, but not with the transcellular route.

The PCP assay consists of the quantification of the passage of a tracer molecule from the apical side to the basolateral side of the *in vitro* cell culture (Ghaffarian and Muro, 2013). To perform PCP, sugar tracers (mannitol or dextran) labeled to a fluorescent dye (e.g., fluorescein isothiocyanate) are normally used (Figure 5). These tracers can pass through the epithelium only using the paracellular way, as they do not have specific transporter on cells, avoiding the influence on the transporter activity (Yeste et al., 2018). Other tracers can be used to analyze the PCP, for example, radiolabeled markers, that provide the required sensitivity, but specific precautions for handling and storage are needed. Radiolabeled markers also have a short half-life and are not suitable for long term storage. It is also important to note that, contrarily to TEER assay, the use of tracer compounds to analyze the PCP can interfere with the transport

process and affect the barrier integrity, making the tested cells unusable for further experiments (Srinivasan et al., 2015).

A healthy monolayer of cells will retain most of the tracer molecule in the apical part, while a damaged one will leave the tracer free to move to the basolateral part. For the same reason, healthy cells will present a higher TEER compared to the damaged one.

THE ENDOCANNABINOID SYSTEM

The integrity and health of the intestinal mucosa are also indirectly related to other systems in the body, amongst which the endocannabinoid system (ECS) is getting an increasing interest since it seems to control several GI functions, as well as the regulation of intestinal inflammation. ECS is composed of three fundamental constituents: receptors, signaling molecules, and the enzymes involved in ligands biosynthesis and degradation (Di Marzo and Piscitelli, 2015). Fifty years ago (in 1964) the psychoactive ingredient of *Cannabis sativa*, Δ^9-tetrahydrocannabinol (THC) was isolated, opening the road to the discovery of an entire system previously unknown. Thirty years later the endogenous counterparts of THC were discovered: N-arachidonoylethanolamine (anandamide, AEA) in 1992, and 2-arachidonoylglycerol (2-AG) in 1995 (Maccarrone et al., 2015).

Presently, in physiological and pathological conditions, the ECS has great importance. Several diseases like inflammation, pain, emesis, anorexia, epilepsy, glaucoma, schizophrenia, cardiovascular disorders, obesity, metabolic syndrome-related diseases, multiple sclerosis, cancer, Parkinson's disease, Huntington's disease, Alzheimer's disease, and Tourette's syndrome could be possibly treated by drugs modulating ECS (Kaur and Singh, 2016). After the first discovery of ECS in the central nervous system (CNS), where it is mostly expressed, its localization was found all along the human body, with a particular interest in the cardiovascular, gastrointestinal, immune, and reproductive systems (Cabral et al., 2015; Maccarrone et al., 2015). Besides humans, the ECS was also identified in other mammals, birds, and fish. This signaling system has been suggested to play several roles in animals (Salzet et al., 2000) and growing evidence indicates that the activation of cannabinoid receptors by endogenous, plant-derived, or synthetic cannabinoids may exert beneficial effects on inflammation and visceral pain. Nevertheless, the studies conducted since now have just

scratched the surface of the possible implication of the ECS, especially about animal medicine and nutrition.

Endocannabinoid system constituents

Receptors

The main endocannabinoid receptors are two G protein-coupled receptors (GPCRs), named cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (Mackie, 2008), although additional receptors may be involved (Morales and Reggio, 2017). When GPCRs were stimulated by hormones, neurotransmitters, or lipids, they change their conformation in the "active" one, leading to a wide range of intracellular responses (Figure 6). CB1 is the most widely expressed receptor protein from the GPCRs family in the brain, mainly in basal ganglia, hippocampus, olfactory bulb, and cerebellum (Mackie, 2005). Besides the various roles of this receptor in the brain, where it is mainly located, CB1 seems involved in feeding behavior. In fact, when activated, it allows an increase of appetite and inhibits satiation, drives the intake of fat-rich foods, interacts with gut microbiota, and controls gut contractility via control the enteric nervous system (ENS) (DiPatrizio, 2016).

CB2, in contrast with CB1, is mainly expressed in immune tissues, like microglia, leukocytes, and in cells of the macrophage lineage (Bie et al., 2018). CB2 has been the subject of considerable attention, due to its promising therapeutic potential for treating various pathologies while avoiding psychotropic effects (Dhopeshwarkar and Mackie, 2014). In the gut, CB2 activation has been shown to inhibit the secretion of pro-inflammatory cytokines, suppressing the levels of cyclooxygenases (COX), and inducible nitric oxide synthase (NOS) (Klein, 2005).

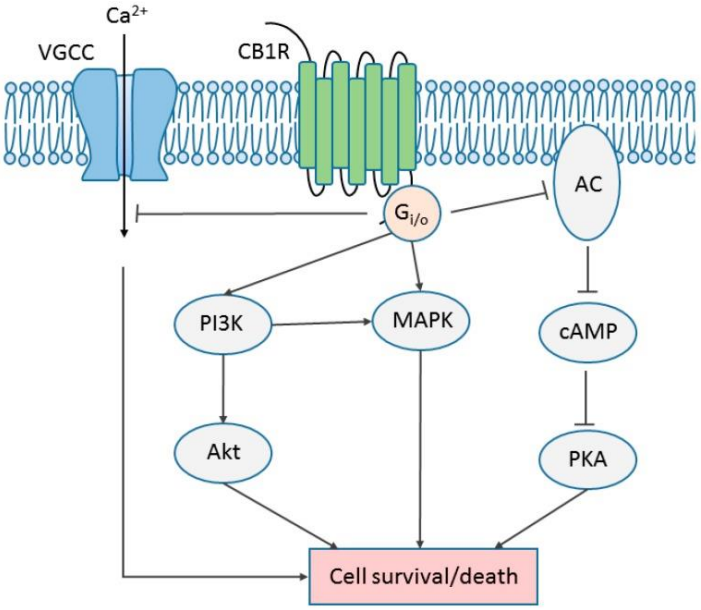

Figure 6. An example of a G-protein coupled receptor (CB1) intracellular signaling (Zou and Kumar, 2018). Depending on the ligand and subcellular nature, the result of CB1-mediated signaling could be promotion of cell survival or cell death. In fact, CB1 is coupled to Gi/o and inhibits the activity of adenylyl cyclase, formation of cyclic adenosine monophosphate (cAMP), and the activity of protein kinase A (PKA). CB1 is able to repress calcium influx via voltage-gated calcium channel (VGCC). Moreover, several mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathways are activated by the CB1.

Other receptors related to the endocannabinoid system belong to peroxisome proliferator-activated receptors (PPAR) and transient receptor potential channel (TRP) families. Concerning TRP, they are integral membrane proteins localized mostly in cellular membranes, with the role of control the entrance of ions Ca^{++} in the cells (Hardie, 2007). The superfamily of TRP contains seven subfamilies (Nilius et al., 2007; Vay et al., 2012) and the subfamilies TRPV, TRPM, and TRPA can interact with cannabinoids, in particular the receptors TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, and TRPA1 (De Petrocellis et al., 2011; Di Marzo and Petrocellis, 2010; Muller et al., 2019).

TRPV1 channel is an ion channel activated by capsaicin, as well as by low pH, noxious heat, eicosanoids, and endocannabinoids (Moran et al., 2011). This receptor is located in brain, skin, pancreas, GIT, and bladder (Nilius and Owsianik, 2011). In the GIT, TRPV1 is mainly expressed by the myenteric plexus (MP), submucosal plexus (SMP), and mucosa (Holzer,

2011). TRPV1 action includes accelerating gastric emptying process, reducing gastric acid secretion, and protecting gastric mucosa (Du et al., 2019).

The nociceptive *TRPA1* channel is an ion channel that reacts to mechanical, thermal (cold), and chemical stimulation. It can be activated by different chemical substances present in herbs and spices, like allicin or cinnamaldehyde (Holzer, 2011). TRPA1 is expressed in thermo-receptive neurons (for cold detection), airway sensory nerves, immune cells, enterochromaffin cells, and in lamina propria. TRPA1 seems to act as a pro-inflammatory regulator, involved in neurogenic inflammation and dermatitis (Meseguer et al., 2014; Wong et al., 2018). Differently, other studies suggest an anti-inflammatory role of TRPA1, via T helper lymphocytes cells involvement (Bátai et al., 2019; Bertin et al., 2017). In the GIT, TRPA1 activation promotes contractility, mechano- and chemo- sensory function, and modify both epithelial and muscular functions (Holzer, 2011).

Signaling molecules

The signaling molecules of the ECS are divided into two different types: endogenous and exogenous. The first are the ones produced by the organism, named endocannabinoid, the second are the ones produced by plants, able to bind and activate the endocannabinoid receptor, named phytocannabinoid.

The endocannabinoids are the endogenous ligands of cannabinoid receptors. These molecules are eicosanoids, mostly derived from arachidonic acid, able to bind and activate the specific cannabinoid receptor with different affinity (Wang and Ueda, 2009). Endocannabinoids are present in the brain and peripheral tissues, as muscle, adipose tissue, and circulating cells, and they have been found also in breast milk (Di Marzo et al., 1998; Hillard, 2018). Endocannabinoids are produced on demand from membrane phospholipids, in contrast with classical neurotransmitters and neuropeptides which are stored in vesicles in the cells. Endocannabinoids are produced by intracellular enzymes and released from cells, then follow

the immediate action as signaling molecules; after the uptake by cells, they are inactivated by specific enzymes (Piomelli, 2003). Numerous stimuli are able to enhance the production of endocannabinoid, as stress, obesity, and inflammation, with different effects on organs and tissues (Figure 7). The main endocannabinoids are anandamide (AEA, N-arachidonylethanolamine) and 2 arachydonyl-glicerol (2-AG), initially isolated from porcine brain and canine intestine respectively (Devane et al., 1992; Mechoulam et al., 1995). Palmitoylethanolamide (PEA) or oleoylethanolamide (OEA) are considered endocannabinoid-like and can directly or indirectly act on cannabinoid receptors (Maccarrone et al., 2015).

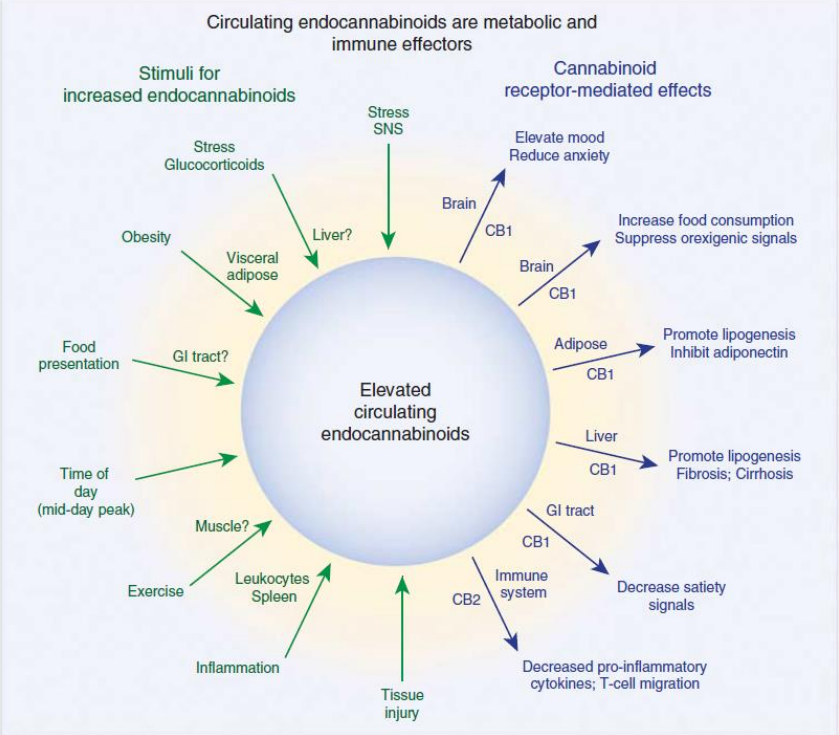

Figure 7. Schematic representing of the stimuli and of the potential targets of endocannabinoids, together with the cannabinoid receptor subtype that is involved (Hillard, 2018). The endocannabinoid system exerts regulatory control on every aspect related to search, intake, metabolism, and storage of calories. For example, activation of CB1 signaling results in increased consumption of food, modulate energy homeostasis via interactions with ghrelin, potentiates hedonic consumption of highly palatable foods, increases energy storage via stimulation of fat mass expansion, and the induction of lipogenesis (Hillard, 2018).

The phytocannabinoids are of plant origin, mainly meroterpenoids derived from *Cannabis spp*. More than 100 phytocannabinoids have been isolated and divided into 11 classes, among which

the most known are THC, cannabidiol (CBD), and cannabinol (CBN) (Fisar, 2009). THC and CBD are the main constituents of *Cannabis sativa*, with various pharmacological profiles; in particular, THC is well known for its psychoactive side effects (Gaoni and Mechoulam, 1964). CBN has slight THC-like effects, while CBD is known as a non-psychotropic constituent with other pharmacological effects (Fisar, 2009) (Figure 8).

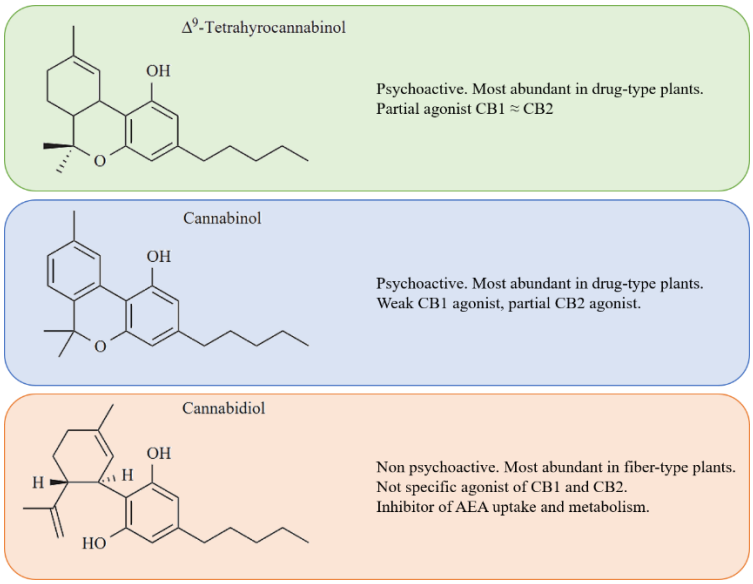

Figure 8. Chemical structure of the 3 main phytocannabinoids: THC, CBN (cannabinol), and CBD (cannabidiol). In particular are reported psychotropic (THC), weakly psychotropic (CBN), and non-psychotropic (CBD) phytocannabinoids with their main characteristics. Adapted from Fisar, 2009.

Enzymes for synthesis and degradation

The endocannabinoids present in the human circulation are subjected to dynamic variations of their concentrations. Circulating endocannabinoids come from multiple organs and tissues, including brain, muscle, adipose tissue, and circulating cells (Hillard, 2018). Once synthesized from specific enzymes, endocannabinoids bind the specific receptors and activate a retrograde signaling pathway. Then, they are quickly inactivated from enzymes specifically involved in endocannabinoid degradation (Fisar, 2009). Both AEA and 2-AG contain arachidonic acid, but

their synthesis and degradation are operated by different enzymes. An example of AEA synthesis and degradation is reported in Figure 9.

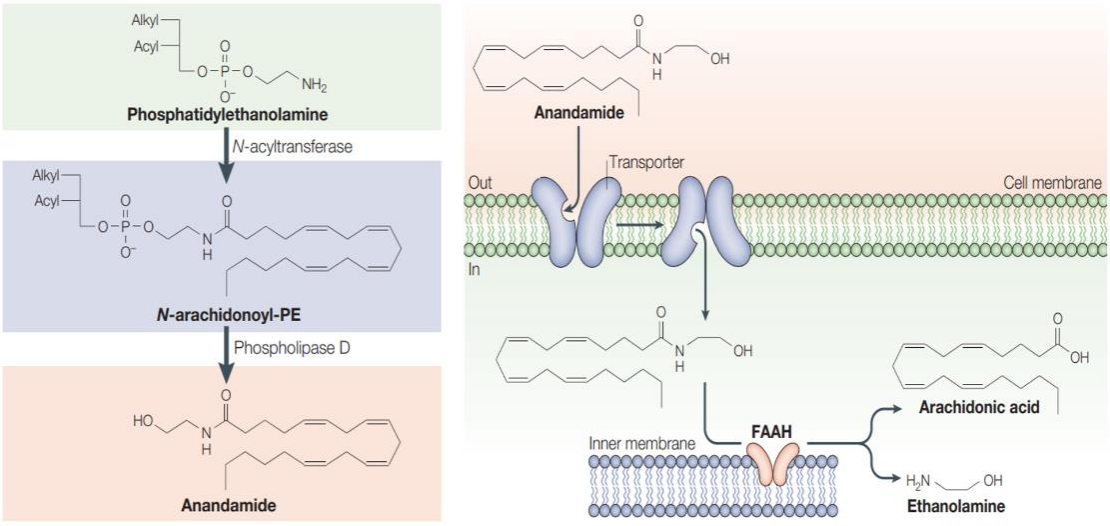

Figure 9. Mechanism of anandamide formation and degradation. Adapted from (Piomelli, 2003). The sequence of reactions includes: first, the synthesis of the anandamide precursor NAPE (N-acyl-phosfatidylethanolamine) followed by its cleavage to yield anandamide, catalyzed by NAPE-PLD (N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D); second, AEA can be internalized by neurons and, once inside cells, can be hydrolyzed by FAAH (fatty acid amino hydrolase) to yield inactive breakdown products.

Endocannabinoid system in the gut

The ECS is involved in the control of several GI functions at both peripheral and central levels (Figure 10). For this reason, it is not surprising that cannabis has been used for millennia to treat symptoms of inflammation and functional disorders of the GIT, as abdominal cramps, pain, diarrhea, nausea, and vomiting (DiPatrizio, 2016). In particular, activation of CB1 and possibly CB2 attenuates the emetic reflex by reducing the release of excitatory transmitters, including serotonin (5-HT) (Sharkey and Wiley, 2016).

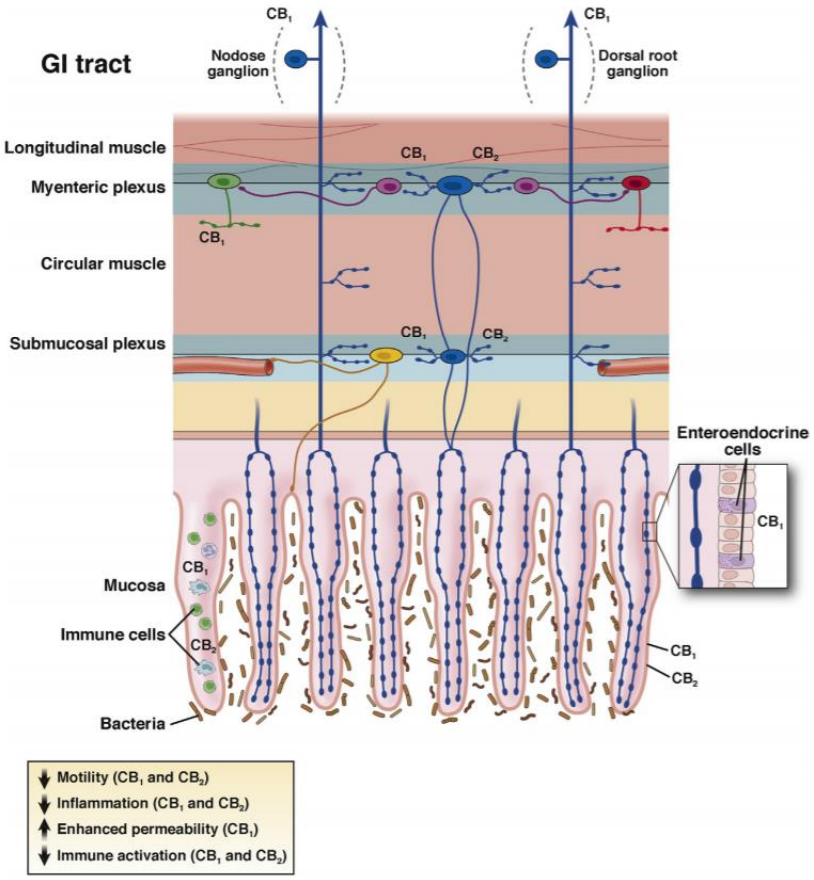

Figure 10. Effects of endocannabinoids in the gastrointestinal tract are mainly mediated by CB1 and CB2 receptors. CB1 is expressed on enteric neurons in the myenteric and submucosal plexuses, and in some enteroendocrine cells in the epithelium. CB2 is expressed by enteric neurons and immune cells. ECS is able to influence motility, inflammation, and immune response in GIT (Sharkey and Wiley, 2016)

Gastrointestinal motility

The control of the GI motility performed by the ECS is mainly mediated by the reduction in the release of acetylcholine (Ach) via the activation of presynaptic CB1 (DiPatrizio, 2016). However, recent evidence suggests that this effect is also related to the inhibition of the peristaltic reflex. In parallel with the inhibition of the release of ACh, on rodents models CB1 agonist AEA was able to significantly inhibit the release of both substance P and VIP, reducing the contractility of longitudinal muscle in a dose-response manner (Baldassano et al., 2008), with a relaxation of the peristaltic reflex (Yuece et al., 2007). Contrarily, CB2 does not seem to play a key role in the control of GI motility under physiological conditions. However, Duncan

and colleagues (2008) shown that intestinal hypermotility caused by lipopolysaccharide (LPS) administration was completely inhibited by CB2 agonist, suggesting a role of CB2 in pathophysiological conditions. Also Bashashati et al. (2012) studied the reaction of ECS after LPS-induced inflammation, showing that the inhibition of FAAH (the enzyme involved in the degradation of AEA) normalized hypermotility in a CB1- and CB2- dependent manner. Additionally, blocking the degradation of 2-AG via inhibiting the activity of MAGL, results in a reduction of whole-gut transit (DiPatrizio, 2016).

Gastrointestinal inflammation

Regulation of intestinal inflammation is one of the homeostatic regulations controlled from the ECS. In fact, the beneficial effects of cannabinoids were more evident in situations where increased GI motility and visceral sensation were induced by an inflammatory stimulus. Positive effects on PCP in Caco-2 cells were recorded when an antagonist of CB1 was administered to cells subjected to an inflammatory challenge with LPS (Alhamoruni et al., 2012). On the other hand, in human colonic epithelial cells HT-29, CB2 receptor agonists inhibited tumour necrosis factor α (TNF-α) and stimulated the production and release of interleukin 8 (IL-8), which are recognized to exert a major influence on maintenance of intestinal homeostasis (Ihenetu et al., 2003). Roles of CB1 and CB2 receptors in modulating inflammatory process was also suggested from some reports showing the suppression of mast cells and macrophages activity, inhibiting proliferation of T helper lymphocytes, and secreting inflammatory cytokines (Alhouayek and Muccioli, 2012; Di Marzo and Izzo, 2006; Smith et al., 2000). The key role of the ECS in the regulation of intestinal inflammation has been confirmed also by the evidence that pharmacological treatment with FAAH inhibitors, with consequent reduction of degradation of endocannabinoid molecules, prevented the development of colitis in mice (Sałaga et al., 2014). The increase of ECS activity results in the reduction of inflammation and improved colon integrity.

Brain-gut axis

The mechanisms of endocannabinoid signaling in the gut have been suggested to participate in the control of energy balance and food intake with the vagus nerve, which bi-directionally communicates between the gut and brain (DiPatrizio, 2016). Several studies suggest that the intake of dietary fat, due to its distinguishable taste properties, is driven by endocannabinoid molecules in the gut (DiPatrizio et al., 2013, 2011; DiPatrizio and Piomelli, 2015). Tasting dietary fat enhances the endocannabinoid molecules levels in jejunum, while inhibiting CB1 drives a reduction to the preference to unsaturated dietary fats (DiPatrizio et al., 2011). Additionally, normal-weight and obese humans showed an increase in circulating endocannabinoid molecules during and after the consumption of highly palatable food (Rigamonti et al., 2015).

STRATEGIES FOR ANIMAL NUTRITION

A growing body of literature demonstrates how different feed additives may play some roles on the integrity of the GIT. Nutritional strategies aimed to target the health status of the intestinal tract of livestock animals got increasing attention after the complete ban of in-feed antibiotic growth promoters in European Union (Regulation (EC) No 1831/2003) and the indication for a judicious use of antimicrobials in food-producing animals in USA ("Veterinary Feed Directive," 2015). These decisions were strongly driven by the urgent problem of antimicrobial resistance, particularly against bacteria which have developed resistance to each new antibiotic coming to market, requiring serious action to prevent a global crisis in health care to human and animals (Prestinaci et al., 2015). In fact, new challenges in the management of pathologies in animal production were posed and the use of alternative molecules to antibiotic and antibiotic growth promoters increased significantly (Karavolias et al., 2018). Nutritional strategies have been aimed to search not only for antimicrobial alternatives, but also for active principles able to directly enhance the health status of the animals, acting directly or indirectly on the integrity of the intestinal barrier.

With this target in mind, in the current post-antibiotic era, numerous alternatives have been studied and gained an increasing interest such as organic acids (OA), inorganic acids, botanicals including nature-identical compounds (NIC), essential oils (EO), plant extracts, and so on. All these categories showed an interesting potential thanks to their important biological activities including antimicrobial potential, as well as anti-inflammatory, antioxidant, and immunomodulatory abilities (Rossi et al., 2020; Tugnoli et al., 2020; Windisch et al., 2008).

Organic acids

OAs have been widely used over the last decades for their positive effect on animal health and growth efficiency. In fact, OAs were reported as effective growth enhancers, although there is a large variation in responses due to various factors such as type and dose of OA used, duration of supplementation, diet composition, and buffering capacity, and age of the animals. Besides the role of acidifiers, OAs are accepted also as antimicrobials, "improvers" of nutrient digestibility, enhancers of the immune system, and growth promoters (Khan and Iqbal, 2016; Tugnoli et al., 2020). Not all OAs have antimicrobial properties, but many of interest from this perspective easily fall into three categories: SCFA, medium-chain fatty acids (MCFA), and TCA (tricarboxylic acid) cycle acids. The antimicrobial activity of OAs depends on various factors, as carbon chain length and degree of unsaturation, but overall the pKa of the acids drives their antimicrobial action (Grilli and Piva, 2012). In particular, when OAs are undissociated, they are able to diffuse across the bacterial cell membrane and dissociate inside the cell, releasing H^+ ions and decreasing cytoplasmatic pH. This causes an activation of the proton, with a consequent consumption of energy. Additionally, the released anion $RCOO^-$ is toxic to DNA replication, triggering the disruption of metabolic functions, and the increase of osmotic cell pressure (Grilli and Piva, 2012). Above "anion model" inhibits bacterial replication and growth, leading to a bacteriostatic or bactericidal effect. Of course, depending on the mode of action, the spectrum of activity of OAs is highly variable in relation to the target microorganism and, in particular, to the complexity and structure of its outer cell membrane. Gram-positive bacteria (i.e., *Clostridium perfringens*, *Streptococcus spp.*) are mainly susceptible to MCFA, while Gram-negative bacteria (i.e., *Escherichia coli*, *Salmonella spp.*) are more sensitive to SCFA (Tugnoli et al., 2020). Interestingly, OAs do not affect beneficial lactic acid bacteria, resulting in a positive modulation of the microbial population (Ferronato and Prandini, 2020).

Botanicals

Chapter modified from: Paper published on Nutrition Research Reviews (2020) 26;1-17. "Single components of botanicals and nature-identical compounds as a non-antibiotic strategy to ameliorate health status and improve performance in poultry and pigs". Barbara Rossi, Andrea Toschi, Andrea Piva, and Ester Grilli

Botanicals represent a wide group of compounds with different biological activities. Based on their origin, botanicals can be obtained from the whole plant or some of its dried parts (powder extract), or by solvent extraction (essential oils, EOs, and oleoresins). EOs are aromatic, volatile and oily, and represent a mixture of complex compounds extracted from different parts of the plant material. The composition may vary in individual chemical compositions and concentrations. The chemical diversity of compounds occurs not only between and within plant families and genera, but also within populations of a single plant species. The variability in the composition of EOs depends not only on several physiological, environmental, and geographical conditions, but also on the part of the plant used for extraction (Figueiredo et al., 2008). Because of the antimicrobial, anti-inflammatory, and antioxidative properties, EOs have been widely used in traditional medicine to improve health (Omonijo et al., 2018). Another possibility is to use the pure bioactive compounds composing the EO, such as the chemically synthesized counterparts of these pure bioactives, referring to them as NICs or pure botanicals or phytochemicals. The difference in using NICs, rather than plant extracts, is represented by the fact that the former are single chemically pure molecules. In the livestock industry, it is important to control intestinal inflammation in order to have healthier animals and better zootechnical performance (Murugesan et al., 2015; Omonijo et al., 2018). In this context, there are many properties of great interest like antibacterial, antifungal, antiviral, antiprotozoal, anti-inflammatory, antioxidant, and immunomodulatory, that may impact on gut functions and gut morphology.

The specific antimicrobial mechanism of action of EOs is still unclear, in fact various mechanisms of antibacterial activity have been proposed. Thanks to their lipophilic nature, EOs

easily penetrate through the bacterial cell membranes. The penetration seems possible as EOs are able to destabilize the cellular architecture, leading to the breakdown of membrane integrity. This causes an increase in bacterial permeability, with the disruptions of several cellular activities, such as membrane transport and energy production. Moreover, another mode of action seems connected to the reduction of membrane potentials, with the disruption of proton pumps, and the depletion of the ATP. All these factors, which alter the cell organization, may cause a cascade effect, resulting in disruption of the arrangement of phospholipids bilayers and polysaccharides molecules (Shaaban, 2020; Swamy et al., 2016).

The antimicrobial mode of action of individual components of EO can have single or multiple targets (Nazzaro et al., 2013b). Several classes of botanicals, such as terpenes, polyphenols, aldehydes, and organosulfur compounds are able to inhibit the bacterial cell division protein FtsZ, a prokaryotic homolog of tubulin, or the quorum sensing (QS), a bacterial intercellular communication system that governs proliferation and virulence (Nazzaro et al., 2013a). Moreover, terpenes can also interact with the cell surface of bacteria (Nazzaro et al., 2013b), binding phospholipids and leading to the disaggregation of membrane structures and ion leakage, reduction of membrane potential, collapse of the proton pump, and depletion of the ATP pool (Ultee et al., 2002).

Several botanical reported also an immunomodulatory effect on the host defenses, thus showing both immunosuppressors or immunostimulators actions depending on the precise target (Anastasiou and Buchbauer, 2017). The effects on the host immune defenses are connected to the antioxidant and anti-inflammatory activity of botanicals. By controlling the production of reactive oxygen species (ROS), which are responsible of a wide spectrum of diseases, botanicals modulate the inflammatory status of the host at many levels, including the overall health status of the GIT (Reuter et al., 2010). This means that the antioxidant mechanism of action mediated by botanical components could also be the explanation of their anti-inflammatory properties. These properties enhance the general health status of animals, thereby

improving intestinal health. Various aromatic plant bioactives were reported to exert beneficial effects on animals by improving gut morphology and expression of TJ proteins (Grilli et al., 2015; Sun et al., 2017; Zou et al., 2016a, 2016b), promoting the integrity of the intestinal barrier (Grilli et al., 2015; Zou et al., 2016b).

AIM OF THE THESIS

The increase of antibiotic resistance posed new challenges in the management of livestock animal species, as the growing sensitivity of the consumers about the problem of antibiotic resistance and antibiotic contamination in food led to an increase of interest in the antibiotic-free (ABF) and no-antibiotic ever (NAE) productions. This led to the discovery of natural antimicrobials alternative to classical antibiotics growth promoters (AGP). Novel natural alternatives can support the management of pathogen infections not only from an antimicrobial perspective, but also thanks to their multi-target mechanism of action. In fact, the exposure to enteric pathogens compromises the integrity of the first line of defense of the organism, that is the intestinal mucosa, so it is strategic to find novel molecules able also to boost mucosa barrier function and preventing oxidative stress and inflammation.

For these reasons, the project behind this thesis was aimed to examine how new potential alternative approaches to the classical antibiotic prophylaxis could also target intestinal health, directly or indirectly. Different experiments were performed in order to assess the potential of natural alternatives to antibiotics against different stressful situations that could normally occur during the livestock management cycles. In fact, these studies are not only limited to find antimicrobials alternative to antibiotics, but also to evaluate the potential of these molecules, such as NICs, EOs, OAs, alone or combined, to support the intestinal maturation and integrity. The studies were conducted both *in vitro,* exploiting the advantageous characteristics of Caco-2 cells model, and *in vivo* in piglets. The possible effects of alternative molecules as antimicrobials, antioxidant, anti-inflammatory, endocannabinoid system activators, and promoters of the intestinal epithelial integrity were evaluated. The purposes of these studies were to evaluate molecules to stimulate and support the GIT and, more in general, the organism at epithelial, immunological, and enteroendocrine level.

EXPERIMENTAL STUDIES

EXPERIMENTAL STUDY 1

Paper published in Molecules (2020) 19;25(18): 4296. doi: 10.3390/molecules25184296.

Nature-Identical Compounds and Organic Acids Ameliorate and Prevent the Damages Induced by an Inflammatory Challenge in Caco-2 Cell Culture

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Abstract

Bioactive compounds, such as organic acids (OA) and nature-identical compounds (NIC), can exert a role in the protection of intestinal mucosa functionality due to their biological properties. The aim of this study was to understand the role of 2 OA (citric and sorbic acid) and 2 NIC (thymol and vanillin), alone or combined in a blend (OA + NIC), on intestinal barrier functionality, either during homeostatic condition or during an inflammatory challenge performed with pro-inflammatory cytokines and lipopolysaccharides (LPS). The study was performed on the human epithelial cell line Caco-2, a well-known model of the intestinal epithelial barrier. The results showed how OA and NIC alone can improve transepithelial electrical resistance (TEER) and mRNA levels of tight junction (TJ) components, but OA + NIC showed stronger efficacy compared to the single molecules. When an inflammatory challenge occurred, OA + NIC blend was able both to ameliorate, and prevent, damage caused by the pro-inflammatory stimulus, reducing or preventing the drop in TEER and improving the TJ mRNA expression. The data support the role of OA + NIC in modulating gut barrier

functionality and reducing the negative effects of inflammation in intestinal epithelial cells, thereby supporting the gut barrier functionality.

Introduction

The intestinal mucosa is composed of a single layer of epithelial cells connected to each other by inter-epithelial junctions, and it is the first line of physical defense against harmful agents (Pitman and Blumberg, 2000). The maintenance of this barrier function contributes to the homeostasis and health of the animals, since barrier dysfunction might play a role in gut inflammation and disease, thereby reducing growth performance (Moeser et al., 2007). Tight junctions (TJ) are one of the junctional multiprotein complexes which help the adhesions of adjacent epithelial cells, functioning as they gate keepers to control the diffusion of solutes, regulating ion transport, blocking of macromolecules and controlling the selective transport of nutrients (Anderson and Van Itallie, 2009). Dysregulation of TJ enables harmful substances to translocate, resulting in a damaged tissue and inflammation. More than 30 structural and functional proteins constitute the TJ complex, among them zonula occludens-1 (ZO-1) and occludin are two crucial proteins to maintain the structural function of TJ (Tsukita et al., 2001). Moreover, recent outcomes highlighted the role of transient potential receptor vanilloid (TRPV) channels in modulating inflammation, while their precise role is still under investigation. In fact, TRPV channels are reported both as pro-inflammatory and as anti-inflammatory (Alaimo and Rubert, 2019; De Petrocellis et al., 2012). When intestinal barrier failure occurs, concentration of pro-inflammatory cytokines produced by the lamina propria increases (Vitale et al., 2017), negatively influencing intestinal integrity, and leading to a reduction in intestinal functionality (Campbell et al., 2013; Capaldo and Nusrat, 2009). For these reasons it is important to develop strategies to prevent intestinal barrier failure, supporting animal healthy growth (especially in the early stages of life like weaning). In this context, bioactive compounds such as organic acids (OA) and nature-identical compounds (NIC) can exert a role in the

maintenance of intestinal functionality due to their numerous biological properties (Khan and Iqbal, 2016; Rossi et al., 2020; Tugnoli et al., 2020). In particular, our research group (Grilli et al., 2015) reported the beneficial effects of a blend of OA (citric and sorbic acid) and NIC (thymol and vanillin), on porcine intestinal barrier functionality, through a stimulation of the local immune response. Moreover, the results from that study demonstrated how these compounds together can improve the intestinal barrier functionality on Caco-2 cells, by increasing transepithelial electrical resistance (TEER) and reducing intestinal permeability to dextran (paracellular permeability, PCP). Nevertheless, little is known about their actual mode of action, whereas understanding the mechanisms underlying the effects of these OA and NIC, and more in general of bioactive compounds, is critical to their safe and effective application in animal nutrition. So far, the alleged mechanism of action of OA and NIC as growth enhancers was mostly connected to their well-documented antimicrobial properties, thereby implicating a possible role of these compounds in modulating the intestinal microflora. On the other side, recent clues are suggesting a direct role of these compounds in priming and boosting the host immune response (Swaggerty et al., 2019).

With the present study we wanted therefore to add a piece of information that could help elucidate the mechanism of action of citric and sorbic acid, thymol, and vanillin in preventing or improving intestinal barrier failure. To exclude any possible effect/interference mediated by the microflora, we performed *in vitro* experiments and used Caco-2 cells as a model to study the intestinal epithelium. As *in vitro* model, Caco-2 cells is frequently used due to its morphological, ultrastructural, and biochemical similarities with small intestinal epithelial cells for both pigs and humans (Lea, 2015). Two sets of experiments were performed, one to assess the role of these molecules in "healthy conditions" (Experiment 1) and the second set to understand their role in "challenged conditions" that potentially mimic stressful events like inflammation at weaning (Experiment 2).

Materials and Methods

Chemicals and Reagent

Cell culture reagents and chemicals were provided by Sigma-Aldrich (Milan, Italy), unless something different specified. All chemicals were analytical grade: citric acid (99%), thymol (≥98.5%), and vanillin (99%) were obtained from Sigma-Aldrich (Milan, Italy), sorbic acid (99%) was purchased from Chem-Lab (Chem-Lab NV, Zedelgem, Belgium).

Cell line and culture conditions

The human colon adenocarcinoma cell line (Caco-2) was obtained from DSMZ (DSMZ-German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Germany). Caco-2 cells were maintained at 37 °C, in an atmosphere containing 5% CO_2 at 95% relative humidity, in a medium (*basal medium*) consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 1% penicillin/streptomycin (P/S) and 1% L-Glutamine. Then Caco-2 cells were cultured in two different systems based on the analysis subsequently performed: 1) 96-well plates, where cells were seeded at density of 1.5×10^4 cells/well and cultured in basal medium; 2) 24 well transwell polyethylene terephthalate inserts (0.4 µm pore; Corning, Amsterdam, Nederland), where cells were seeded at a density of 5×10^4 cells/transwell.

Viability assay on Caco-2

Screening of cell viability was assessed with PrestoBlue reagent (Invitrogen, ThermoFisher Scientific, Milan, Italy). Briefly, Caco-2 cells were seeded onto 96-well plates and cultured in basal medium. When cells reached the confluence, the medium was supplemented with specific treatments and their relative control. Treated cells were tested for their viability 24 hours and 7

days after each treatment, following the manufacturer instructions. Fluorescence values were recorded with Varioskan LUX (Thermofisher Scientific). The % of cell viability was calculated by the following formula, where *mean fluo* is the fluorescence of cells with substances and *mean control* represent the fluorescence of the control group (adapted from Sharma (Sharma et al., 2016)):

$$\text{Cell Viability \%} = \text{Mean Fluo} / \text{Mean control} \times 100$$

Measurement of transepithelial electrical resistance (TEER)

Cells were seeded onto 24 transwell and TEER was measured every other day after cells reached confluence, using an epithelial tissue voltohmmeter (Millicell ERS-2, Merk, Merckmillipore, Germany). The experiment started 28 days after the seeding on filters once TEER values were stable (~150 $\Omega \cdot cm^2$), then cells were treated with the substances for 15 days. During the treatment TEER was measured at 0, 2, 4, 7, 9, 11, 14 and 15 days.

Measurement of paracellular permeability (PCP)

Cells were seeded onto 24 transwell and PCP was recorded as apical-to-basolateral passage of dextran. PCP was measured on day 15 by applying fluorescein isothiocyanate–dextran (FD4) (100 mg/mL) on the apical well of the transwell and collecting basal media after 24 hours for measurements of FD4 fluorescence. Fluorescence values for PCP were recorded with Varioskan™ LUX (Thermofisher Scientific, Milan, Italy).

Gene expression analysis

At the end of the experiments onto 24 transwell, cells were collected, snap-frozen in liquid nitrogen and stored at -80°C in lysis buffer (Macherey-Nagel, Düren, Germany) until gene expression analysis. RNA was isolated using NucleoSpin RNA Kit (Macherey-Nagel) and genomic DNA contamination was removed with deoxyribonuclease (rDNA RNase-Free; Macherey-Nagel), according to the manufacturer's instruction. RNA yield and quality were determined spectrophotometrically using A260 and A280 nm measurements (Microvolume Mode with SmartPath Technology, Denovix). A total of 450 ng of RNA was reverse-transcribed for each sample with iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), according to the manufacturer's instructions. Real-time PCR was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and iTaq Universal SYBR Green Supermix (Bio-Rad). Gene expression was normalized using two reference genes, such as ribosomal protein lateral stalk subunit P0 (RPLP0) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. The sequences, expected product length, accession number in the GenBank database and reference primers are shown in Table 1. Primers were obtained from Sigma-Aldrich.

Table 1. Primer sequence used for gene expression analysis.

Gene	Primer sequence (F and R) 5' → 3'	Product length (bp)	Accession N.	Reference
ZO-1	F: CGGGACTGTTGGTATTGGCTAGA R: GGCCAGGGCCATAGTAAAGTTTG	184	NM_001301025.3	(Park et al., 2015)
OCCL	F: TCCTATAAATCCACGCCGGTTC R: CTCAAAGTTACCACCGCTGCTG	105	NM_001205254.2	(Park et al., 2015)
TRPV1	F: GACCACCTGGAACACCAACG R: TGAGCAGACTGCCTATCTCG	177	NM_080704.3	(Hofmann et al., 2014)
TRPV3	F: GAGCAGATTCCGGATGGGA R: CCGCAAACACAGTCGGAAA	64	NM_001258205.1	(Xu et al., 2002)
RPLP0	F: GCAATGTTGCCAGTGTCTG R: GCCTTGACCTTTTCAGCAA	142	NM_001002.3	(Dydensborg et al., 2006)
GAPDH	F: TGCACCACCAACTGCTTAGC R: GGCATGGACTGTGGTCATGAG	87	NM_02046	(Vandesompele et al., 2002)

F = forward; R = reverse; ZO-1 = zonula occludens-1; OCCL = occludin; TRPV1 = Transient Receptor Potential Vanilloid 1; TRPV3 = Transient Receptor Potential Vanilloid 3; OR1G1 = Olfactory Receptor Family 1 Subfamily G Member 1; RPLP0 = Ribosomal Protein Lateral Stalk Subunit P0; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

Experiment 1 – Dose response

The aim was to investigate whether OA and NIC, alone or combined in a blend (OA+NIC), have an impact on intestinal mucosa barrier function. A viability assay in 96-well plates was performed to determine the response of Caco-2 to increasing concentrations of any single bioactive, or the blend OA + NIC. Concentrations of the substances were selected to meet the composition of a product used in our previous research study (Grilli et al., 2015). Citric acid was tested at 130, 260, 391, 651, 911, 1172, and 1300 µM; sorbic acid was tested at 149, 298, 447, 745, 1040, 1340, and 1490 µM; concentrations of thymol tested were 11, 23, 35, 58, 81, 104, and 110 µM, while vanillin was tested at 7, 13, 20, 35, 46, 59, and 70 µM. OA + NIC was evaluated at the following concentrations: 100, 200, 300, 500, 700, 900, and 1000 ppm (consistent with the inclusions of the single compounds, as reported in Table 2).

Table 2. Composition of the blend OA + NIC at the concentration tested for the viability assay. The inclusions of the single compounds in each row correspond to the amount of the molecules in the mixture (OA + NIC).

OA + NIC (ppm)	Citric acid (µM)	Sorbic acid (µM)	Thymol (µM)	Vanillin (µM)
100	130	149	11	7
200	260	298	23	13
300	391	447	35	20
500	651	745	58	35
700	991	1040	81	46
900	1172	1340	104	59
1000	1300	1490	110	70

Concerning the effect of the substances on the intestinal functionality, on day 28 cells on transwell filters were divided in groups and treated with different doses of citric acid, sorbic acid, thymol, vanillin, or a blend of all these molecules (OA + NIC). Citric acid was tested at 260, 455, and 651 µM, sorbic acid was tested at 298, 521, and 745 µM, thymol was tested at 23, 40, and 58 µM, and vanillin was tested at 13, 23, and 35 µM. Concerning the blend, cells were treated with OA + NIC at 200 or 1000 ppm (Grilli et al., 2015) (subsequently named as OA+NIC 200 and OA+NIC 1000). OA+NIC 200 is composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin, while OA+NIC 1000 is composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin. At the end of the experiments cells were collected for gene expression analysis.

Experiment 2 – Inflammatory challenge

The purpose was to assess the preventive or ameliorative potential of the blend OA+NIC against an inflammatory challenge. Inflammatory challenge was induced for 24 hours and consisted in the exposure to a cocktail of pro-inflammatory cytokines such as IL-1β (25 ng/mL), TNF-α (50

ng/mL), IFN-γ (50 ng/mL), and LPS from *E. coli* O55:B5 (10 ug/mL) (adapted from Van de Walle (Van De Walle et al., 2010)).

Concerning the effect on the intestinal functionality, on day 28 Caco-2 cells on filters were divided into 3 groups: control (CTR), OA+NIC 200 and OA+NIC 1000. The challenge was performed at 2 different time-point to evaluate the enhancing or preventive properties of the blend.

1 *Therapeutic approach*: the challenge was performed at day 0, along with treatments, and stopped after 24 hours, then cells continued to receive the different treatments for 15 days.

2 *Preventing approach*: the challenge was performed at day 14, after treatments, and stopped after 24 hours. Cells received the different treatments for a total of 15 days.

At the end of the experiments cells were collected for gene expression analysis.

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). ANOVA repeated measures was used for TEER. One-way ANOVA followed by Bonferroni and Tukey's post-test was used for PCP, gene expression, and viability assay. The experimental unit was the well (n = 6). Differences were considered significant at $p < 0.05$, trends at $0.05 < p \leq 0.10$.

Results

Experiment 1 – Dose response

Experiment 1 was performed to investigate the potential effect of OA and NIC, alone or combined in a blend, on intestinal epithelium. The results are divided as follows: citric acid, sorbic acid, thymol, vanillin, and the blend of OA+NIC. These molecules were tested at increasing concentrations to determine viability, TEER, PCP, and gene expression were evaluated.

1. *Citric acid*

An increase in viability was reported with 260 µM of citric acid after 24 hours of treatment, while after 7 days a significant reduction of the vitality (up to 10%) was reported with 651 µM of citric acid (Figure S1). Compared to control (CTR), 651 µM negatively impacted TEER on days 2, 7, 11, 14 and 15 (Figure 1). A reduction in PCP was reported for any concentration of citric acid (Figure 2). Results about gene expression are reported in Figure 3. Compared to CTR citric acid did not affect the expression of TJ proteins at any concentration.

2. *Sorbic acid*

Data obtained from viability assay reported a dose-dependent increase after 24 hours of treatment with sorbic acid, starting from the lowest level of inclusion. After 7 days cell viability was significantly decreased in a dose dependent manner, compared to CTR (Figure S1). Figure 1 shows the results of TEER. Compared to CTR, TEER was consistently increased by the supplementation of 298 µM of sorbic acid on day 7 and thereafter. Treatment with 521 µM of sorbic acid significantly increased TEER at day 9 and 14, compared to CTR. Sorbic acid at 745 µM did not differ from CTR. Consistently with TEER, PCP was significantly reduced by the supplementation of 298 µM of sorbic acid (Figure 2). Results about gene expression are

reported in Figure 3. Compared to CTR mRNA level of ZO-1 was lower in the group treated with 298 μM. No differences were reported for occludin.

3. Thymol

Viability of Caco-2 cells was not affected by thymol at any concentration or time point (Figure S1). Compared to CTR, 23 μM of thymol increased TEER at day 4, while 40 μM increased TEER starting from day 4 and constantly until day 15. Thymol at 58 μM increased TEER at day 11 and 14, compared to CTR (Figure 1). The PCP was significantly reduced by the supplementation of 40 μM and 58 μM of thymol (Figure 2). Results of gene expression analysis of TJ markers are represented in Figure 3. The mRNA level of ZO-1 and occludin showed no difference between CTR and treated groups.

4. Vanillin

No variation in terms of viability was observed at any of the tested doses (Figure S1). Compared to CTR, at day 4 an increase in TEER was reported for cells supplemented with 13 μM of vanillin, while an increase for group treated with 23 μM was registered at day 14, and at day 15 for 35 μM (Figure 1). No variations of PCP were reported (Figure 2). A trend of increase in mRNA levels of ZO-1 was reported ($p = 0.07$) (Figure 3).

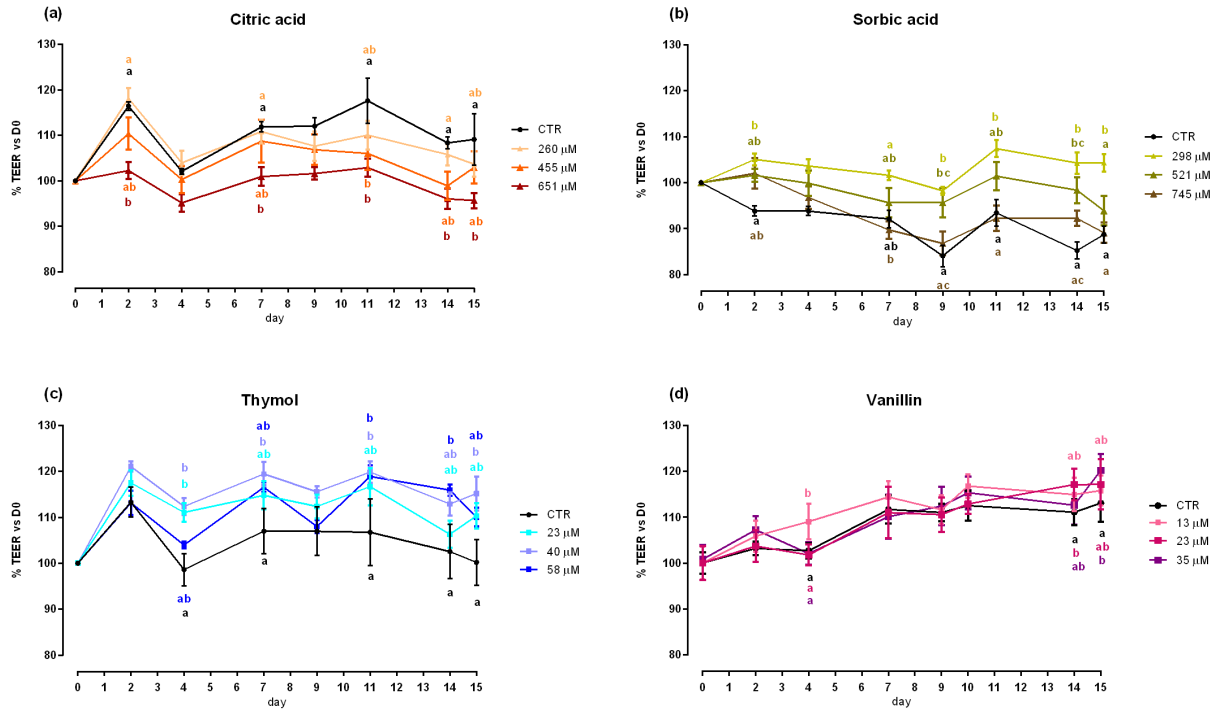

Figure 1. TEER of Caco-2 cells cultured with single OA and NIC. Data in the graph are represented as percentage over the initial TEER value and given as means (n = 6) ± SEM, represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab), colors of the letters refer to the different treatments. TEER = transepithelial electrical resistance (Ω·cm2). (**a**) Cells treated with citric acid; CTR = control group; 260 = treated group with 260 µM of citric acid; 455 = treated group with 455 µM of citric acid; 651 = treated group with 651 µM of citric acid. (**b**) Cells treated with sorbic acid; CTR = control group; 298 = treated group with 298 µM of sorbic acid; 521 = treated group with 521 µM of sorbic acid; 745 = treated group with 745 µM of sorbic acid. (**c**) Cells treated with thymol; CTR = control group; 23 = treated group with 23 µM of thymol; 40 = treated group with 40 µM of thymol; 58 = treated group with 58 µM of thymol. (**d**) Cells treated with vanillin; CTR = control group; 13 = treated group with 13 µM of vanillin; 23 = treated group with 23 µM of vanillin; 35 = treated group with 35 µM of vanillin.

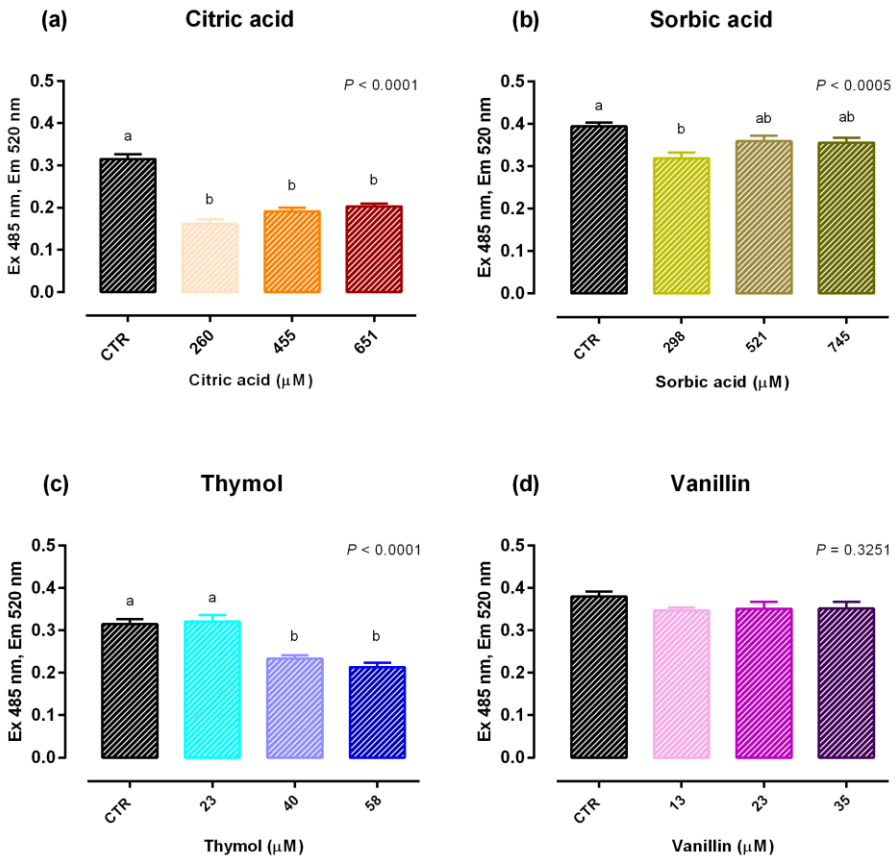

Figure 2. PCP of Caco-2 cells grown with single OA and NIC. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). PCP = paracellular permeability (excitation 485 nm, emission 520 nm). (**a**) Cells treated with citric acid; CTR = control group; 260 = treated group with 260 μM of citric acid; 455 = treated group with 455 μM of citric acid; 651 = treated group with 651 μM of citric acid. (**b**) Cells treated with sorbic acid; CTR = control group; 298 = treated group with 298 μM of sorbic acid; 521 = treated group with 521 μM of sorbic acid; 745 = treated group with 745 μM of sorbic acid. (**c**) Cells treated with thymol; CTR = control group; 23 = treated group with 23 μM of thymol; 40 = treated group with 40 μM of thymol; 58 = treated group with 58 μM of thymol. (**d**) Cells treated with vanillin; CTR = control group; 13 = treated group with 13 μM of vanillin; 23 = treated group with 23 μM of vanillin; 35 = treated group with 35 μM of vanillin.

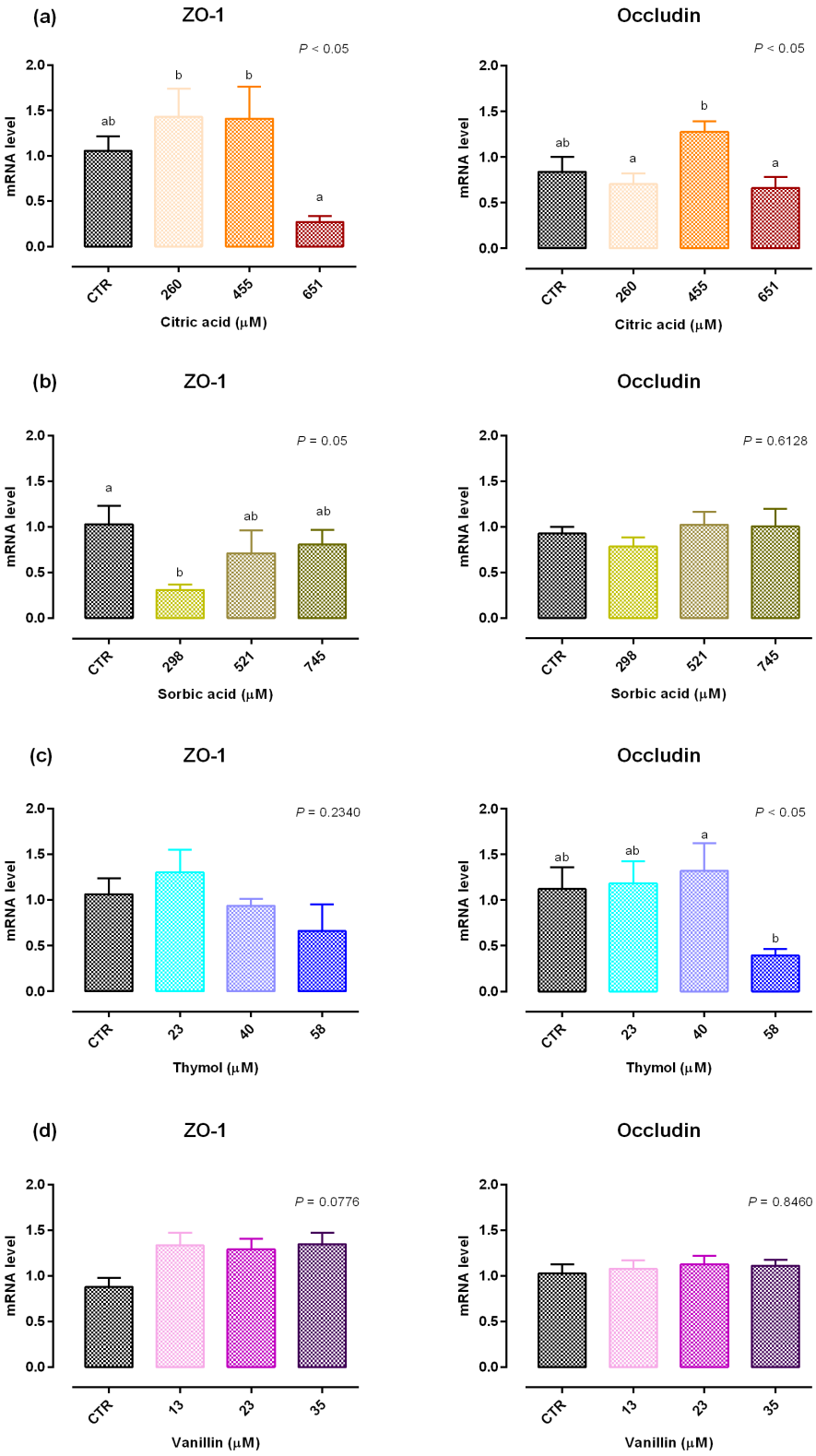

Figure 3. Gene expression in Caco-2 cells after 15 days of treatment with single OA and NIC. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). (**a**) Cells treated with citric acid; CTR = control group; 260 = treated group with 260

μM of citric acid; 455 = treated group with 455 μM of citric acid; 651 = treated group with 651 μM of citric acid. (**b**) Cells treated with sorbic acid; CTR = control group; 298 = treated group with 298 μM of sorbic acid; 521 = treated group with 521 μM of sorbic acid; 745 = treated group with 745 μM of sorbic acid. (**c**) Cells treated with thymol; CTR = control group; 23 = treated group with 23 μM of thymol; 40 = treated group with 40 μM of thymol; 58 = treated group with 58 μM of thymol. (**d**) Cells treated with vanillin; CTR = control group; 13 = treated group with 13 μM of vanillin; 23 = treated group with 23 μM of vanillin; 35 = treated group with 35 μM of vanillin. ZO-1 = zonula occludens.

5. Blend of organic acids and nature-identical compounds

Cell viability was increased after 24 hours and 7 days of treatment with 300 ppm and 700 ppm (Figure S2). TEER data are reported in Figure 4. Compared to CTR, TEER was increased by the supplementation of OA+NIC at 200 ppm (OA+NIC 200), starting from day 4, and the increment remained significant throughout the study, until day 15. TEER was increased also by the supplementation of OA+NIC at 1000 ppm (OA+NIC 1000) at day 14 and day 15 (compared to CTR). About TJ, ZO-1 was increased by OA+NIC 1000 compared to CTR, while OA+NIC 200 showed an intermediate value. No variation of PCP was reported (Figure 5). Occludin mRNA level showed a numerical increase ($p = 0.20$) in the group treated with OA+NIC 1000, compared to control (Figure 6). Moreover, TRPV1 (Transient Receptor Potential Vanilloid 1) was increased by OA+NIC 1000 compared to CTR, while OA+NIC 200 showed an intermediate value. The mRNA level of TRPV3 (Transient Receptor Potential Vanilloid 3) was tendentially reduced by the treatments ($p = 0.06$) (Figure 7).

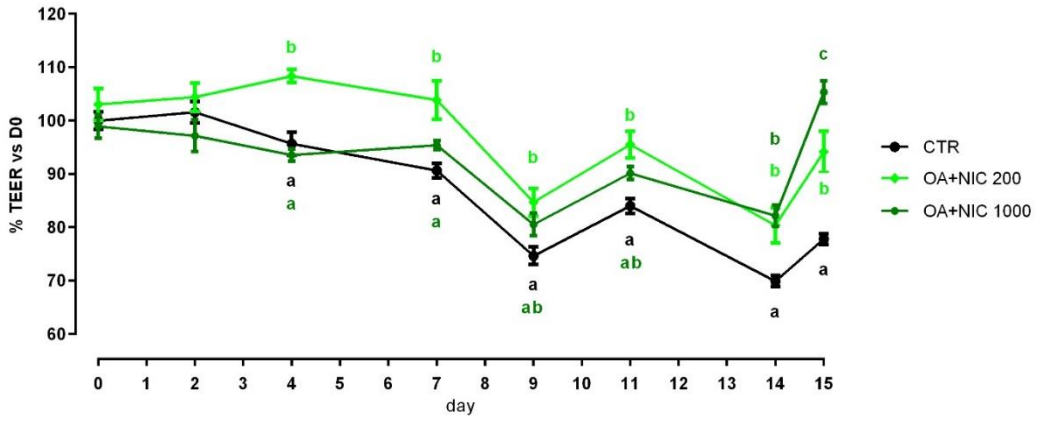

Figure 4. TEER of Caco-2 cells cultured with OA + NIC. Data in the graph are represented as percentage over the initial TEER value and given as means (n = 6) ± SEM, represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab), colors of the letters refer to the different treatments. TEER = transepithelial electrical resistance ($\Omega \cdot cm^2$); CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin.

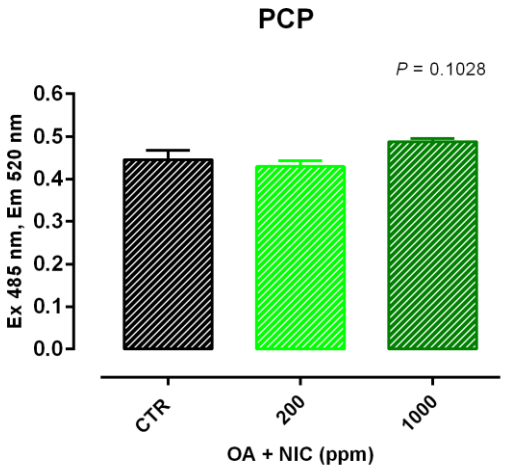

Figure 5. PCP of Caco-2 cells after 15 days of treatment with OA + NIC. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). PCP = paracellular permeability (excitation 485 nm, emission 520 nm). CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin.

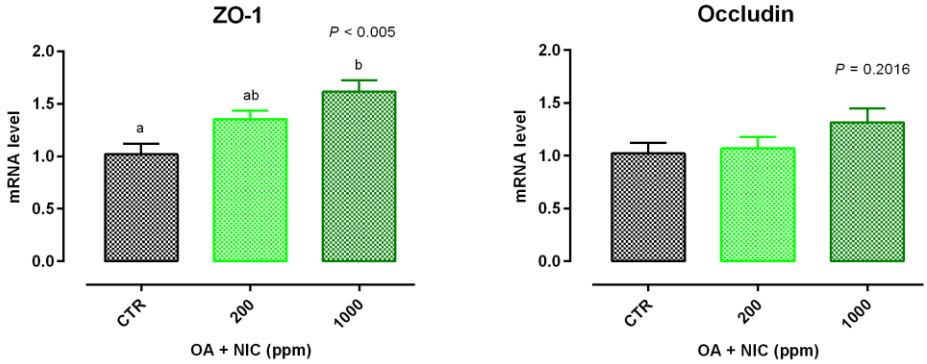

Figure 6. Gene expression in Caco-2 cells after 15 days of treatment with OA + NIC. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with p < 0.05 (a, b); means with at least one common letter are not significantly different (ab). CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin; ZO-1 = zonula occludens 1.

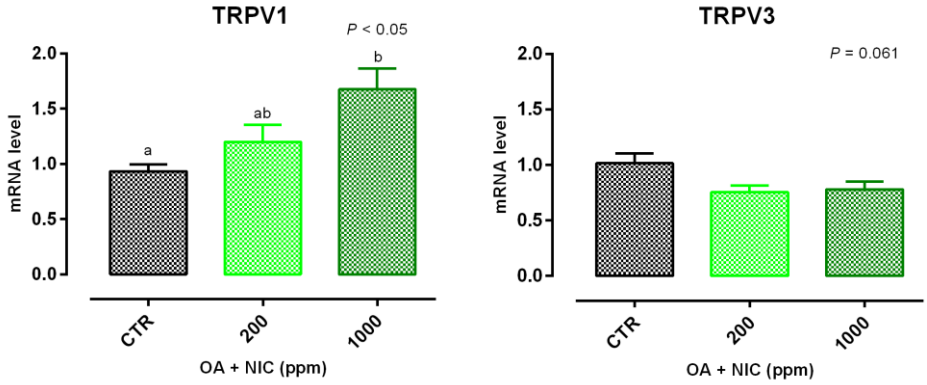

Figure 7. Gene expression in Caco-2 cells after 15 days of treatment with OA + NIC. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with p < 0.05 (a, b); means with at least one common letter are not significantly different (ab). CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin; TRPV1 = Transient Receptor Potential Vanilloid 1; TRPV3 = Transient Receptor Potential Vanilloid 3.

Experiment 2 – Inflammatory challenge

The purpose of the experiment 2 was to assess the role of the blend of OA+NIC in "challenging conditions" induced by the addition of a cocktail of inflammatory cytokines and LPS. The results of the ameliorative or preventing potential of OA+NIC against the inflammatory challenge were divided in 2 subsections. The *therapeutic approach* was defined as the ability of the blend to re-establish or ameliorate the parameters evaluated after the challenge. The *preventive approach* was defined as the capacity of OA+NIC to help cells to prevent the negative effects induced by the inflammatory stimuli.

1. *Therapeutic approach*

When the inflammatory challenge was performed on day 0, TEER was increased by the supplementation of OA+NIC 1000 immediately within 24 hours from the challenge, compared to CTR, and then constantly throughout all the study. Cells treated with OA+NIC 200 showed a significant increase of the TEER compared to CTR at days 4, 7, 9, 12, and 15 (Figure 8). No variations in PCP were recorded (Figure 9). mRNA level of ZO-1 was significantly increased in both the supplemented groups, while occludin resulted upregulated only in OA+NIC 1000 (Figure 10). A trend of increase in mRNA levels of TRPV1 was reported (p = 0.10), while TRPV3 showed no difference between CTR and treated groups (Figure 11).

2. *Preventive approach*

When the inflammatory challenge was performed on day 14, TEER was increased by OA+NIC 200 starting from day 2 and the increment remained significant until the challenge. Cells pre-treated with OA+NIC 1000 showed an increase in TEER at days 5, 9 and 14 before the challenge. After the challenge, TEER remained significantly higher in the cells treated with OA+NIC 1000 compared to CTR (Figure 8). No variations in PCP were recorded (Figure 9). An upregulation of both ZO-1 and occludin was recorded for cells receiving the pre-treatment

with OA+NIC 1000 (Figure 10). Moreover, TRPV1 was tendentially increased by the treatments (p = 0.08), while TRPV3 showed no difference compared to CTR (Figure 11).

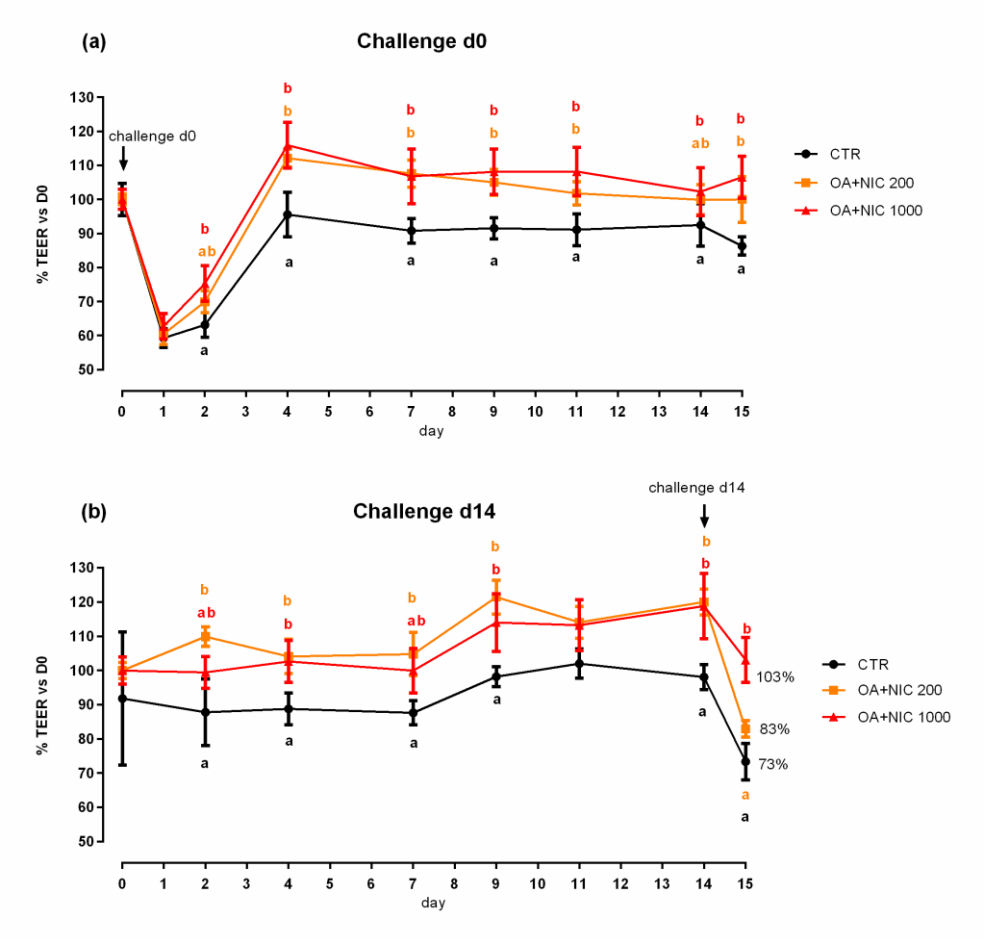

Figure 8. TEER of Caco-2 cells cultured with OA + NIC and subjected to an inflammatory challenge. Data in the graph are represented as percentage of the initial TEER value and given as means (n = 6) ± SEM, represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab), colors of the letters refer to the different treatments. TEER = transepithelial electrical resistance (Ω·cm^2); CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin. (**a**) Cells challenged at day 0 of the study. (**b**) Cell challenged at day 14 of the study.

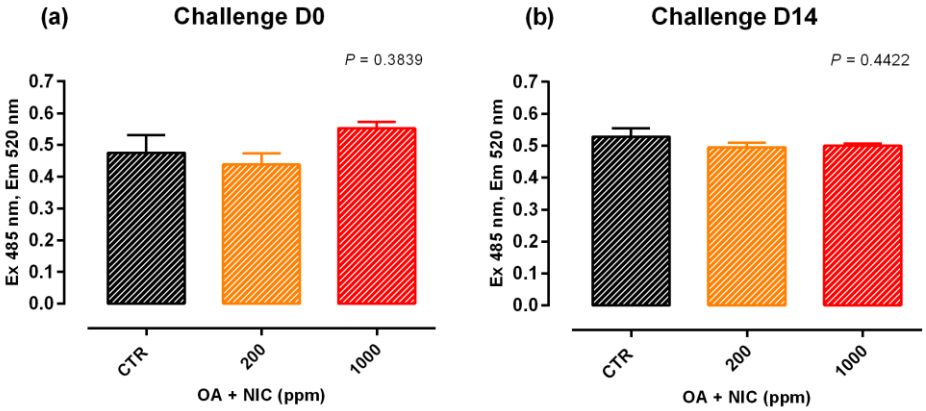

Figure 9. PCP of Caco-2 cells after 15 days of treatment with OA + NIC and subjected to an inflammatory challenge. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$. PCP = paracellular permeability (excitation 485 nm, emission 520 nm). CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin. (**a**) Cells challenged at day 0 of the study. (**b**) Cell challenged at day 14 of the study.

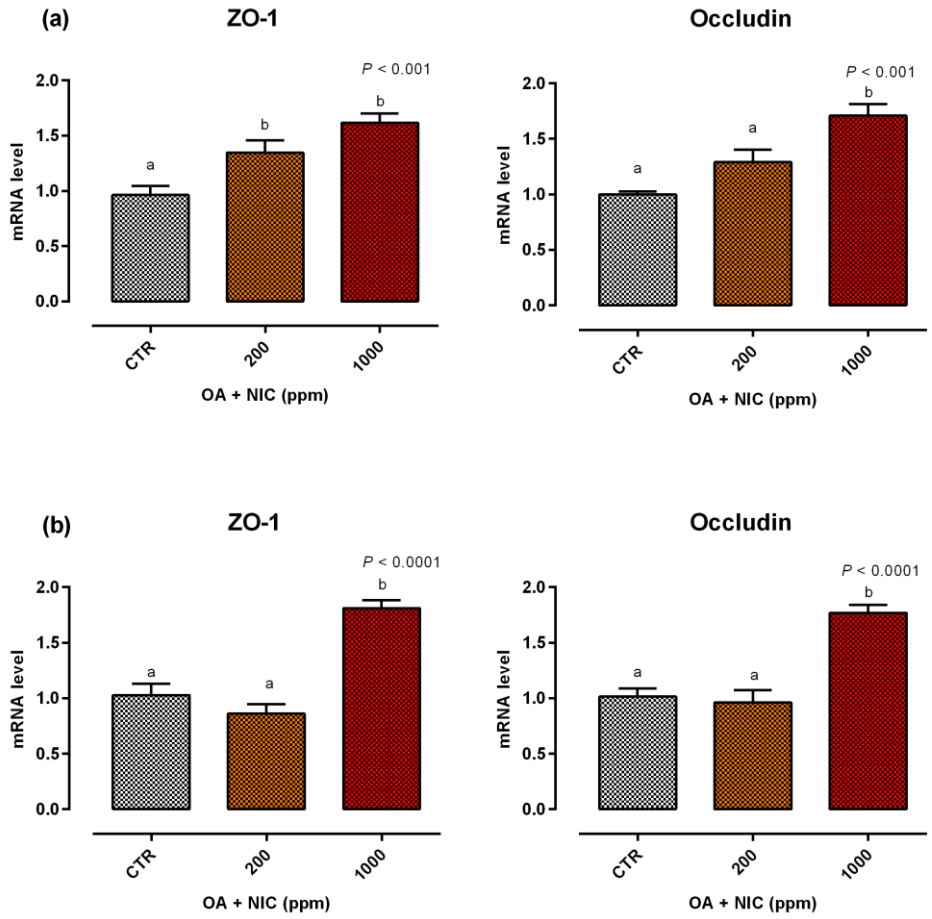

Figure 10. Gene expression in Caco-2 cells after 15 days of treatment with OA + NIC and subjected to an inflammatory challenge. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with $p < 0.05$ (a, b). CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed from 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed from 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin; ZO-1 = zonula occludens. (**a**) Cells challenged at day 0 of the study. (**b**) Cell challenged at day 14 of the study.

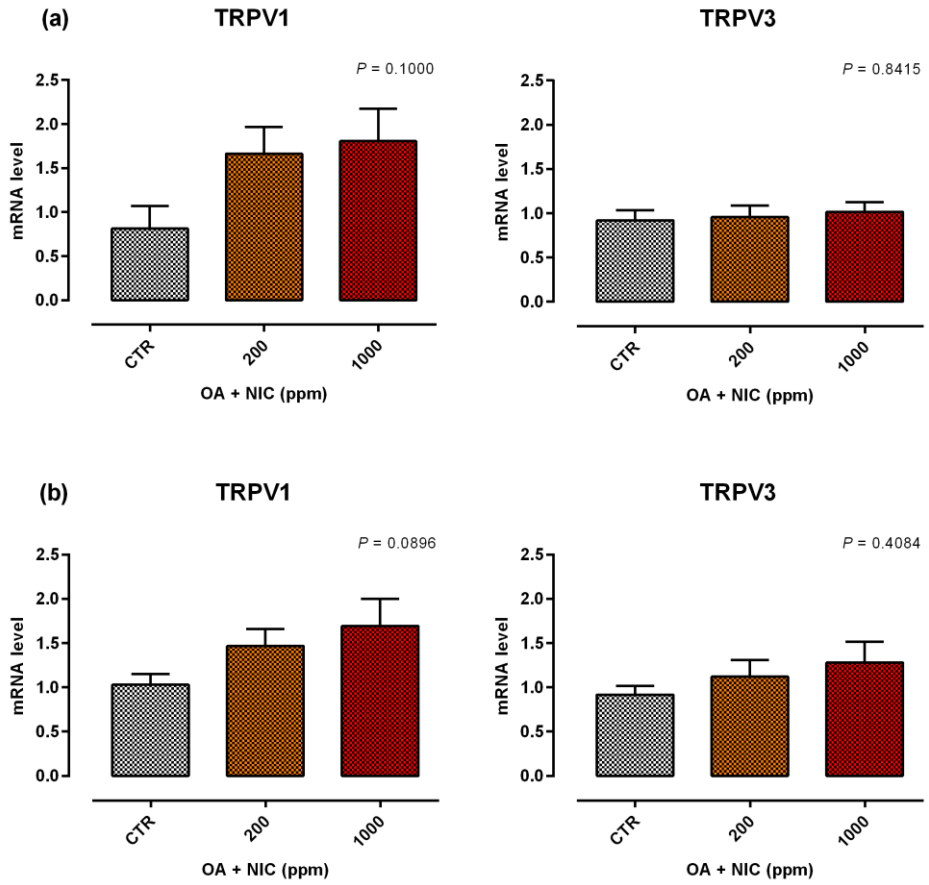

Figure 11. Gene expression in Caco-2 cells after 15 days of treatment with OA + NIC and subjected to an inflammatory challenge. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with $p < 0.05$. CTR = control group; OA + NIC 200 = treated group with 200 ppm of OA + NIC, composed by 260 µM of citric acid, 298 µM of sorbic acid, 23 µM of thymol, and 13 µM of vanillin; OA + NIC 1000 = treated group with 1000 ppm of OA + NIC, composed by 1300 µM of citric acid, 1490 µM of sorbic acid, 110 µM of thymol, and 70 µM of vanillin; TRPV1 = Transient Receptor Potential Vanilloid 1; TRPV3 = Transient Receptor Potential Vanilloid 3. (**a**) Cells challenged at day 0 of the study. (**b**) Cell challenged at day 14 of the study.

Discussion

OA and NIC are widely used as feed additives in animal nutrition because of their beneficial effects on growth performance and intestinal morphology, as well as their antibacterial, antioxidant and anti-inflammatory properties (Rossi et al., 2020; Tugnoli et al., 2020). In this study the attention was focused on the activity of selected OA, citric and sorbic acid, and NIC, thymol and vanillin on human Caco-2 intestinal cell line. First, the aim was to assess the safety and efficacy of these molecules tested individually. Second, we wanted to verify a possible synergistic effect given by the combination of these molecules and last, we wanted to investigate the effects of the blend of OA and NIC before and after an inflammatory challenge. A previous study conducted by our research group found a reduction in mRNA level of inflammatory cytokines, together with a decrease in paracellular permeability in piglets fed OA + NIC (Grilli et al., 2015). As OA and NIC are mostly known for their ability to positively modulate gut microbial population (Biagi and Piva, 2010; Fitzgerald et al., 2004; Grilli and Piva, 2012; Lambert et al., 2001; Llana-Ruiz-Cabello et al., 2015; Shen et al., 2005; Yu et al., 2019; Zhao et al., 2017), *in vivo* results might be mediated by the immunomodulatory and epigenetic interplay between host and intestinal microflora (Felix et al., 2018). As a consequence, we wanted to verify the effect of each compound directly on the epithelium.

The most striking result is that each of the molecules tested individually had little or no effect on Caco-2, whereas when they were combined together they improved epithelial barrier by increasing TEER and TJ components gene expression. Interestingly, the OA+NIC blend at 200 ppm, corresponding to the lowest dose of the individual compounds, had a minor effect on the measured parameters, whereas the blend at 1000 ppm consistently improved TEER and ZO-1 expression despite the individual compounds at much lower doses started to have some cytotoxic effect. We previously reported the synergy of OA and NIC in terms of antibacterial action (Grilli et al., 2013) and intestinal mucosa functionality in weaned pigs (Grilli et al.,

2015). Additionally, it was demonstrated a positive effects on TEER on Caco-2 cells (Grilli et al., 2015). In the present study we wanted to add a piece of information about the role of the single components of the blend and, moreover, the distinct responses of OA+NIC against an inflammatory challenge. Caco-2 are a validated model to study intestinal function as the cells are able to polarize and form the brush border. Once differentiated they express a phenotype common to both enterocytes and colonocytes and they are a useful tool for absorption and intestinal transport studies (Lea, 2015). Moreover, Caco-2 preserve many characteristics of the live tissue they are derived from, as they are able to synthetize enzymes and cytokines (Lea, 2015). Nevertheless, this model as few limitations as, by lacking the lamina propria, it doesn't possess the underlying functions that a more complex organism is able to perform, including, but not limited to, blood perfusion and circulation as well as immune response. Citric acid is an intermediate of the Krebs cycle and might have a role in cell metabolism (Krebs and Johnson, 1980) although *in vitro* is a strong chelator and can interfere with calcium transport thereby affecting TEER and TJ expression (Gonzalez-Mariscal et al., 1990; Noach et al., 1993). Sorbic acid is a medium chain unsaturated fatty acid that is metabolized via beta oxidation but can have a role in the signaling of insulin-like growth factor (IGF) pathway (Luo et al., 2011). In the current study, citric and sorbic acid exerted a slight cytotoxic effect at high doses when used individually, but when combined with thymol and vanillin no negative effects were reported. Thymol and vanillin are botanical compounds that have *in vitro* and *in vivo* anti-inflammatory and anti-oxidant activity and are widely used in animal nutrition (Rossi et al., 2020; Windisch et al., 2008). The effects of thymol on intestinal secretory activity were studied ex vivo by Boudry and Perrier (2008) and Michiels et al. (2010a): it was demonstrated that thymol induces ion secretions in the small intestine via the nervous system. As electrolytes and water secretion is usually a mean to eliminate bacterial toxins from the lumen, thymol may play a role as a mechanism of defense of the organism and have an effect, as a consequence, on the barrier function.

Although the present study was limited to the analysis of only few aspects of barrier function, it is interesting to note how the selected OA and NIC conferred a stronger trait of resistance to polarized Caco-2 under healthy or challenged conditions. The measurement of TEER reflects the ionic conductance of the paracellular pathway, while PCP indicates the paracellular dextran flow, as well as the pore size of the TJ (Srinivasan et al., 2015). Despite the absence of variation on PCP mediated by OA+NIC, the blend showed ameliorative effects, in terms of TEER and TJ, both in normal and challenging conditions, most likely driven by thymol and vanillin. In fact vanillin, beside anti-inflammatory and anti-microbial properties, is also potentially targeting TRPV channel. These TRP channels are nonselective cation channels expressed on the cell membrane and exhibit variable permeability ratios for Ca^{2+} versus Na^{+}. They mediate many functions among which divalent cation flux (Premkumar, 2014). The same potential to act as possible ligand of these channels is empowered by thymol (Premkumar, 2014). Because cation flux regulation is essential during a challenge induced by toxins secreted by *Escherichia coli* for instance, the contribution of these compounds in maintaining intestinal homeostasis can be relevant. Furthermore, the modulation of these channels from OA+NIC, with an increased expression of TRPV1 and a reduction of TRPV3, support the hypothesis of the anti-inflammatory potential of the mixture. In fact, TRPV1 and TRPV3 are known for their anti-inflammatory (Bujak et al., 2019) and pro-inflammatory (De Petrocellis et al., 2012) action, respectively. Moreover, the cytokine cascade induced by the host inflammatory response has a direct effect on TJ proteins. It is well documented in fact, that IFN-γ and TNF-α can cause a direct disruption of ZO-1 and occludins (Al-Sadi et al., 2009) so that the increased expression of these proteins by Caco-2 cells during an inflammatory challenge might be the direct consequence of the beneficial and anti-inflammatory effect given by the mix of OA+NIC.

Another interesting piece of information from this experiment resulted from the dualistic approach, either preventive or therapeutic. In the first case, the application of the mix of OA+NIC for 14 days before the challenge allowed the cells to be more prepared to the damages

deriving by the inflammatory challenge. In particular, the preventive administration of OA+NIC led the cells to deal with the challenging stimuli, avoiding the fall in TEER and TJ gene expression correlated with an inflammatory challenge. Whereas in the second case, the application of the OA+NIC mix right at the time of the challenge allowed the cells to recover their functionality properties already after few days, with a re-establishment or an improvement of the parameters evaluated. If translated *in vivo*, these results could constitute the starting point of a possible use of these compounds either as to imprint the epithelium and the mucosa of the newly weaned animal, to make it more resistant to future challenges, or as non-antibiotic molecules to support conventional medical treatments and therapies that are commonly used at the onset of enteric disorders.

Conclusions

To conclude, OA and NIC have different chemical and biological properties that are best exploited when in combination as they address a wide range of molecular targets. By modulating the antioxidant and anti-inflammatory status (Hoffman-Pennesi and Wu, 2010; Xu et al., 2002), cell metabolism (Grilli et al., 2013; Shen et al., 2005) and ion transport (Dydensborg et al., 2006; Vandesompele et al., 2002) these molecules have the potential to ameliorate intestinal barrier function under normal or challenged conditions *in vitro*. This would justify their use *in vivo* as feed additives although more studies to assess the mode of action of these bioactive compounds *in vivo* need to be performed. In fact, *in vitro* systems are not predictive of the response of the immune system, nor of digestion and absorption, as well as they do not take into consideration the complicated interplay between the host and microflora.

Nevertheless, this study highlighted some specific features of this combination of sorbic, citric acid and thymol and vanillin in preventing and ameliorating intestinal epithelium loss of integrity that might be at the core of the efficacy of these compounds *in vivo*.

Supplementary materials

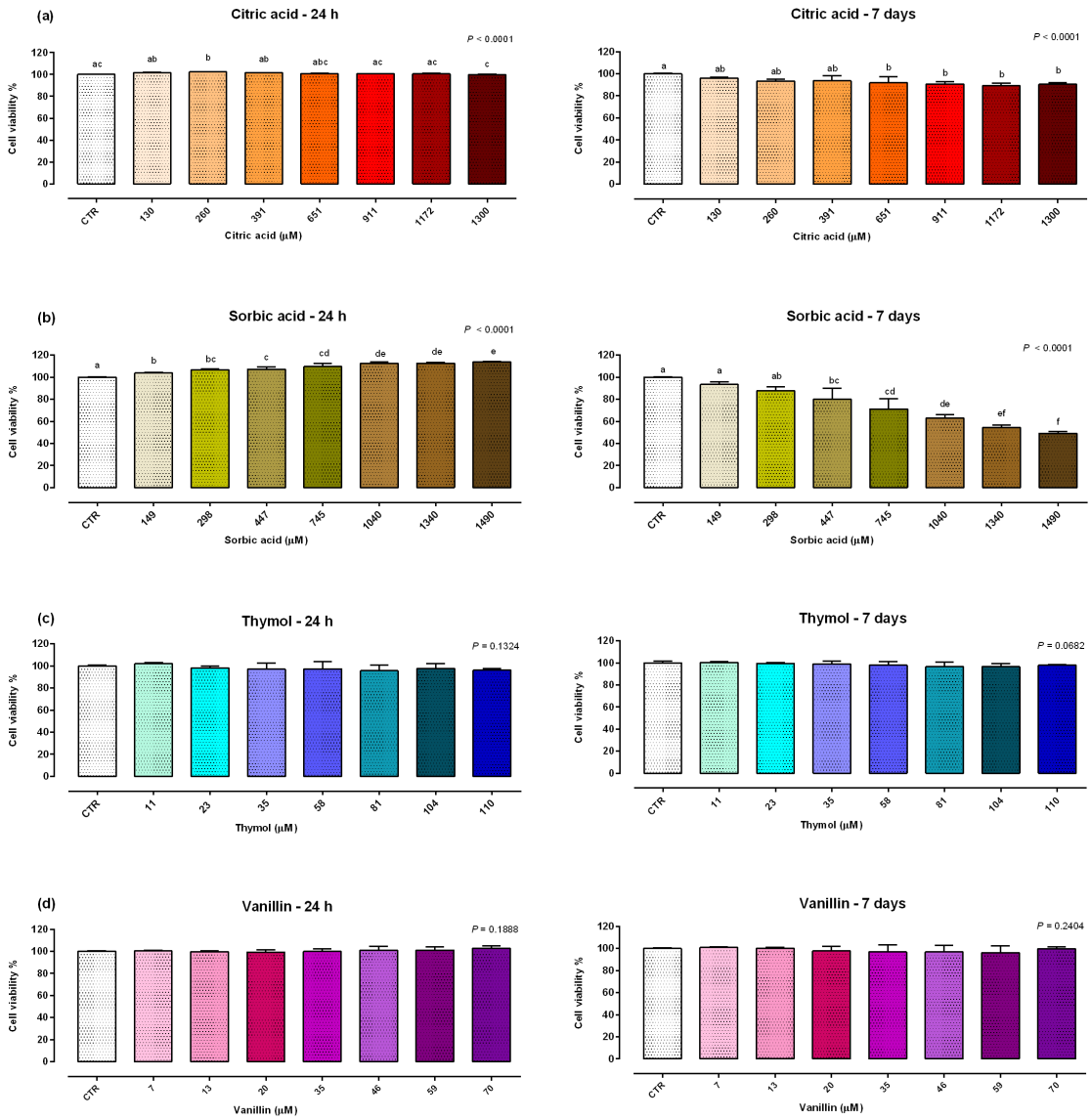

Figure S1. Viability of Caco-2 cells treated for 24 hours and 7 days with incremental doses of single OA and NIC. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). (**a**) Cells treated with incremental doses of citric acid. CTR = control group; 130 = treated group with 130 μM of citric acid; 260 = treated group with 260 μM of citric acid; 391 = treated group with 391 μM of citric acid; 651 = treated group with 651 μM of citric acid; 911 = treated group with 911 μM of citric acid; 1172 = treated group with 1172 μM of citric acid; 1300 = treated group with 1300 μM of citric acid. (**b**) Cells treated with incremental doses of sorbic acid. CTR = control group; 149 = treated group with 149 μM of sorbic acid; 298 = treated group with 298 μM of sorbic acid; 447 = treated group with 447 μM of sorbic acid; 745 = treated group with 745 μM of sorbic acid; 1040 = treated group with 1040 μM of sorbic acid; 1340 = treated group with 1340 μM of sorbic acid; 1490 = treated group with 1490 μM of sorbic acid. (**c**) Cells treated with incremental doses of thymol. CTR = control group; 11 = treated group with 11 μM of thymol; 23 = treated group with 23 μM of thymol; 35 = treated group with 35 μM of thymol; 58 = treated group with 58 μM of thymol; 81 = treated group with 81

µM of thymol; 104 = treated group with 104 µM of thymol; 110 = treated group with 110 µM of thymol. (**d**) Cells treated with incremental doses of vanillin. CTR = control group; 7 = treated group with 7 µM of vanillin; 13 = treated group with 13 µM of vanillin; 20 = treated group with 20 µM of vanillin; 35 = treated group with 35 µM of vanillin; 46 = treated group with 46 µM of vanillin; 59 = treated group with 59 µM of vanillin; 70 = treated group with 70 µM of vanillin.

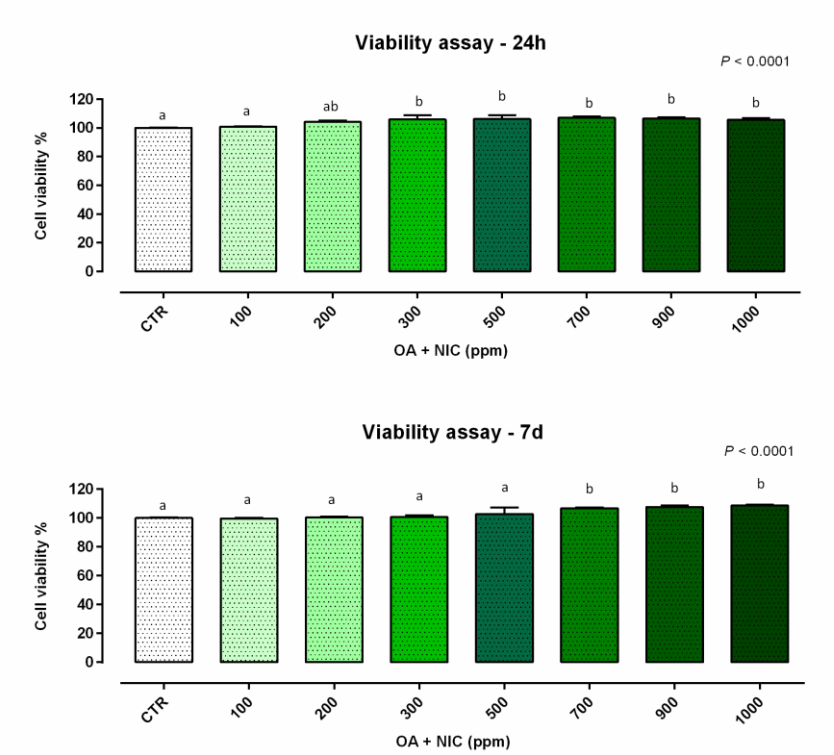

Figure S2. Viability of Caco-2 cells treated for 24 hours and 7 days with incremental doses of the blend of OA + NIC. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$; means with at least one common letter are not significantly different. CTR = control group; 100 = treated group with 100 ppm of the blend; 200 = treated group with 200 ppm of the blend; 300 = treated group with 300 ppm of the blend; 500 = treated group with 500 ppm of the blend; 700 = treated group with 700 ppm of the blend; 900 = treated group with 900 ppm of the blend; 1000 = treated group with 1000 ppm of the blend.

EXPERIMENTAL STUDY 2

Student thesis. Manuscript ready for submission. Modified from:

Effects of Ginger Oil, Tea Tree Oil and Grape Seed Extract on Intestinal Integrity, Oxidation, and Microbial activity

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Abstract

Essential oils (EOs) are known for their role as antiseptic, antimicrobial, sedative, anti-inflammatory, and locally anesthetic remedies. Moreover, EOs seem able to support intestinal health improving the intestinal barrier function. The aim of the study was to analyze the positive effects of ginger essential oil (GEO), tea tree oil (TTO), and grape seed extract (GSE) on intestinal barrier function and antioxidant activity on human Caco-2 cell line. Caco-2 cells were treated with 1, 10, and 100 ppm of each EOs, then transepithelial electrical resistance (TEER), tight junctions' (TJs) gene expression, paracellular permeability (PCP), reactive oxygen species (ROS), and glutathione (GSH) was measured. Secondly, the antimicrobial activity of GEO, TTO, and GSE was assessed against the main intestinal pathogens of poultry. Minimal inhibitory concentrations (MICs) were assessed treating pathogens with concentrations of EOs up to 10,000 ppm. The results showed how GEO, TTO, and GSE can improve TEER and mRNA levels of TJ components, but also affected the PCP. When oxidative stress occurred, EOs were able both to reduce the cytoplasmatic ROS and prevent the drop of GSH. Unfortunately, only TTO seems effective against the intestinal poultry's pathogens. The data

support the role of EOs in modulating gut barrier function and reducing the negative effects of oxidative stress in intestinal epithelial cells, thereby supporting the gut barrier functionality.

Introduction

Essential oils (EOs) are natural complex compounds constituted by aromatic plants secondary metabolites. Known for their antiseptic properties and their fragrance, they are also used in the preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, and locally anesthetic remedies (Bakkali et al., 2008). In nature, EOs play an important role in the protection of the plants as antibacterial and insecticides (Bakkali et al., 2008). The composition of EOs is highly variable since it depends on the species, the stage of development, and the part of the plant used for the extraction, as well as temperature, humidity, composition of the soil, and chemotypes (Figueiredo et al., 2008). Normally, these complex natural mixtures are characterized by 2-3 major components with a high concentration (up to 70%), among the 20-60 components which compose the mixture (Eslahi et al., 2017). In terms of effects and biological activity, each chemical constituent has its own characteristic properties. This suggests that EOs are of a complex character with rather various effects (Tabashsum and Biswas, 2019). In animal nutrition, among feed additives, natural extracts, EOs, and botanicals have gained increasing interest, thanks to their various biological activities (Rossi et al., 2020; Windisch et al., 2008). In the last years, the number of studies conducted to investigate the benefits of EOs on the performance of swine and poultry considerably increased. The use of natural products seems useful not only to increase the voluntary feed intake, but also for nutrients absorption and to support the immune status (Zeng et al., 2015). Moreover, EOs seem to be able to support intestinal health improving the intestinal barrier function, which clearly plays a key role in animal health and productivity (Kraimi et al., 2019; Pham et al., 2020). For these reasons, it is important to understand the role of EOs on intestinal function and microbiota. In this study, the attention was focused on the potential role of natural products in improving

the barrier function of the intestinal epithelium and in reducing the proliferation of the intestinal pathogens, with particular interest to ginger, tea tree, and grape seed. The EO obtained from *Zingiber officinale*, normally named ginger essential oil (GEO), is well known for various activities such as antioxidants, antibacterial, anti-inflammatory, antiseptic, antiparasitic, and immunomodulatory. The positive effects of GEO are demonstrated both *in vitro* (Singh et al., 2008; Stoyanova et al., 2006) and *in vivo* (Herve et al., 2019; Zeng et al., 2015). GEO counts between its composition α-zingiberene, β-sesquiphyllandrene, α-curcumene, and 1,8-cineole (Feng et al., 2018; International Organization for Standardization, 2014). *Melaleuca alternifolia* EO, commonly known as tea tree oil (TTO), has a long history of use as a topical antiseptic, and in recent times, it has gained a reputation as a safe, natural, and effective antimicrobial agent (Carson et al., 2006). Terpinen-4-ol, γ-terpinene, and α-terpinene represent the main components of TTO (Groot and Schmidt, 2016; International Organization for Standardization, 2017). Grape seed extract (GSE), obtained from *Vitis vinifera*, is a rich source of phenolic compounds, such as anthocyanins, quercetin derivatives, catechin, epicatechin, resveratrol, and chlorogenic acid (Mandic et al., 2008). As for GEO and TTO, also GSE is widely known for its broad range of properties, like anti-inflammatory, cardioprotective, antimicrobial, and anticancer (Garavaglia et al., 2016). This study has the dual purpose to evaluate the potential of these substances both on the mucosa and against pathogens, to better characterize the potential of GEO, TTO, and GSE in animal nutrition. It was decided to use an *in vitro* approach in both the case. The human epithelial cell line Caco-2, thanks to their advantageous properties to spontaneously differentiate into a monolayer of cells (Lea, 2015), was used to verify the possible positive effects of GEO, TTO, and GSE on intestinal epithelium. Moreover, the antimicrobial capacity of these natural compounds was assessed against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Clostridium perfringens*, and *Enterococcus cecorum.*

Materials and Methods

Chemicals and Reagents

Chemicals and cell culture reagents were provided by Sigma-Aldrich (Milan, Italy), unless something different specified. Ginger essential oil (GEO), tea tree oil (TTO), and grape seed extract (GSE) were purchased from Frey&Lau (Frey + Lau GmbH, Immenhacken 12, D-24558 Henstedt-Ulzburg, Germany). Stock solutions of GEO, TTO, and GSE were prepared in medium (DMEM or Brain Heart Infusion broth, BHI) supplemented with 100% (v/v) ethanol to ensure the solubility of EOs, the final concentration of ethanol was ≤ 5% (v/v).

Experiment 1 - In vitro analysis on Caco-2 cells

Cell line and culture conditions

The human colon adenocarcinoma cell line (Caco-2) was obtained from DSMZ (DSMZ-German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Germany). Caco-2 cells were maintained at 37 °C, in an atmosphere containing 5% CO_2 at 95% relative humidity, in a medium (*basal medium*) consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 1% penicillin/streptomycin (P/S) and 1% L-Glutamine. Then Caco-2 cells were cultured in two different systems based on the analysis subsequently performed: 1) 96-well plates, where cells were seeded at a density of 1.5×10^4 cells/well, to determine viability and oxidative response; 2) 24 well transwell polyethylene terephthalate inserts (0.4 µm pore; Corning, Amsterdam, Nederland), where cells were seeded at a density of 5×10^4 cells/transwell, to determine electrophysiological parameters. For all the subsequent analysis control groups were incubated in medium containing ≤ 0.4% (v/v) of ethanol to exclude the possibility of inhibitory effects by the ethanol.

Cell line and culture conditions

Screening of cell viability to GEO, TTO, and GSE was assessed with PrestoBlue™ reagent (Invitrogen, ThermoFisher Scientific, Milan, Italy). Caco-2 cells seeded onto 96-well plates were cultured in basal medium supplemented with specific treatments and their relative control. Concentrations of the substances were selected to meet and exceed the suggestion of the producer for the use of these compounds as feed additives, these concentrations were: 1, 10, 100, 200, 300, 500, 600, 800, and 1,000 ppm. Treated cells were tested for their viability both 24 hours and 7 days after each treatment, following the manufacturer instructions. It was not possible to test the viability after 14 days of treatment because Caco-2 tend to create multilayers when growing on wells (instead of transwells filters), and this may affect the result. Fluorescence values were recorded with Varioskan™ LUX (Thermofisher Scientific, Milan, Italy). The % of cell viability was calculated by the following formula, where *mean fluo* is the fluorescence of cells with substances and *mean control* represent the fluorescence of the control group (adapted from Sharma et al. (2016)):

$$\text{Cell Viability \%} = \text{Mean Fluo} / \text{Mean control} \times 100$$

Measurement of Reactive Oxygen Species (ROS) Level

ROS (Reactive Oxygen Species) were measured using CellROX® Deep Red Reagent (ThermoFisher Scientific, Milan, Italy) as described by the manufacturer's instructions. Briefly, CellROX® Deep Red Reagent is a fluorogenic probe for measuring cellular oxidative stress in living cells, in particular the signal is localized in the cytoplasm. The cell-permeable reagent is non-fluorescent while in a reduced state, whereas it becomes fluorescent upon oxidation by reactive oxygen species, with emission maxima ~665 nm. Caco-2 cells seeded onto 96-well plates were treated with 1, 10, and 100 ppm of GEO, TTO, or GSE for 24 hours. CellROX® Deep Red Reagent was added at a final concentration of 5 μM to the cells and then incubated

for 30 min at 37 °C. Subsequently, the medium was removed, and the cells were washed three times with DPBS (Dulbecco's phosphate buffered saline). Fluorescence values were recorded with Varioskan™ LUX (Thermofisher Scientific, Milan, Italy). Hydrogen peroxide 750 μM was added 60 min before treatment with CellROX® as ROS-inducer molecule.

Measurement of Reduced Glutathione (GSH) Level

The cellular glutathione level (GSH) was estimated using ThiolTracker™ Violet dye (Invitrogen, ThermoFisher Scientific, Milan, Italy), which reacts with reduced thiols in intact cells. Caco-2 cells seeded onto 96-well plates were treated with 1, 10, and 100 ppm of GEO, TTO, or GSE for 24 hours. Then, following the manufacturer's instructions, cells were incubated with 20 μM of ThiolTracker Violet dye for 30 min and then washed with DPBS C/M (Dulbecco's phosphate buffered saline, containing Ca^{++} and Mg^{++}). The fluorescence excitation and emission wavelength for ThiolTracker™ Violet dye are 404/526 in nm and were recorded with Varioskan™ LUX (Thermofisher Scientific, Milan, Italy). Hydrogen peroxide 750 μM was added 60 min before treatment with ThiolTracker™ Violet as a ROS-inducer molecule.

Dose response on Caco-2

The experiment started on day 28 after the seeding on filters, once Caco-2 cells were completely differentiated into enterocytes and TEER (transepithelial electrical resistance) values were stable (~150 $\Omega \cdot cm^2$). Cells on transwell filters were divided into groups and treated with different doses of GEO, TTO, and GSE for 15 days. The range of concentrations to be tested was selected considering the inclusion of these essential oils suggested by the producer for the use as feed additives. For this reason, all the substances were tested at 1, 10, and 100 ppm. During the treatment, TEER was measured at 0, 2, 4, 7, 9, 11, 14, and 15 days, using an

epithelial tissue voltohmmeter (Millicell ERS-2, Merk, Merckmillipore, Germany). Paracellular permeability (PCP) was recorded as apical-to-basolateral passage of dextran. PCP was measured on day 15 by applying fluorescein isothiocyanate–dextran (FD4) (100 mg/mL) on the apical well of the transwell and collecting basal media after 24 hours for measurements of FD4 fluorescence. Fluorescence values for PCP were recorded with Varioskan™ LUX (Thermofisher Scientific, Milan, Italy).

Gene expression analysis

At the end of the experiments onto 24 transwell, cells were collected, snap-frozen in liquid nitrogen and stored at −80 °C in lysis buffer (Macherey-Nagel, Düren, Germany) until gene expression analysis. RNA was isolated using NucleoSpin RNA Kit (Macherey-Nagel) and genomic DNA contamination was removed with deoxyribonuclease (rDNA RNase-Free; Macherey-Nagel), according to the manufacturer's instruction. RNA yield and quality were determined spectrophotometrically using A260 and A280 nm measurements (Varioskan™ LUX, Thermofisher Scientific, Milan, Italy). A total of 450 ng of RNA was reverse-transcribed for each sample with iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), according to the manufacturer's instructions. Real-time PCR was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and iTaq Universal SYBR Green Supermix (Bio-Rad). Gene expression was normalized using two reference genes, such as ribosomal protein lateral stalk subunit P0 (RPLP0) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. The sequences, expected product length, accession number in the GenBank database and reference primers are shown in Table 1. Primers were obtained from Sigma-Aldrich.

Table 1. Primer sequence used for gene expression analysis.

Gene	Primer sequence (F and R) 5' → 3'	Product length (bp)	Accession N.	Reference
ZO-1	F: CGGGACTGTTGGTATTGGCTAGA R: GGCCAGGGCCATAGTAAAGTTTG	184	NM_001301025.3	(Park et al., 2015)
OCCL	F: TCCTATAAATCCACGCCGGTTC R: CTCAAAGTTACCACCGCTGCTG	105	NM_001205254.2	(Park et al., 2015)
CLDN-2	F: ATTGTGACAGCAGTTGGCTT R: CTATAGATGTCACACTGGGTGATG	86	NM_001171092.1	(Hichino et al., 2017)
CLDN-3	F: ACATCATCACGTCGCAGAACATC R: AGTGCCAGCAGCGAGTCGTA	103	NM_001306.4	(Park et al., 2015)
CLDN-15	F: AGGAAGCAGAGAGACCCACA R: AGAACCCCTAGGGAACTGGA	155	NM_001185080.2	(Tatsuta et al., 2019)
JAM-A	F: CAGAGGTGATTCATGGCTCTGTG R: TTCCAGGCTGGCAATAACTGAC	96	NM_001382727	(Park et al., 2015)
RPLP0	F: GCAATGTTGCCAGTGTCTG R: GCCTTGACCTTTTCAGCAA	142	NM_001002.3	(Dydensborg et al., 2006)
GAPDH	F: TGCACCACCAACTGCTTAGC R: GGCATGGACTGTGGTCATGAG	87	NM_02046	(Vandesompele et al., 2002)

F = forward; R = reverse; ZO-1 = zonula occludens-1; OCCL = occludin; CLDN-2 = claudin 2; CLDN-3 = claudin 3; CLDN-15 = claudin 15; JAM-A = Junctional adhesion molecule A; RPLP0 = Ribosomal Protein Lateral Stalk Subunit P0; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

<u>Experiment 2 - In vitro antimicrobial assay</u>

Bacterial strains and culture conditions

All the strains used in this study were a field strain originally obtained from the intestine of symptomatic poultry. The strains were stored at -80°C in Brain Heart Infusion broth (BHI) supplemented with 20% (v/v) glycerol, and they were recovered in BHI at 37°C.

Minimal inhibitory concentration of natural extracts

The minimal inhibitory concentrations (MICs) of GEO, TTO, and GSE were determined in triplicate using a broth macrodilution method in a 10-ml disposable tube containing appropriate dilution of each substance with the bacteria strain. Each solution was buffered to ensure a final pH 6.5, filter-sterilized, and diluted in sterile BHI to reach the final concentration tested. GEO, TTO, and GSE were tested at final concentrations of 10,000, 8,000, 6,000 ppm, and concentrations ranging from 4,000 to 62.5 ppm following a 2-fold dilutions. An overnight bacterial culture was diluted to obtain an inoculum of 10^5 cfu/mL. The bacterial strains were incubated with the tested substances under aerobic (*Escherichia coli, Salmonella enteritidis, and Staphylococcus aureus*) or anaerobic (*Clostridium perfringens and Enterococcus cecorum*) conditions at 37 °C for 24 h. Control strains were grown in medium containing 5% (v/v) of ethanol to exclude the possibility of inhibitory effects by the ethanol. Bacterial growth was indicated by the presence of turbidity in the tube and confirmed by spreading 100 µl of broth of each tube onto BHI agar. After 48 h of incubation, the growth of viable cells was observed. The MIC was defined as the lowest concentration effective in killing more than 99.9% of the initial inoculum.

Statistical analysis

Data were analyzed using GraphPad Prism® (GraphPad Software, Inc., La Jolla, CA, USA). ANOVA repeated measures was used for TEER. One-way ANOVA followed by Bonferroni and Tukey's post-test was used for PCP, PrestoBlue™ reagent, CellRox® Deep Red, ThiolTracker™ Violet dye assay, and gene expression analysis. The experimental unit was the well (n = 6). Concerning the antimicrobial assay, the experimental unit was the tube (n = 3). Differences were considered significant at $p < 0.05$, trends at $0.05 < p \leq 0.10$.

Results

Ginger essential oil

1. Viability and dose response

A significant reduction in viability was reported with 1000 ppm of GEO starting from 24 hours of treatment. After 7 days, a reduction was reported starting from 300 ppm of inclusion (Figure S1). Concerning TEER, 100 ppm of GEO significantly increased the resistance starting from day 2 and the increment remained significant throughout all the study, compared to control and the other 2 inclusions (Figure 1). Conversely, an increase in PCP was recorded for cells treated with 100 ppm of GEO (Figure 2). Results about gene expression are reported in Figure 3. Compared to the control, mRNA levels of ZO-1 and occludin were significantly increased by the treatments, with a major extent for the inclusion of 100 ppm of GEO ($p < 0.0005$). Also, JAM-A was tendentially increased by the treatments ($p = 0.09$). Conversely, both claudin-2 and claudin-15 mRNA levels were significantly reduced by the addition of GEO, with a stronger reduction with 100 ppm of inclusion ($p < 0.05$). No differences were reported for claudin-3.

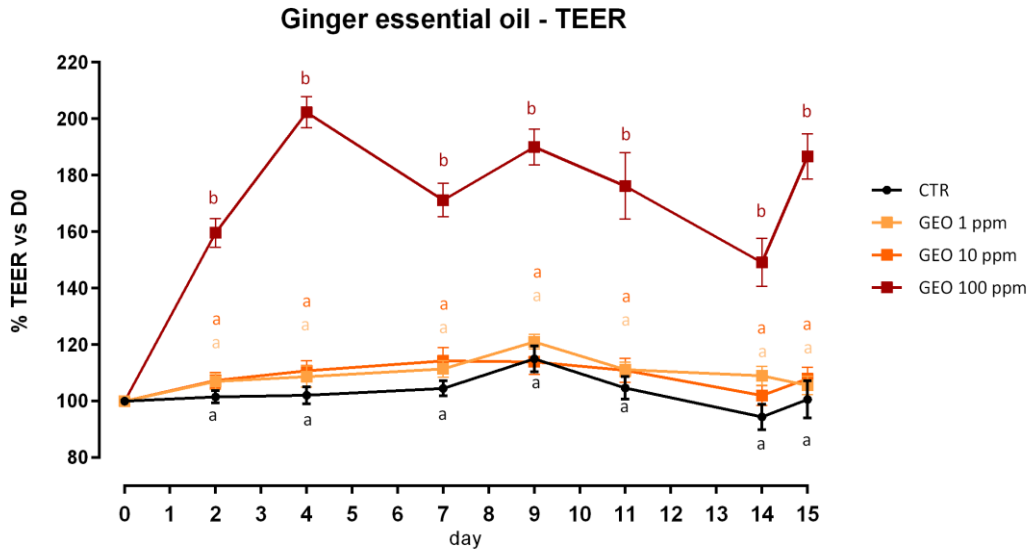

Figure 1. TEER of Caco-2 cells cultured with 1, 10, or 100 ppm of ginger essential oil. Data in the graph are represented as percentage over the initial TEER value and given as means (n = 6) ± SEM, represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b), colors of the letters refer to the different treatments. TEER = transepithelial electrical resistance ($\Omega \cdot cm^2$).

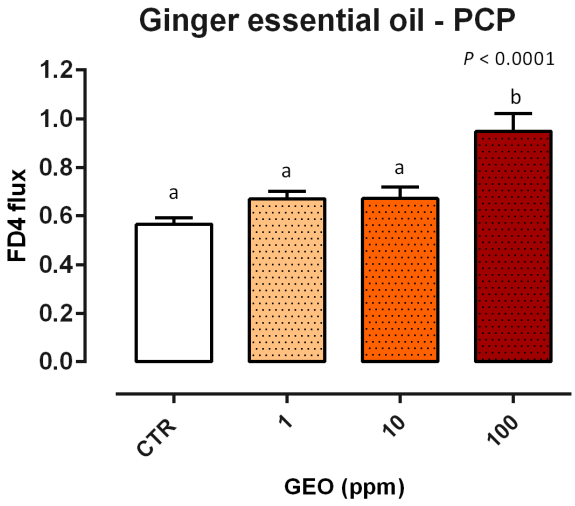

Figure 2. PCP of Caco-2 cells grown with 1, 10, or 100 ppm of ginger essential oil. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b). PCP = paracellular permeability (excitation 485 nm, emission 520 nm).

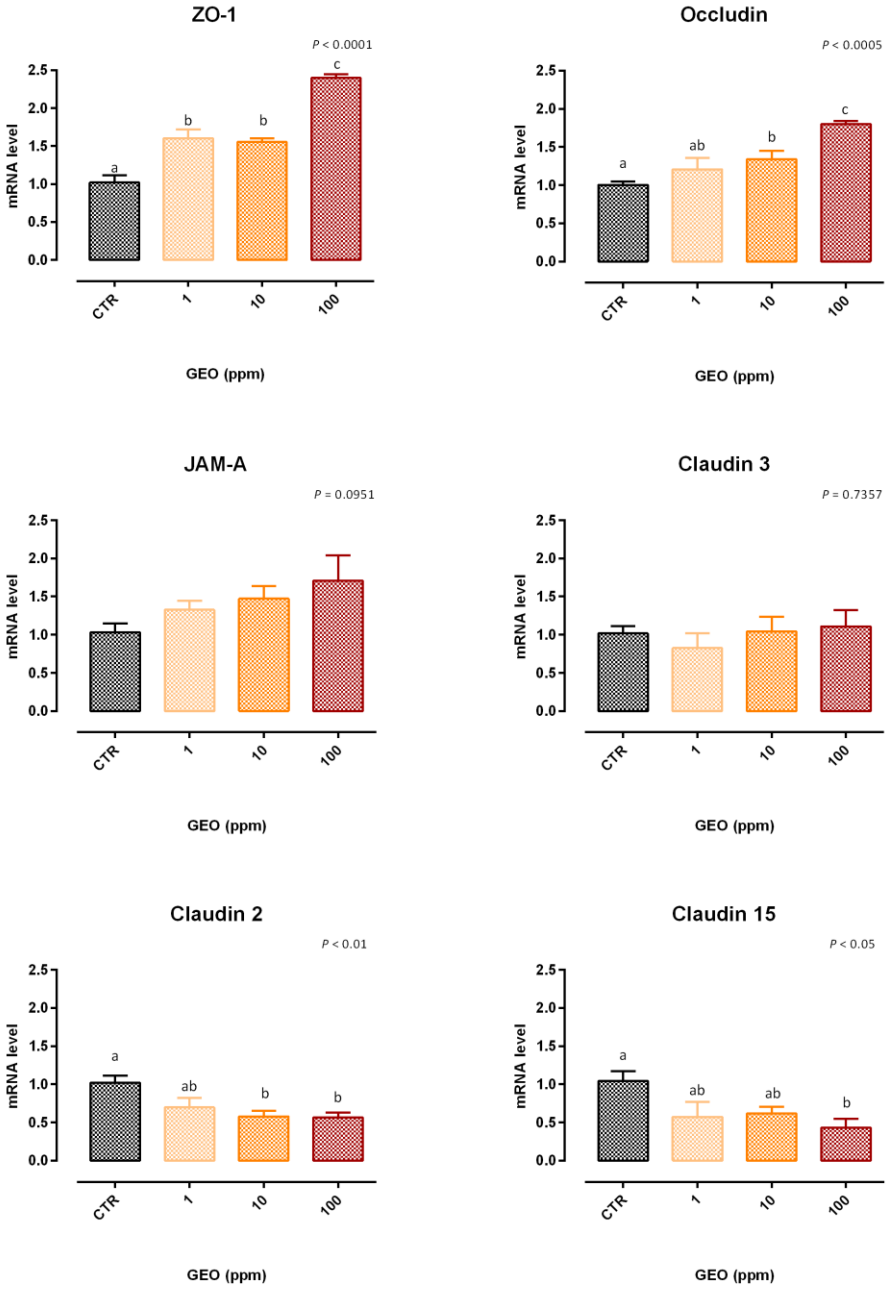

Figure 3. Gene expression in Caco-2 cells after 15 days of treatment with ginger essential oil. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). ZO-1 = zonula occludens 1, JAM-A = Junctional adhesion molecules A.

2. Antioxidant activity

After 24 hours of incubation, GEO did not induce ROS production for all the concentrations tested (data not shown). Moreover, when cells were challenged with H_2O_2 for 60 min (ROS-production challenge, positive control), the treatment with 100 ppm of GEO allowed to significantly reduce the ROS presence compared to the positive control, maintaining the same level of ROS of the unchallenged group (Figure 4a). Concerning ThiolTracker™ Violet assay, no significant differences were reported after 24 hours of treatment (data not shown). Moreover, cells treated with 1 and 10 ppm of GEO and challenged with H_2O_2 showed GSH level comparable with the negative control, while 100 ppm showed results comparable with the positive control (H_2O_2 challenge) (Figure 4b).

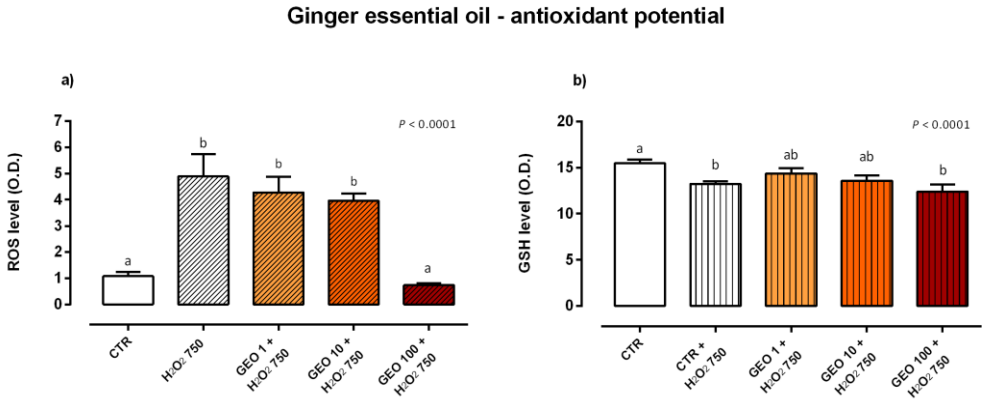

Figure 4. Effects of ginger essential oil on intracellular ROS and GSH production: (**a**) ROS level of Caco-2 cells treated with 1, 10, or 100 ppm of GEO and challenged with 750 µM of hydrogen peroxide (H_2O_2) for 60 min. ROS production was assessed by CellROX® deep red reagent assay and expressed as optical density (O.D.). (**b**) GSH level of Caco-2 cells treated with 1, 10, or 100 ppm of GEO and challenged with 750 µM of hydrogen peroxide (H_2O_2) for 30 min. GSH level was assessed by ThiolTracker™ Violet reagent assay and expressed as optical density (O.D.). Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). 750 µM of hydrogen peroxide (H_2O_2) was used as a positive control.

3. Antimicrobial activity

No MIC was found for the pathogens tested for any concentration of GEO tested.

Tea tree oil

1. Viability and dose response

After 7 days, a significant reduction in viability was reported starting from 300 ppm of inclusion (Figure S2). Concerning the dose response, 100 ppm of TTO positively impacted the TEER immediately after the start of the treatment and the increment remained constant throughout all the study, until day 15, compared to control (Figure 5). No significant variations were reported for PCP (Figure 6). Results about gene expression are reported in Figure 7. Compared to the control, mRNA level of ZO-1 was significantly increased by the supplementation of 100 ppm of TTO ($p < 0.05$). Also, occludin was tendentially increased by the treatments ($p = 0.09$). Conversely, claudin-15 was tendentially reduced by the treatments ($p = 0.09$). No differences were reported for JAM-A, claudin-2, and claudin-3.

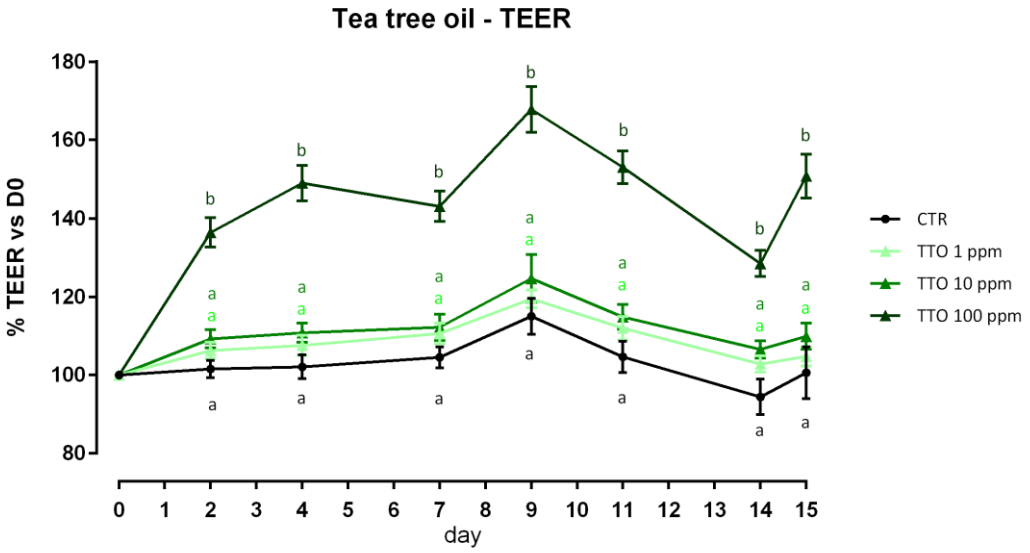

Figure 5. TEER of Caco-2 cells cultured with 1, 10, or 100 ppm of tea tree oil. Data in the graph are represented as percentage over the initial TEER value and given as means (n = 6) ± SEM, represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b), colors of the letters refer to the different treatments. TEER = transepithelial electrical resistance ($\Omega \cdot cm^2$).

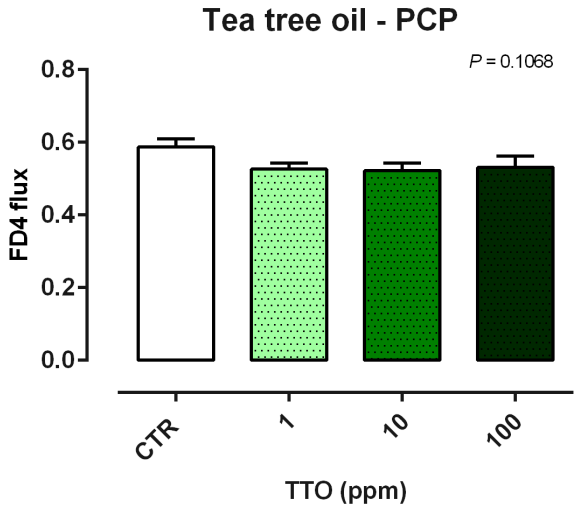

Figure 6. PCP of Caco-2 cells grown with 1, 10, or 100 ppm of tea tree oil. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b). PCP = paracellular permeability (excitation 485 nm, emission 520 nm).

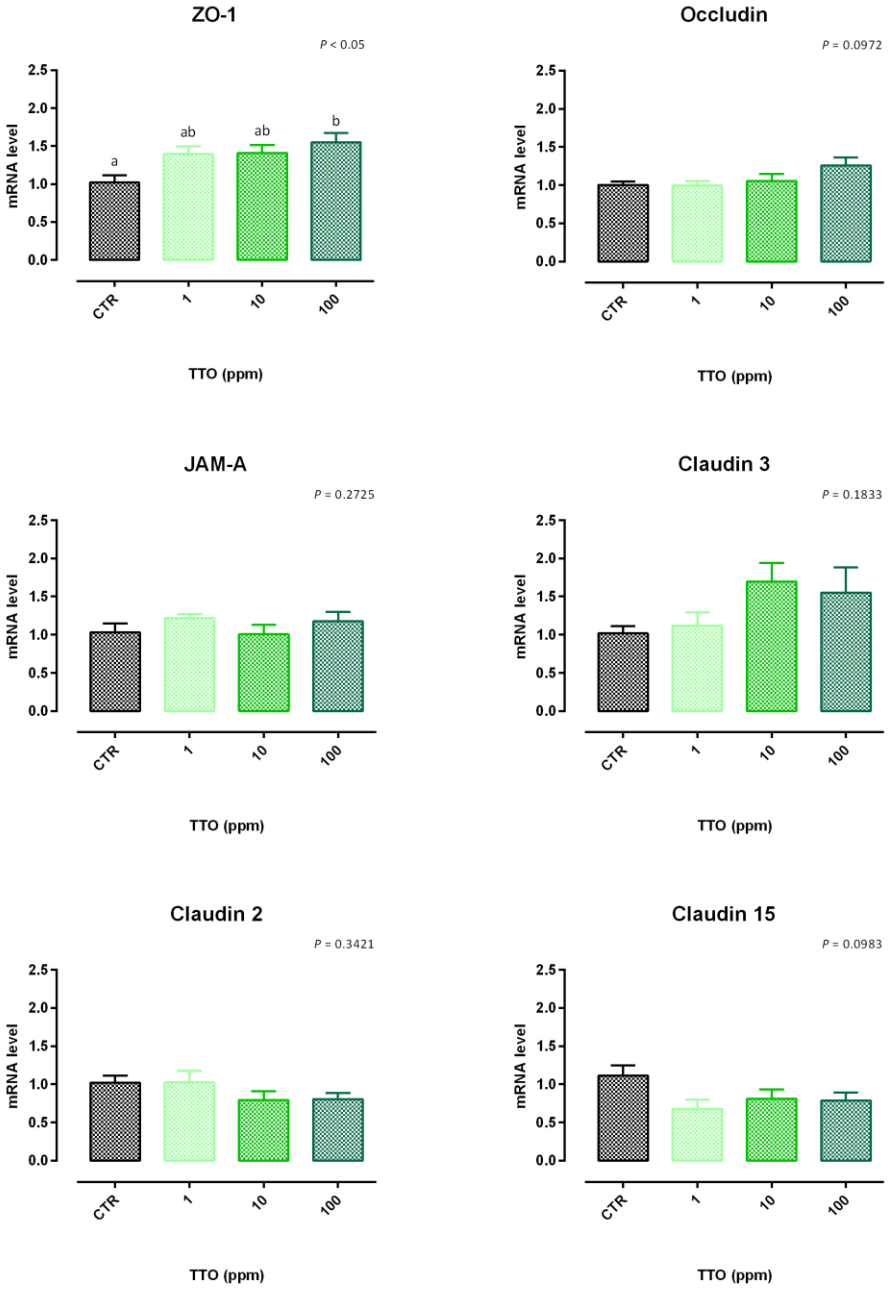

Figure 7. Gene expression in Caco-2 cells after 15 days of treatment with tea tree oil. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). ZO-1 = zonula occludens 1, JAM-A = Junctional adhesion molecules A.

2. Antioxidant activity

For what concerns the ROS levels, after 24 hours of incubation TTO did not induce ROS production for all the concentration tested in Caco-2 cells (data not shown). When cells were subjected to a challenge with 750 µM of H_2O_2, the addition of TTO positively impacted the ROS production, with a reduction compared to the positive control with TTO 100 (Figure 8a). About the GSH level, after 24 hours of treatment, a significant reduction in total thiol level was reported for cells treated with 1 ppm of TTO compared to control, while no differences were reported in other groups (data not shown). When cells were subjected to a challenge with 750 µM of H_2O_2, the preventive treatment with TTO positively impacted the GSH level, with a restoring to control condition with TTO 100 (Figure 8b).

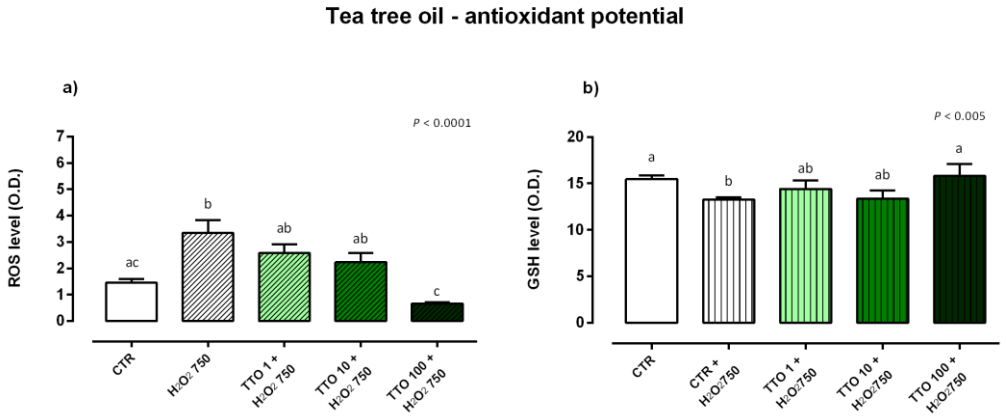

Figure 8. Effects of tea tree oil on intracellular ROS and GSH production: (**a**) ROS level of Caco-2 cells treated with 1, 10, or 100 ppm of TTO and challenged with 750 µM of hydrogen peroxide (H_2O_2) for 60 min. ROS production was assessed by CellROX® deep red reagent assay and expressed as optical density (O.D.). (**b**) GSH level of Caco-2 cells treated with 1, 10, or 100 ppm of TTO and challenged with 750 µM of hydrogen peroxide (H_2O_2) for 30 min. GSH level was assessed by ThiolTracker™ Violet reagent assay and expressed as optical density (O.D.). Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). 750 µM of hydrogen peroxide (H_2O_2) was used as a positive control.

3. Antimicrobial activity

The results of the antimicrobial assays are reported in Table 2. No MIC was found for *Staphylococcus aureus*, though a slight reduction in growth rate was recorded at 10,000 and 8,000 ppm. The concentration of 6,000 ppm was effective to completely inhibit the growth of *Salmonella enteritidis* and *Enterococcus cecorum*, while the MIC of *Escherichia coli* was 4,000 ppm of TTO.

Table 2. Minimal inhibitory concentration (MIC) of tea tree oil against various poultry pathogens (n = 3).

Bacteria strain	**MIC – TTO (ppm)**
Escherichia coli	4,000
Salmonella enteritidis	6,000
Clostridium perfringens	6,000
Enterococcus cecorum	6,000
Staphylococcus aureus	Not found

Grape seed extract

1. Viability and dose response

Data obtained from the viability assay reported a significant reduction after 7 days of treatment starting from 200 ppm of the extract (Figure S3). Concerning TEER, cells treated with GSE up to 100 ppm showed an increase in TEER compared to control (Figure 9) starting from day 2. Moreover, the addition of 100 ppm showed an increase in TEER at day 4 and 15 compared to the other treated groups. Concerning PCP, a reduction of apical-to-basolateral passage of FD4 was recorded in groups treated with 1 ad 10 ppm of GSE, while the addition of 100 ppm caused an increase in PCP (Figure 10). Results about gene expression are reported in Figure 11. Compared to control mRNA level of ZO-1 and JAM-A were tendentially increased by the

treatments (p = 0.07 and p = 0.06 respectively). No differences were reported for occludin, claudin-2 and claudin-3.

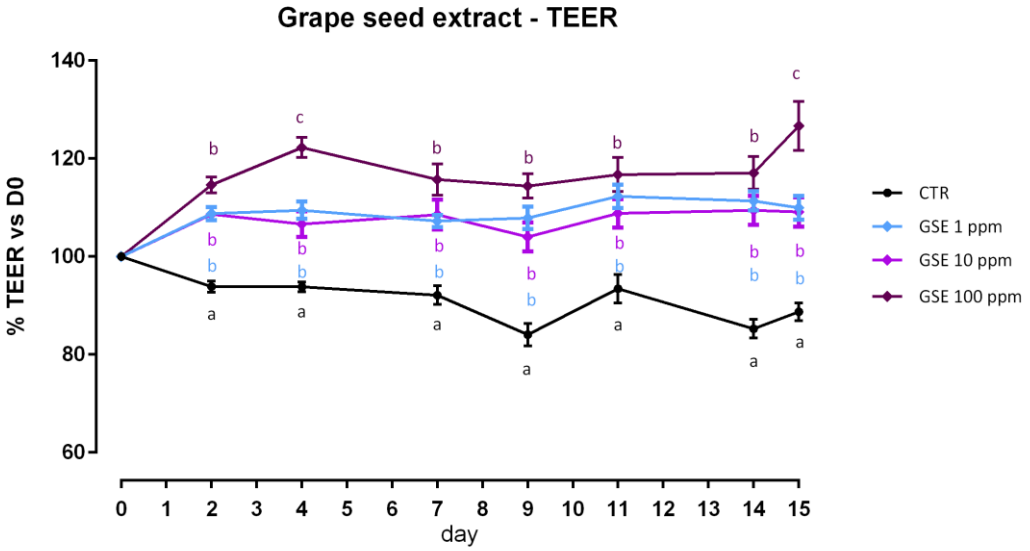

Figure 9. TEER of Caco-2 cells cultured with 1, 10, or 100 ppm of grape seed extract. Data in the graph are represented as percentage over the initial TEER value and given as means (n = 6) ± SEM, represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b), colors of the letters refer to the different treatments. TEER = transepithelial electrical resistance ($\Omega \cdot cm^2$).

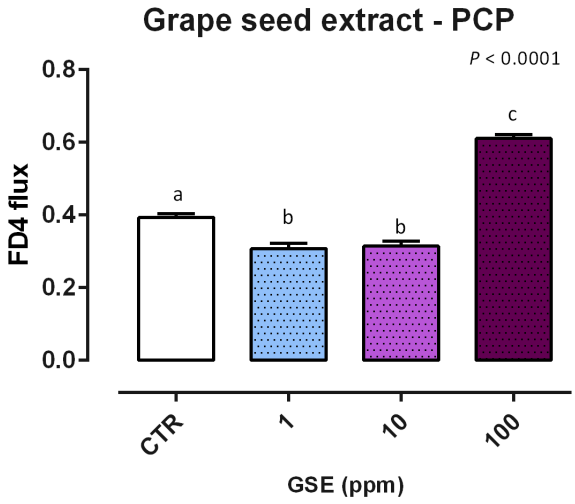

Figure 10. PCP of Caco-2 cells grown with 1, 10, or 100 ppm of grape seed extract. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b). PCP = paracellular permeability (excitation 485 nm, emission 520 nm).

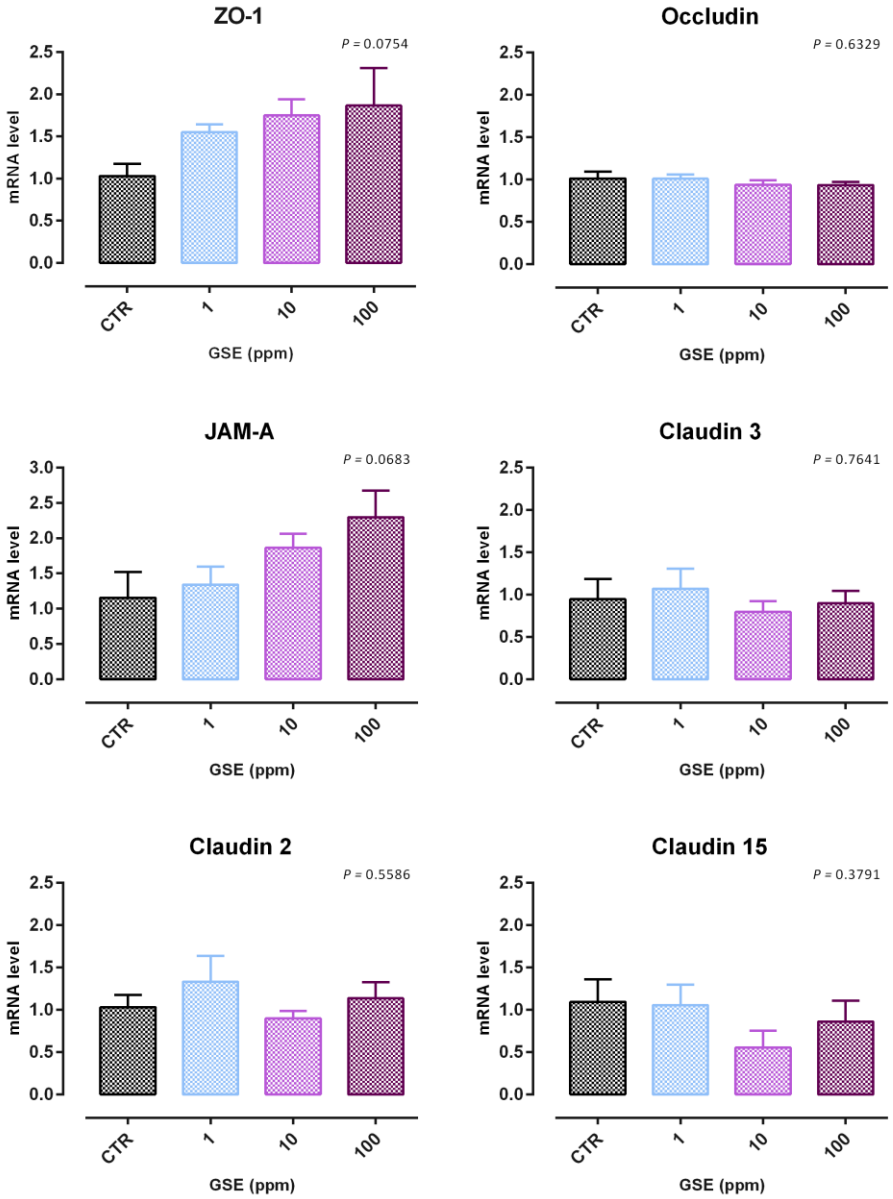

Figure 11. Gene expression in Caco-2 cells after 15 days of treatment with grape seed extract. Values are least square means (n = 6) ± SEM represented by vertical bars. A modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), calculated relative to the control group. Means with different letters indicate statistical significance with $p < 0.05$ (a, b). ZO-1 = zonula occludens 1, JAM-A = Junctional adhesion molecules A.

2. Antioxidant activity

After 24 hours of incubation with the different dosages of GSE (1, 10, 100 ppm), no variations in ROS productions were reported (data not shown). Moreover, when cells were -challenged with H_2O_2 for 60 min, the preventive treatment with 100 ppm of GSE significantly ameliorate the ROS presence compared to the positive control, maintaining the same level of ROS of the unchallenged group (Figure 12a). About the ThiolTracker™ Violet assay, after 24 hours of treatment, a significant reduction in GSH level was reported for cells treated with 1 ppm of GSE compared to control, while no differences were reported in other groups (data not shown). When cells were subjected to a challenge with 750 µM of H_2O_2, the addition of GSE positively impacted the GSH level, with a restoring to the control condition in cells treated with 100 ppm of GSE (Figure 12b). A lower dose of GSE allowed a less restorative effect against the ROS-inducer challenge.

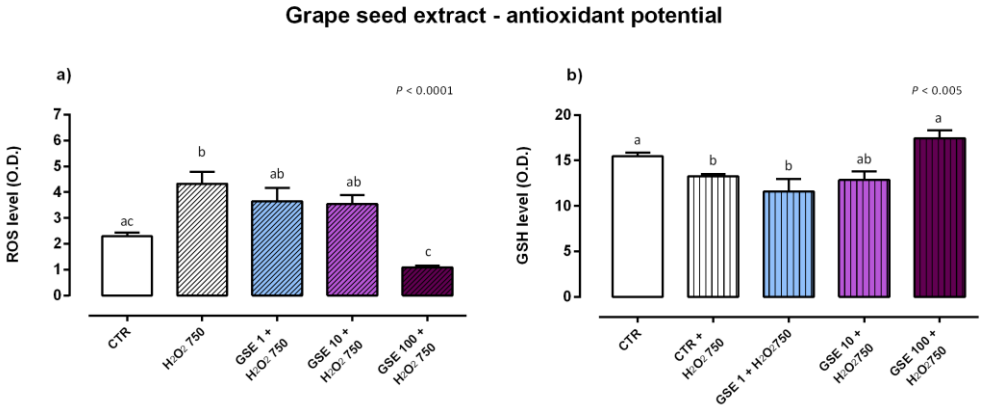

Figure 12. Effects of grape seed extract on intracellular ROS and GSH production: (**a**) ROS level of Caco-2 cells treated with 1, 10, or 100 ppm of GSE and challenged with 750 µM of hydrogen peroxide (H_2O_2) for 60 min. ROS production was assessed by CellROX® deep red reagent assay and expressed as optical density (O.D.). (**b**) GSH level of Caco-2 cells treated with 1, 10, or 100 ppm of GSE and challenged with 750 µM of hydrogen peroxide (H_2O_2) for 60 min. GSH level was assessed by ThiolTracker™ Violet reagent assay and expressed as optical density (O.D.). Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab). 750 µM of hydrogen peroxide (H_2O_2) was used as a positive control.

3. Antimicrobial activity

For all the pathogens tested, no MIC was found for any concentration tested of GSE.

Discussion

The present study was conducted to investigate the effects of natural extracts from ginger, tea tree, and grape seed on the intestinal function and oxidative stress in Caco-2 cells, as well as antimicrobial activity against the main poultry intestinal pathogens. EOs are one of the complementary and alternative medicine that continues to increase in popularity, even in animal production, thanks to the request for a reduction of the use of antibiotics (*Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance)*, 2003, "Veterinary Feed Directive," 2015) and to the therapeutic potential of natural substances (Aziz et al., 2018).

Ginger, tea tree, and grape seeds are widely used for their countless characteristics, mainly antimicrobial, antioxidant, and anti-inflammatory (Herve et al., 2019; Libera et al., 2020; Liju et al., 2015; Puvača et al., 2019; Qu et al., 2019; Weseler and Bast, 2017). To the best of our knowledge, this is the first approach assessing the role of these EOs on enterocytes, as there are no or few studies of this kind conducted on Caco-2 cells in the scientific literature. Caco-2 is a validated model to study intestinal function due to their characteristics, as the ability to polarize and form the brush border, and the possibility to synthesize enzymes and cytokines, becoming a useful tool for absorption and intestinal transport studies (Lea, 2015). In our study, all the EOs tested significantly impacted the viability of the cells after 7 days of treatments, with a reduction of the viability in concentrations ranging from 200 to 300 ppm. The cytotoxicity levels of the EOs analyzed are probably related to the relative percentage of bioactive compounds that compose the oils. In fact, many reports demonstrated that α-zingiberene, α-terpineol, and

procyanidins, showed cytotoxic activity against various cell types, associated with an increased number of lysed cells, and ruled out the possibility of apoptotic cell death (Collett et al., 1996; Hayes et al., 1997; Laurent et al., 2004; Lee, 2016). Lower concentrations seemed safe and were chosen for the subsequent analyses.

Although the present study was restricted to the analysis of only some aspects of intestinal barrier function, it is interesting to note how the selected concentration, 1, 10, and 100 ppm of each EOs tested, conferred a stronger trait of resistance to polarized Caco-2 with a similar pattern for both GEO, TTO, and GSE. In particular, it appears clear that the concentration of 100 ppm was the most helpful for both GEO and TTO, while GSE seems efficient also at the lower concentrations tested. However, the concentration that conferred the stronger trait of TEER also led to an increase in the value of the PCP recorded. These differences resulted superior for GSE, where 1 and 10 ppm allowed an increase in TEER and, in agreement, a reduction of PCP, in accordance with Arie et al. (2019). Unfortunately, for 100 ppm the enhanced resistance matched with an increased flux of dextran. While the measures of TEER and of the passage of marker molecules are both indicators of the integrity of the tight junctions and of the cell monolayer, they determine different entities. The measurement of TEER reflects the ionic conductance of the paracellular pathway, while PCP indicates the paracellular dextran flow of non-electrolyte tracers, as well as the pore size of the tight junctions (Zucco et al., 2005). These discrepancies in the results are normally correlated with a change in the composition of tight junctions, especially for the flavonoid-reach extracts (Anderson and Van Itallie, 2009; Sharma et al., 2016; Suzuki et al., 2011). In fact, the increase of PCP not correlated with a decrease in TEER is normally linked with a depletion in the expression of ZO-1, without any changes for claudin (Van Itallie et al., 2009). Results of gene expression do not support this hypothesis, in fact, both ZO-1 and JAM-A were positively affected by treatments. Also, the gene expression of TJs corroborates the hypothesis that both GEO and GSE can enhance the intestinal barrier.

This study showed how the positive modulation of 100 ppm of GEO, TTO, and GSE is not limited only to the stimulation of the intestinal integrity, but is also effective in reducing oxidative stress. In fact, GEO, TTO, and GSE are well known for their antioxidant potential (Afshari et al., 2007; Brand et al., 2001; Hosseinzadeh et al., 2017; Jayaprakasha et al., 2001; Kim et al., 2004). The production of ROS is a key event in the progression of many inflammatory disorders, including those involving the gastrointestinal tract. In the gut, disruption of the mucosal barrier will rapidly activate an acute inflammatory response that begins in the lamina propria. Uncontrolled and persistent oxidative stress with overproduction of ROS, and/or inadequate removal of ROS by antioxidant systems, will cause apoptosis and tissue injury, highlighting the importance of maintaining the redox balance (Aviello and Knaus, 2017; Rezaie et al., 2007). An increase in ROS production following a GSH depletion represents a crucial event that irreversibly activates apoptotic signaling (Armstrong et al., 2002). The glutathione redox cycle involves two enzymes: glutathione peroxidase, which uses glutathione to reduce organic peroxides and H_2O_2, and glutathione reductase, which reduces the oxidized form of GSH. In addition, GSH can react directly with free radicals (Jozefczak et al., 2012; Patlevič et al., 2016).

The results obtained from the current study indicate that GEO, TTO, and GSE could decrease the concentration of H_2O_2-induced ROS in Caco-2 cells. As for TEER, the strongest results were obtained with the inclusion of 100 ppm of the EOs, which allowed the restoration of the levels of ROS, helping the maintenance of the oxidative stress under physiological conditions. Many studies reported that GEO has a strong antioxidant activity attributed to the enhancement of the activity of antioxidant enzymes like superoxide dismutase and catalase (Afshari et al., 2007; Hamed et al., 2012; Hosseinzadeh et al., 2017). Moreover, flavonoids might exert direct antioxidant effects by scavenging reactive species, but also by other mechanisms such as modulation of antioxidant enzymes (Halliwell, 2007). Results obtained from GSE found support in the study performed by Pinent et al. (2016), which reported the ability of GSE to

prevent oxidative stress in pre-treated Caco-2 cells. In addition, the presence of 100 ppm of both TTO and GSE helped also to recover the GSH level after the challenge with hydrogen peroxide. The restoration of the GSH level suggests the stimulation of the glutathione redox cycle as a partial explanation of their antioxidant activity. Considering that 100 ppm of GSE also helped to recover the normal level of GSH after the challenge with H_2O_2, it is plausible to assume that the antioxidant activity of GSE could be related to the modulation of the GSH cycle, promoting glutathione peroxidase (GPx) activity (Yang et al., 2014). On the other hand, GEO seems only slightly effective in restore the GSH levels after a pro-oxidative stimulus, suggesting a marginal role of GEO in the modulation of GSH levels, in accordance with Mohamed et al. (2015), that demonstrated a marginal role of GEO in the modulation on GSH after a pro-oxidative stimulus in rats.

Moreover, also the antimicrobial power of these EOs against the main pathogens of poultry (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Clostridium perfringens*, and *Enterococcus cecorum*) was analyzed. These pathogenic bacteria are known to trigger severe infections of economic importance in poultry, such as colibacillosis, staphylococcosis, necrotic enteritidis, bacterial chondronecrosis with osteomyelitis (BCO), through decreasing productivity of the infected birds and increasing their mortality worldwide, (Jung et al., 2018; Kabir, 2010; Kuramasu et al., 1967; Shojadoost et al., 2012). Interestingly, this experiment showed significant results only about TTO, with a well-known mode of action related to the ability to alter cell membrane structure and to the leakage of potassium ions (Cox et al., 2000; Swamy et al., 2016). It was effective in reducing the proliferation of *Escherichia coli* and *Salmonella enteritidis*, accordingly to McMahon et al. (2006). Contrasting results are related to *Staphylococcus aureus,* but it could be related to the susceptibility of the pathogens (Carson et al., 2006; Cox et al., 2000). TTO seems also effective against *Clostridium perfringens* and *Enterococcus cecorum*, suggesting its potential use for BCO and necrotic enteritis in poultry. Both GEO and GSE were unsuccessful against the pathogens analyzed. These results are in

divergence with some literature, where these natural compounds were reported as promising alternatives to antibiotics (Abd El-Hack et al., 2020; Elizondo et al., 2010; Mahboubi, 2019; Reyes et al., 2017; Shrestha et al., 2012). In most of these studies, MICs were found using higher concentration compared to the ones tested in our study. Moreover, variation in EO composition, mainly related to the origin and source of *Zingiber officinale* or the mode of extraction from *Vitis vinifera*, could explain the differences belonging to the various report in the literature.

If translated *in vivo*, these results could add a piece of information for the possible use of these EOs not only limited as non-antibiotic substances to support conventional therapies, but either to support the epithelium and the mucosa of a young animal, and to make it more resistant to oxidative and inflammatory challenge. Indeed, ginger, tea tree, and grape seed showed an increase of TEER correlated with an augmented gene expression of TJs, allowing modulation of the transcellular and paracellular pathway and reducing the risk of translocation of noxious molecules or pathogens. Also, these EOs exhibit the ability to reduce the ROS, as well as restore GSH level. Further markers should be studied to better identify the specific pathway activated and the active ingredient mainly related to it, but these outcomes validate the suggestion to use GEO, TTO, and GSE mainly as modulators of intestinal barrier functionality, rather than antimicrobials. Their ability to regulate the intestinal barrier reduces the risk of pathogen infections, resulting in better growth performance. Of course, a correct evaluation of the dosage is needed, as the positive antimicrobial effects mediated by high concentration could be linked with negative effects on the intestinal epithelium.

Conclusions

To conclude, EOs have different chemical and biological properties with a wide range of molecular targets, showing the potential to ameliorate intestinal barrier function and intestinal oxidative status *in vitro*. This would validate their use as feed additives, although more studies need to be performed to uniform the scientific opinion of EOs. In fact, it is clear that, besides their advantageous characteristics, the variability in composition and formulation represents a limit that needs to be overcome, in order to apply these substances more safely.

Supplementary materials

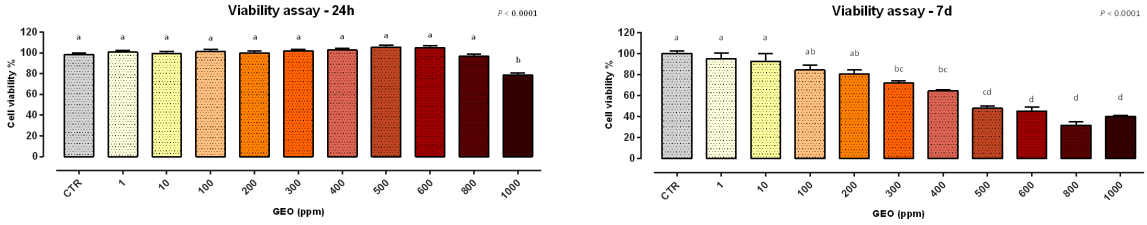

Figure S1. Viability of Caco-2 cells treated for 24 hours and 7 days with incremental doses of ginger essential oil. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab).

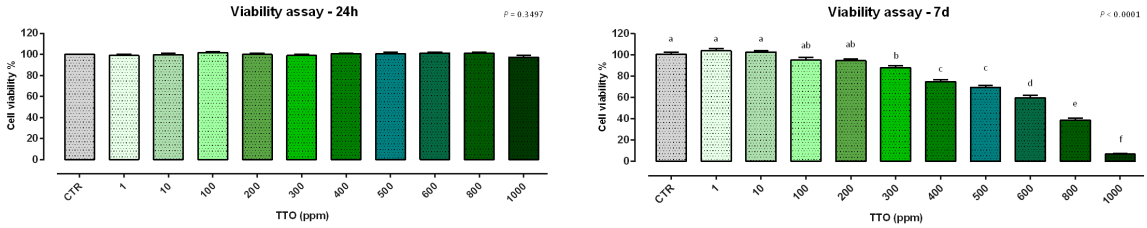

Figure S2. Viability of Caco-2 cells treated for 24 hours and 7 days with incremental doses of tea tree oil. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab).

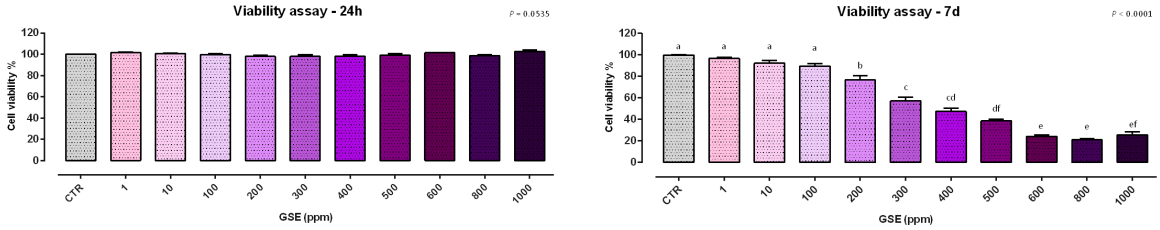

Figure S3. Viability of Caco-2 cells treated for 24 hours and 7 days with incremental doses of grape seed extract. Data in the graph are means (n = 6) ± SEM represented by vertical bars. Means with different letters indicate statistical significance with $p < 0.05$ (a, b); means with at least one common letter are not significantly different (ab).

EXPERIMENTAL STUDY 3

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Thymol modulates the endocannabinoid system and gut chemosensing of weaning pigs

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Abstract

Background: The recent identification of the endocannabinoid system in the gastrointestinal tract suggests a role in controlling intestinal inflammation. In addition, the gut chemosensing system has therapeutic applications in the treatment of gastrointestinal diseases and inflammation due to the presence of a large variety of receptors. The purposes of this study were to investigate the presence of markers of the endocannabinoid system and the chemosensing system in the pig gut and, second, to determine if thymol modulates these markers. One hundred sixty 28-day-old piglets were allocated into one of 5 treatment groups (n = 32 per treatment): T1 (control), T2 (25.5 mg thymol/kg feed), T3 (51 mg thymol/kg feed), T4 (153 mg thymol/kg feed), and T5 (510 mg thymol/kg feed). After 14 days of treatment, piglets were sacrificed (n = 8), and then duodenal and ileal mucosal scrapings were collected. Gene expression of cannabinoid receptors (CB1 and CB2), transient receptor potential vanilloid 1 (TRPV1), the olfactory receptor OR1G1, diacylglycerol lipases (DGL-α and DGL-β), fatty acid amine hydrolase (FAAH), and cytokines was measured, and ELISAs of pro-inflammatory cytokines levels were performed.

Results: mRNAs encoding all markers tested were detected. In the duodenum and ileum, the CB1, CB2, TRPV1, and OR1G1 mRNAs were expressed at higher levels in the T4 and T5

groups compared to the control group. The level of the FAAH mRNA was increased in the ileum of the T4 group compared to the control. Regarding the immune response, the level of the tumor necrosis factor (TNF-α) mRNA was significantly increased in the duodenum of the T5 group, but this increase was not consistent with the protein level.

Conclusions: These results indicate the presence of endocannabinoid system and gut chemosensing markers in the piglet gut mucosa. Moreover, thymol modulated the expression of the CB1, CB2, TRPV1, and OR1G1 mRNAs in the duodenum and ileum. It also modulated the mRNA levels of enzymes involved in the biosynthesis and degradation of endocannabinoid molecules. Based on these findings, the effects of thymol on promoting gut health are potentially mediated by the activation of these receptors.

Introduction

The endocannabinoid system (ECS) comprises three fundamental constituents: receptors, signaling molecules, and enzymes involved in ligand biosynthesis and degradation. The main endocannabinoid receptors are two G protein-coupled receptors (GPCRs) named cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (Mackie, 2008; Matsuda et al., 1990), but additional receptors may also be involved, such as transient receptor potential vanilloid 1 (TRPV1) (Muller et al., 2019). The two most widely investigated endocannabinoid signaling molecules are anandamide (AEA) and 2- arachidonoylglycerol (2-AG), which are lipid molecules generated from the breakdown of arachidonic acid (Castillo et al., 2012). N-Acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) is currently considered the major enzyme responsible for AEA synthesis, while a specific diacylglycerol lipase (DGL) is responsible for 2-AG synthesis (Castillo et al., 2012; Okamoto et al., 2009). The biological activity of ligands is regulated by intracellular enzymes unique to each endocannabinoid ligand: fatty acid amide hydrolase (FAAH) is the principal enzyme responsible for degrading AEA, whereas monoacylglycerol lipase (MAGL) is responsible for degrading 2-AG (Bisogno et al.,

2005; Dinh et al., 2002; McKinney and Cravatt, 2005). In non-neuronal tissues, endocannabinoids act as hormone-like messengers in an autocrine or paracrine mode of action, which is thought to be temporally and spatially restricted (Piomelli, 2003). When endocannabinoid molecules are released, they bind receptors with different affinity. After the binding of ligands, receptors mediate the effects, including the stimulation of extracellular-regulated kinases and the inhibition of adenylyl cyclase (Massa et al., 2005). The ECS controls a variety of gastrointestinal (GI) functions: it is presumed to regulate GI motility via the enteric nervous system (ENS) (Vera et al., 2017), to inhibit the secretion of pro-inflammatory cytokines and to attenuate the lipopolysaccharide (LPS) induced increase in GI transit (Klein, 2005). For example, CB1 receptors are colocalized with acetylcholine transferase, which is a marker for cholinergic neurons. This observation supports the role of endocannabinoids as inhibitors of intestinal motility and secretion by inhibiting cholinergic neurotransmission (Massa et al., 2005). For all these reasons, dysregulation of the ECS clearly plays a role in intestinal disorders, including irritable bowel disease (IBD), irritable bowel syndrome, and obesity (Izzo and Sharkey, 2010).

Gut chemosensation represents the ability of the gut to sense chemical and nutrient stimuli at the GI level through the action of enteroendocrine cells (EEC) (Bertrand, 2009), and it appears to be connected to the presence of chemosensory receptors in the mouth and all along the GI tract (Höfer et al., 1999). This activity is mediated by a large variety of receptors, most of which are GPCRs, including TRP (Transient Receptor Potential) channels and olfactory receptors (ORs). Transient Receptor Potential channels comprise six related protein subfamilies (Takahashi et al., 2012) that include TRPV1, which was previously mentioned as one of the secondary endocannabinoid receptors, whereas ORs have a role in recognizing odorant molecules in the olfactory sensory system (Touhara, 2009). Botanicals such as thymol play a role in regulating the integrity of the intestinal mucosa because of their anti-inflammatory and antioxidant properties (Du et al., 2016). In particular, thymol is a monoterpene and it is a

predominant component of several essential oils derived from plant species belonging to the *Lamiaceae* family. Thymol showed therapeutic potential in reducing oxidative stress, boosting the immune system and fighting against pathogenic bacteria (Nagoor Meeran et al., 2017), and it is also well known in animal nutrition as a key component of many botanical feed additives (Rossi et al., 2020). Therefore, the purposes of this study were 1) to investigate the presence of ECS and gut chemosensory markers in the GI tract of piglets and 2) to evaluate the possible modulation of these systems by treatment with a thymol supplement.

Materials and Methods

Ethics statement

The study was conducted at the facilities of the Research Center for Animal Production and Environment (CERZOO), which is Good Laboratory Practices-certified and operates according to the Procedure of Animal Protection and Welfare (Directive No 86/609/EEC). Animals used in the study were raised and treated according to European Union Directive 2010/63/EU. The study was authorized by Italian Health Ministry according to art. Thirty-one of the Italian Legislative Decree No. 26/2014 and to the recommendation of Commission 2007/526/ CE, covering the accommodation and care of animals used for experimental and other scientific purposes (authorization n. 341/2017-PR issued on May 3, 2017). Animals were obtained from a breeding farm in Cascina Mandellina, Bergamo, Italy.

Animals and diets

One hundred sixty piglets (Duroc × Large White) weaned at 28 days of age and with a BW of 7.71 ± 1.00 kg were divided into 40 pens (4 piglets per pen, castrated males and females were blocked in separate pens) and randomly assigned to one of the following experimental groups (n = 32): control group fed the basal diet (T1), a group fed the basal diet supplemented with 25.5 mg of thymol/kg feed (T2), a group fed the basal diet supplemented with 51 mg of

thymol/kg feed (T3), a group fed the basal diet supplemented with 153 mg of thymol/kg feed (T4) and a group fed the basal diet supplemented with 510 mg of thymol/kg feed (T5). Thymol was provided in a form microencapsulated in a lipid matrix (Vetagro SpA, Reggio Emilia, Italy). Concentrations of thymol were selected to meet or exceed the upper limit of inclusion in food and feed established by the European Agency for the Evaluation of Medicinal Products (EFSA 2012; EMA 1996). The basal feed was formulated to meet or exceed the nutritional requirements of pigs according to National Research Council (2012), and feed and water were provided *ad libitum* (the composition of the basal diet is reported in Table 1). The health status of animals was monitored throughout the study. Piglets were individually weighed at the beginning (day 0) and end of the study (day 14). Growth parameters, such as FI, ADFI, ADG, and F:G, were measured in the animals housed in each pen on d14 of the experiment.

At the end of the study, 8 animals from each treatment group were selected for sacrifice, sample collection, and analysis. Piglets were euthanized by a penetrating captive bolt followed by exsanguination. Duodenal and ileal mucosal scrapings were collected. The duodenum and ileum were longitudinally cut to expose the mucosa, washed with a phosphate-buffered saline solution to remove mucus and digesta, then scraped gently with a glass slide, packed, immediately frozen in liquid N2 and stored at − 80 °C until the analyses of gene and protein expression.

Table 1. Basal diet composition (%) and nutrient profile.

Ingredient (% of dry matter)	
Corn meal	59.25
Soybean meal, 44%	21.90
Sweet milk whey	8.00
Fish meal (Herring 999)	7.00
Soybean oil	1.98
Calcium carbonate	0.35
Vitamin and mineral premix[1]	0.25
L-Lysine HCl	0.54
NaCl	0.16
L-Threonine	0.24
DL-Methionine	0.26
L-Tryptophan	0.08
Total	100
Calculated nutrient composition	
Digestible Energy[2] (kcal/kg feed)	3523
Net Energy[2-3] (kcal/kg feed)	2549
Dry matter (%)	88.53
Crude protein (%)	21.00
Crude fiber (%)	2.64
Crude fat (%)	5.32
Calcium (g/kg)	7.00
Phosphorus – Total (g/kg)	5.60
Phosphorus – Available (g/kg)	1.45
Sodium (g/kg)	1.80

[1]Content of vitamins and Oligo minerals/kg feed: vit. A: 10,000. UI; vit. D3: 1,000UI; vit. E: 100 mg; vit. B1: 3.0 mg; vit. B2: 10.0 mg; vit. B6:5.8 mg; vit. B12: 0.04 mg; Biotin: 0.19 mg; vit. K: 4.8 mg; vit. PP: 35.0 mg; folic acid: 1.4 mg; D-pantothenic acid: 26.1 mg; choline chloride: 120 mg; 49.7 mg Mn from Manganese oxide; 224 mg Fe from Ferrous carbonate; 75.8 mg Cu from Copper sulphate pentahydrate; 139 mg Zn from Zinc oxide; 0.89 mg I from Calcium iodide; 0.64 mg Se from Disodium selenite.

[2]Aritmetic mean.

[3]Net Energy was calculated according to the procedure and equation proposed by Noblet and colleagues (1994).

Gene expression analysis

Gene expression was analyzed using the method reported by Herfel et al. (2011). Duodenal and ileal scraping samples obtained on d14 of the study were disrupted by grinding them in liquid nitrogen with mortar and pestle, and then homogenized using a TissueLyser (Qiagen, Hilden, Germany). Total RNA was isolated using a NucleoSpin® RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed by treating the samples with the deoxyribonuclease supplied in the extraction kit (rDNase, RNase-free; Macherey-Nagel). The RNA yield and quality were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm (A260 and A280 nm, respectively) (Microvolume Mode with SmartPath® Technology, Denovix). One microgram of RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed using an iCycler Thermal Cycler system and Sybr-Green Supermix (Bio-Rad Laboratories Inc.). The thermocycling protocol included an initial denaturation step for 1 min and 30 s at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and 30 s of annealing and extension at 60 °C. After amplification, a melting curve analysis was performed for all the samples, with slow heating from 55 °C to 95 °C at a rate of 0.5 °C/s to validate the absence of non-specific products. Gene expression was normalized to a housekeeping gene (HK) encoding portions of porcine ribosomal subunit 60 S, in particular ribosomal protein L35 (RPL35). The average threshold cycle (CT) was determined for each gene of interest, and the geometric average was calculated for HK by assuming that CT is the number of cycles needed to reach a fixed arbitrary threshold. Delta CT was calculated, then a modification of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression (fold changes), which was calculated relative to the control group. The sequences, accession numbers in the EMBL database/GenBank, expected product lengths, and references for porcine primers are provided in Table 2. Primer oligonucleotides for CB1, DGL-β, and OR1G1 were designed using the

Primer-BLAST tool (NCBI National Center for Biotechnology Information, www.ncbi.nlm.nih.gov). Primers were obtained from Life Technologies (Life Technologies Italia).

Table 2. Primer sequence used for gene expression analysis.

Gene	Primer sequence (F and R) 5' → 3'	Product length (bp)	Accession N.	Reference
CB1	F: TTCCCCACTTCTTTTCCGCC R: GGGAGTCCCTTCGCATCC	208	XM_013992672.2	Present study
CB2	F: TTTATAGCCTGGCCTCCCCT R: TTTTCCCGTCTGCCTCTGTC	240	XM_021095530.1	(Sampaio et al., 2015)
FAAH	F: TGCCACCGTGCAAGAAAATG R: CCACTGCCCTAACAACGACT	234	XM_013999418.2	(Sampaio et al., 2015)
DGL-α	F: GAAACCAAACACGCCTCCAC R: CAACCCAGCAGCAAAGGAAC	211	XM_021082924.1	(Sampaio et al., 2015)
DGL-β	F: TTTGTAATCCCGGACCACGG R: GACCTGCCGAGGAATACGGA	255	XM_021086077.1	Present study
TRPV1	F: TCACCAACAAGAAGGGGCTC R: GGATAGGTGCCTGCACTCAG	116	XM_005669121.1	(Brown et al., 2015)
OR1G1	F: CTTGGTTTGTGTGCTCTGCC R: GAAAAGGCTTTCCGCTTCCC	96	XM_013990010.1	Present study
INF-γ	F: GGCCATTCAAAGGAGCATGGATGT R: TGAGTTCACTGATGGCTTTGCGCT	149	NM_213948.1	(Grilli et al., 2015)
TNF-α	F: GCCCACGTTGTAGCCAATGTCAAA R: GTTGTCTTTCAGCTTCACGCCGTT	99	NM_214022.1	(Grilli et al., 2015)
RPL35	F: AACCAGACCCAGAAAGAGAAC R: TTCCGCTGCTGCTTCTTG	146	NM_214326.2	(Alexander et al., 2012)

F = forward; R = reverse; CB1 = cannabinoid receptor 1; CB2 = cannabinoid receptor 2; FAAH = fatty acid amide hydrolase; DGL-α = diacylglycerol lipase alpha; DGL-β = diacylglycerol lipase beta; TRPV1 = transient receptor potential vanilloid 1; OR1G1 = olfactory receptor 1G1; IFN-γ = interferon-γ; TNF-α = tumor necrosis factor-α; RPL35 = ribosomal protein L35.

ELISA quantification of inflammatory cytokine concentrations

Duodenal and ileal mucosal scrapings were disrupted by grinding the samples in liquid nitrogen with a mortar and pestle, followed by the addition of lysis buffer (10 mM-2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)- HCl, 1 mM-ethylenediaminetetraacetic acid (EDTA), and 0.5%-Triton X100) and homogenization using a Tissue- Lyser (Qiagen). Protein levels of inflammatory cytokines (TNF-α, INF-γ, IL-1β, and IL-8) were analyzed using ELISA kits specific for porcine cytokines (Quantikine ELISA, R&D Systems Inc., Minneapolis, MN, USA). Analyses were performed according to the manufacturer's instructions. ELISA quantification was performed for samples obtained from the T1 and T5 groups. Results are reported as picograms of cytokine per 100 mg of tissue (pg/100 mg).

Statistical analysis

Animals were blocked in a completely randomized design and data were analyzed using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test to detect differences among treatments. The pen was the experimental unit for growth performance, whereas the pig was the experimental unit for gene expression and ELISA data. Differences were considered significant at $p < 0.05$, and trends were defined at $0.05 \leqslant p < 0.1$.

Results

Growth performance

Piglets maintained a good health status throughout the experiment and no mortality was recorded. During the experiment, differences in body weight (BW), feed intake (FI), average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F:G) were not observed among the treatment groups (data not shown).

Endocannabinoid system

Figure 1 summarizes gene expression data for cannabinoid receptors in the duodenal and ileal mucosa at d14. Cannabinoid receptor 1 and 2 mRNAs were detected in both the duodenal and ileal mucosa. The level of the CB1 mRNA was significantly increased in the duodenum of the T5 group ($p = 0.0209$) and in the ileum of the T4 and T5 groups ($p = 0.0054$) compared to the control group. Significantly increased levels of the CB2 mRNA were detected in both the duodenum and ileum of groups T4 and T5 compared to the control group ($p = 0.0004$ and $p = 0.0162$ respectively). Data on gene expression for ECS enzymes are reported in Figure 2. The presence of mRNA for all the enzymes tested was confirmed. The expression of the DGL-α mRNA in both duodenum and ileum was not affected by the treatments. The expression of the DGL-β mRNA was significantly increased in the duodenum of animals fed 51 mg of thymol/ kg of feed (T3) compared to animals fed 510 mg of thymol/kg of feed ($p = 0.0262$). No significant differences were identified in the levels of the DGL-β mRNA in the ileal mucosa. Differences in the levels of the FAAH mRNA were not observed in the duodenum, while mRNA levels were significantly increased in the ileum of the T4 group compared to the control group ($p = 0.0028$).

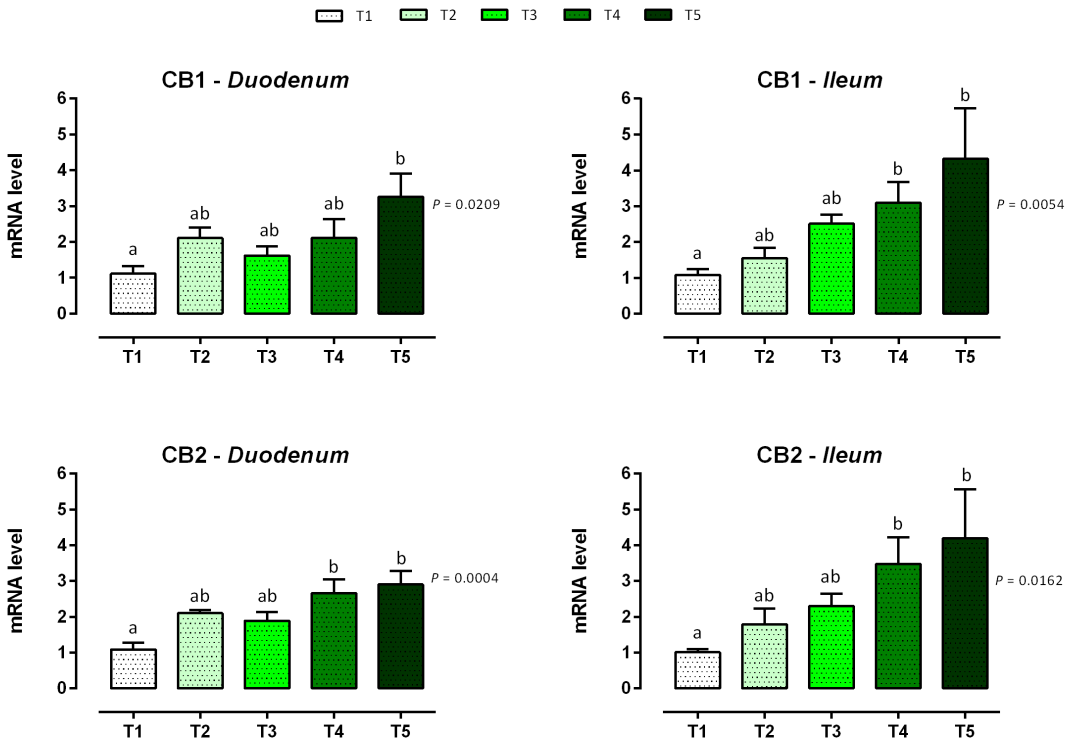

Figure 1. Gene expression of cannabinoid receptors in duodenal and ileal mucosa of piglets. Data are expressed as means (n = 8) and S.E.M. represented by vertical bars. [a,b] Values with different superscripts differ significantly at $p < 0.05$. T1 = basal diet; T2= basal diet + 25.5 mg of thymol/kg feed; T3= basal diet + 51 mg of thymol/kg feed; T4= basal diet + 153 mg of thymol/kg feed; T5 = basal diet + 510 mg of thymol/kg feed (Vetagro SpA, Reggio Emilia, Italy). A modification of the $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control; Livak and Schmittgen, 2001). CB1 = cannabinoid receptor 1; CB2 = cannabinoid receptor 2.

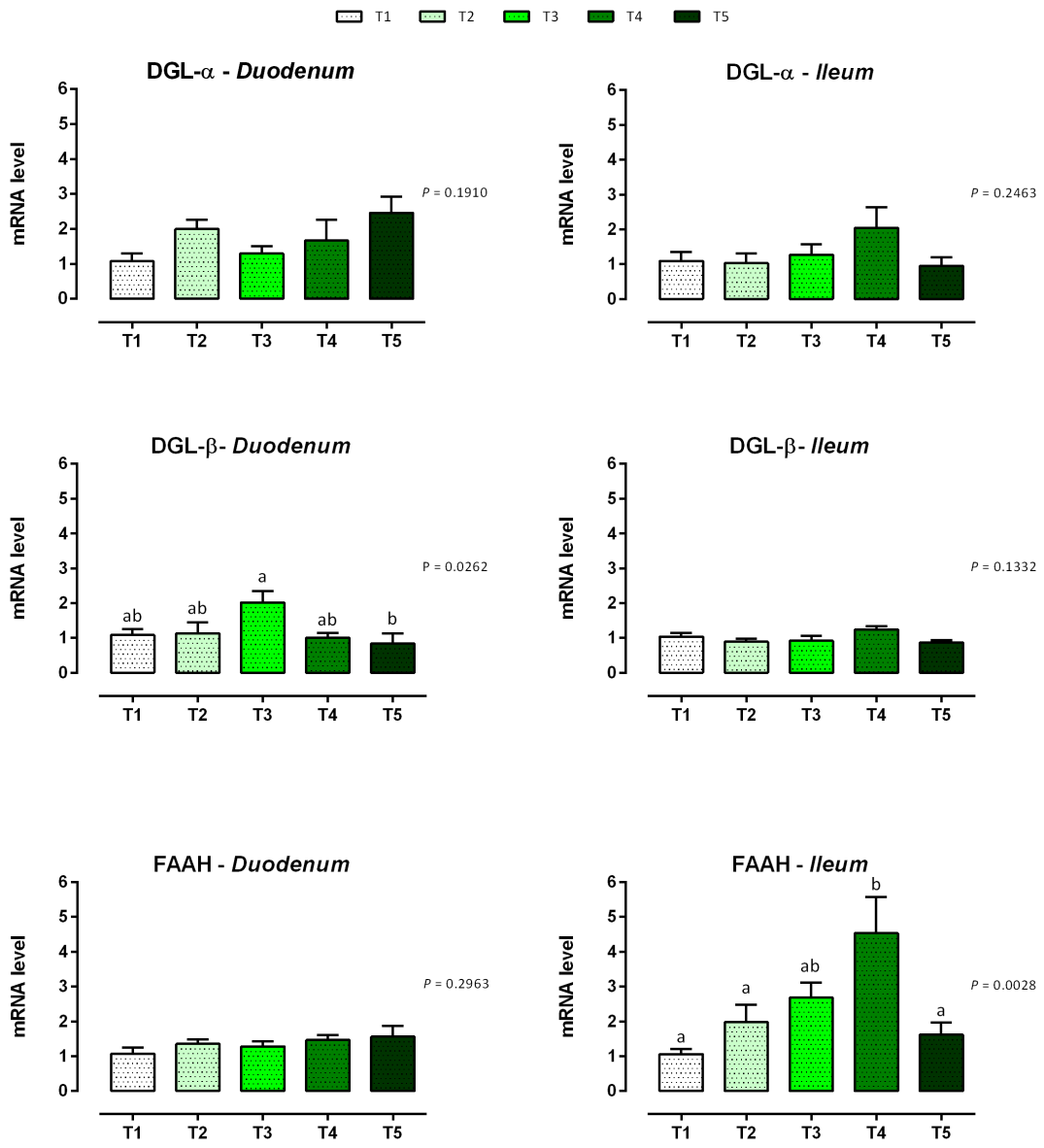

Figure 2. Gene expression of endocannabinoid enzymes in duodenal and ileal mucosa of piglets. Data are expressed as means (n = 8) and S.E.M. represented by vertical bars. a,b Values with different superscripts differ significantly at $p < 0.05$. T1 = basal diet; T2= basal diet + 25.5 mg of thymol/kg feed; T3= basal diet + 51 mg of thymol/kg feed; T4= basal diet + 153 mg of thymol/kg feed; T5 = basal diet + 510 mg of thymol/kg feed (Vetagro SpA, Reggio Emilia, Italy). A modification of the $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control; Livak and Schmittgen, 2001). DGL-α = diacylglycerol lipase alpha; DGL-β = diacylglycerol lipase beta; FAAH = fatty acid amide hydrolase.

Gut chemosensing system

Results for the gut chemosensing are reported in Figure 3. Concerning the gut chemosensing markers, both the TRPV1 and OR1G1 (Olfactory receptor 1G1) mRNAs were detected in the duodenal and ileal mucosa. Moreover, the supplementation of 510 mg of thymol/kg of feed increased the level of the TRPV1 mRNA in the duodenum ($p = 0.0382$), while increased mRNA levels were observed in the ileum of the T4 and T5 groups compared to the control group ($p = 0.0183$). The OR1G1 mRNA was expressed at higher levels in the duodenum of animals provided feed supplemented with 510 mg of thymol/kg of feed (T5) ($p = 0.0210$) and in the ileum of animals fed 153 mg of thymol/kg of feed (T4) ($p = 0.0235$) than in the control group.

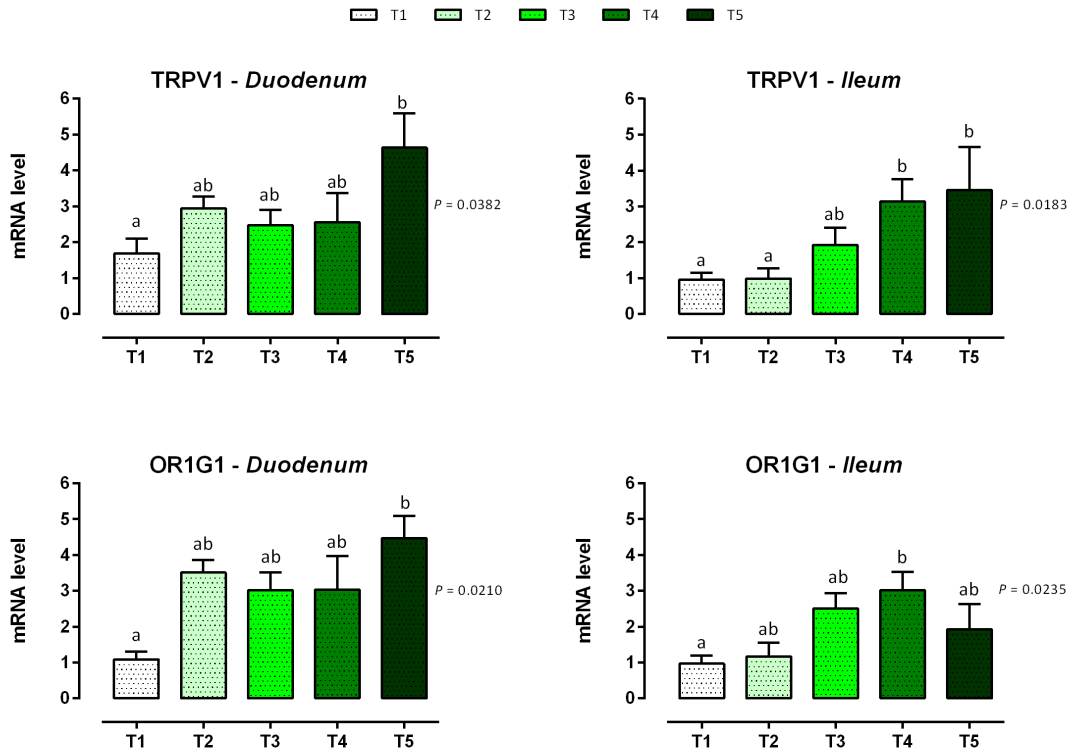

Figure 3. Gene expression of chemosensory receptors in duodenal and ileal mucosa of piglets. Data are expressed as means (n = 8) and S.E.M. represented by vertical bars. a,b Values with different superscripts differ significantly at $p < 0.05$. T1 = basal diet; T2= basal diet + 25.5 mg of thymol/kg feed; T3= basal diet + 51 mg of thymol/kg feed; T4= basal diet + 153 mg of thymol/kg feed; T5 = basal diet + 510 mg of thymol/kg feed (Vetagro SpA, Reggio Emilia, Italy). A modification of the 2–ΔΔCT method was used to analyze the relative expression (fold changes), calculated relative to the control group (control; Livak and Schmittgen, 2001). TRPV1 = transient receptor potential vanilloid 1; OR1G1 = Olfactory receptor 1G1.

Inflammatory cytokines

Figures 4 and 5 show the mRNA and protein levels of inflammatory cytokines in the duodenal and ileal mucosa at d14, respectively. Significantly increased levels of the Tumor necrosis factor (TNF)-α mRNA were detected in the duodenum of animals fed thymol; in particular, the T5 group displayed the highest expression of TNF-α (p = 0.0289). On the other hand, the level of the TNF-α mRNA in the ileal mucosa was not affected by the treatments. No difference was reported in interferon (IFN)-γ expression in both the duodenum and ileum. No statistically significant differences in the levels of these proteins were observed among the treatment groups (Figure 5).

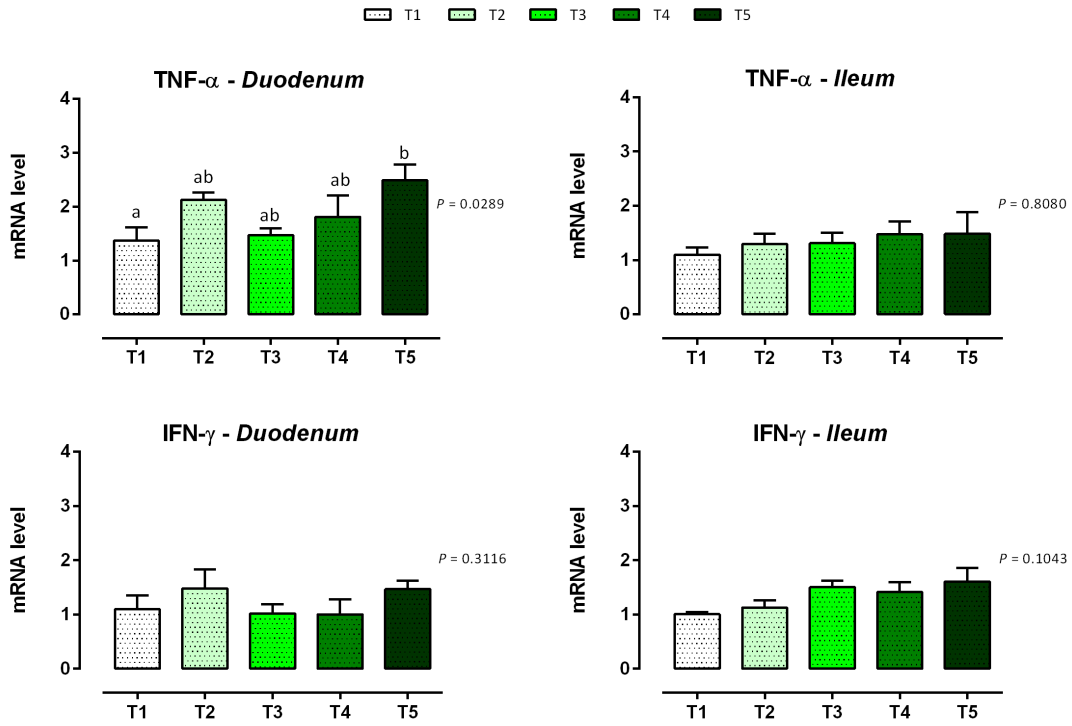

Figure 4. Gene expression of inflammatory cytokines in duodenal and ileal mucosa of piglets. Data are expressed as means (n = 8) and S.E.M. represented by vertical bars. a,b Values with different superscripts differ significantly at $p < 0.05$. T1 = basal diet; T2= basal diet + 25.5 mg of thymol/kg feed; T3= basal diet + 51 mg of thymol/kg feed; T4= basal diet + 153 mg of thymol/kg feed; T5 = basal diet + 510 mg of thymol/kg feed (Vetagro SpA, Reggio Emilia, Italy). A modification of the 2–ΔΔCT method was used to analyze the relative expression (fold changes), calculated relative to the control group (control; Livak and Schmittgen, 2001). TNF-α = tumor necrosis factor-α; IFN-γ = interferon-γ.

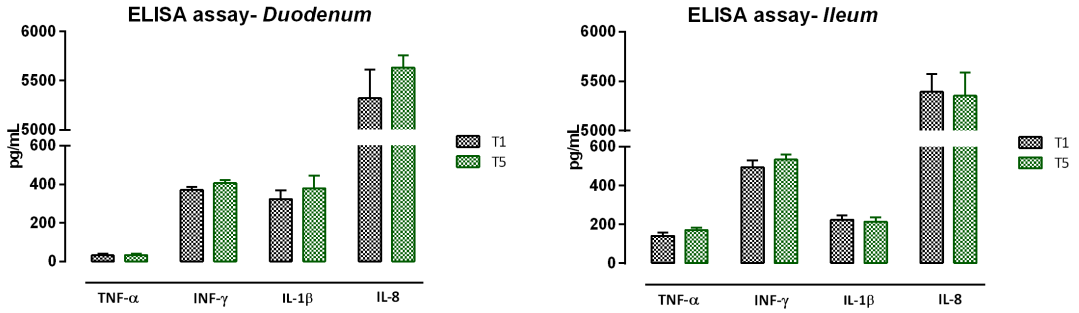

Figure 5. Protein expression of inflammatory cytokines in duodenal and ileal mucosa of piglets. Data are expressed as means (n = 8) and S.E.M. represented by vertical bars. Data refer to picograms of cytokine per 100 mg of tissue (pg/100 mg). T1 = basal diet; T5 = basal diet supplemented with 510 mg of thymol / kg feed (Vetagro SpA, Reggio Emilia, Italy). TNF-α = tumor necrosis factor-α; IFN-γ =interferon-γ; IL-1β = interleukin-1β; IL-8 = interleukin-8.

Discussion

Over the last 50 years, the ability of the ECS to modulate the inflammatory status has been investigated in human medicine, due to the pharmacological potential of cannabinoid therapy to treat clinical conditions such as IBD, Crohn's disease and ulcerative colitis (Ambrose and Simmons, 2019). Similarly, the gut chemosensory system also shows potential as a target for IBD, gluten sensitivity, and obesity (Depoortere, 2014). In this paper, we focused our attention on the presence of ECS and gut chemosensing markers in the duodenal and ileal mucosa of piglets, with a particular focus on both the receptors, namely, CB1, CB2, TRPV1, and OR1G1, and the main enzymes involved in the synthesis and degradation of AEA and 2-AG by performing a molecular analysis. To our knowledge, this paper is the first to describe the alleged roles of the ECS and gut chemosensing system on the intestinal functionality of swine and their possible modulation by thymol.

Interesting results were obtained from the analysis of gene expression of ECS markers, as we detected mRNA for all of these markers. Since it was first described in the central nervous system, the ECS is now proposed to regulate different physiological mechanisms. More

information is now available about this signaling system, but little evidence is available for its role in the organism. Recently, an increasing number of papers examining the role of the ECS in various pathologies has been published, but the possible roles of ECS in animal medicine, production, and nutrition have not been investigated in depth. Notably, the activation of CB1 receptors is involved in inflammation and cell death in different experimental models of disease (Ambrose and Simmons, 2019), but evidence for a possible role of nutrition in modulating this system has not been available until now. Interestingly, we detected the CB1 and CB2 mRNAs in mucosa scrapings, confirming the presence of these receptors in the mucosa of piglets and suggesting that these receptors are not exclusively localized in the ENS. The classically recognized location of the endocannabinoid receptors is in the ENS, and the only report analyzing their presence on the mucosa was performed in mice. In mouse, Sykaras and colleagues (2012) described the presence of the CB1 mRNA in the EEC, where it is suggested to drive the intake of fat-rich foods by inhibiting the release of cholecystokinin (CCK), which normally induces satiation after meals. In addition, CB1 activation inhibits acetylcholine (Ach) and is accompanied by a reduction in GI muscle contraction, resulting in decreased intragastric pressure and the inhibition of gastric emptying, pyloric contraction, and intestinal motility (Vera et al., 2017).

Cannabinoid receptor 1 also regulates food intake in rats, particularly during starvation periods, by increasing 2-AG synthesis through the activity of DGL (DiPatrizio et al., 2015, 2011), suggesting a possible role during weaning. Thymol appears to modulate the expression of these receptors and enzymes. The trend in the use of botanicals in animal feed has increased during the last two decades. Thymol is one of the most well-studied and frequently used molecules in animal nutrition due to its countless beneficial properties (Abd El-Hack et al., 2016). Considering the increase in the levels of the CB1 and CB2 mRNAs in both the duodenum and ileum, we postulated that thymol stimulates the synthesis of the receptors in the GI tract. Cannabinoid receptor 1 and cannabinoid receptor 2 are recognized to play a protective role in

IBD, namely, by functioning to control the inflammatory status and are normally present during weaning and starvation (DiPatrizio, 2016). Therefore, the ECS may play a role in the response to stressful situations, such as weaning for piglets, and thymol potentially exerts a positive effect by stimulating the expression of endocannabinoid receptors. Unfortunately, no studies examining this topic have been published to date, but the use of thymol may plausibly support piglets during weaning by activating the ECS.

Additionally, levels of the DGL and FAAH mRNAs were increased; as mentioned above, DGL is involved in the synthesis of 2-AG, while FAAH is responsible for AEA degradation. According to Bashashati and colleagues (2015), the inhibition of the degradation of 2-AG decreased whole-gut transit in mice; conversely, Izzo and colleagues (2009) concluded that a decrease in the small intestinal content of AEA leads to an increase in transit, suggesting that 2-AG modulates CB1-controlled gut contractility. Thymol may also play a role in modulating gut contractility by regulating enzyme expression and, consequently, the concentrations of the bioactive lipids, resulting in the modulation of gut contractility.

Other interesting data include the detection of mRNAs encoding receptors involved in the gut chemosensory system. Again, we not only detected the TRPV1 and OR1G1 mRNAs in piglet mucosa scrapings but also observed increased levels of the TRPV1 and OR1G1 mRNAs when the diet was supplemented with thymol. The colonic mucosa of both humans and rats express OR mRNAs, and luminal odorants induce serotonin secretion in isolated duodenal enterochromaffin cells and enterochromaffin cell lines (Braun et al., 2007). Moreover, the activation of OR1G1 by luminal thymol increases $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ may modulate Ca^{2+}-activated basolateral K^+ channels, providing a driving force for the exit of Cl^-/HCO_3^- (Kuwahara, 2015). As result, thymol activates certain types of the apical odorant receptor OR1G1 and stimulates Cl− secretion in colonic epithelial cells. Recently, human OR1G1 was reported to participate in glucose homeostasis during meal ingestion by inducing gut peptide secretion (Oh, 2018). A reasonable conclusion is that OR1G1 may play a role in controlling

intestinal permeability and nutrient absorption, although only a few studies examining the role of this OR are available, and, moreover, its presence in the swine GI tract is not well documented. Considering that in this study the mRNA of OR1G1 was detected in both duodenum and ileum, it is clear that this receptor, and probably other receptors of this class, are also present in the pig GI tract.

Thymol has also been linked to the immune system due to its anti-inflammatory and antioxidant properties (Nagoor Meeran et al., 2017). Omonijo et al. (2018) reported a reduction in LPS-induced ROS (reactive oxygen species) and TNF-α levels in cells pretreated with thymol. In the present study, piglets fed microencapsulated thymol exhibited increased levels of the TNF-α mRNA; this result might appear surprising because this cytokine has a proinflammatory function, but no adverse effects on the piglets' health were observed. Moreover, the level of the TNF-α protein was not altered by the treatments. Interestingly, at the dose where we observed an increase in the level of the TNF-α mRNA, we also registered an increase in the expression of the CB1 and CB2 mRNAs. The cannabinoid receptors act on TNF-α in two opposite directions. Stimulation of CB1 reduces TNF-α release from activated microglia (Rossi et al., 2011); conversely, CB2 is known to induce the expression of pro-inflammatory cytokines to promote a TH_1 immune response (Gertsch, 2008), which may explain the increase in the gene expression of this cytokine. Finally, thymol increases the levels of the CB1 and CB2 mRNAs, which controls the release and the expression of TNF-α, respectively, providing an explanation for the regulation of the protein and mRNA levels of this pro-inflammatory cytokine. Moreover, the absence of variations in terms of protein levels of inflammatory cytokines obtained with the ELISA assay is not unexpected, considering that all the piglets were healthy and, without a specific challenge or stressors, proinflammatory and anti-inflammatory cytokines are normally in equilibrium (Cicchese et al., 2018).

Concerning the zootechnical performance, in this study the administration of thymol to weaned piglets at different levels of inclusion did not modify any parameters. Thymol was well tolerated

by the animals also at the higher level of inclusion (10x the highest recommended dose in T5 group). This is an interesting result, mainly regarding FI and ADG, which are normally reduced when thymol is supplemented at a high dosage (Jugl-Chizzola et al., 2006; Michiels et al., 2010b; Trevisi et al., 2007). This high tolerability of thymol could be explained thanks to the microencapsulation of the terpene in a lipid matrix. It is well documented that encapsulation technologies are useful not only to deliver the bioactive compound along all the GI tract (Hébert et al., 1994; Petrujkić et al., 2013; Piva et al., 2007), but also to increase the palatability of some pungent molecules, such as thymol (Hultquist and Casper, 2016).

Despite the increasing number of studies analyzing the roles of ECS and chemosensation in the organism, additional studies are required to understand how endocannabinoid receptors and molecules control various pathologies and inflammation. Nevertheless, these results represent a first insight into the potential uses of botanical feed additives to control intestinal inflammation and motility. Further investigations are required to obtain a better understanding of the protein expression and localization of these markers, together with the possible mechanism by which thymol modulates the expression of endocannabinoid markers.

Conclusions

In conclusion, our data not only confirm the presence of the ECS and gut chemosensing markers in the duodenal and ileal mucosa of piglets but also suggest that thymol modulates the gene expression of these markers. Thymol increases the expression of the mRNAs encoding the CB1 and CB2 receptors both in the duodenum and ileum. This compound also modulates the mRNA levels of enzymes involved in the biosynthesis and degradation of endocannabinoid molecules. Moreover, the upregulation of OR1G1 and TRPV1 by thymol throughout the intestine implicates a possible role for these receptors as mediators of the effects of thymol as a feed additive on promoting gut health.

EXPERIMENTAL STUDY 4

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Cannabinoid and Cannabinoid-Related Receptors in the Myenteric Plexus of the Porcine Ileum

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Abstract

An important piece of evidence has shown that molecules acting on of cannabinoid receptors influence gastrointestinal motility and induce effects on gastrointestinal inflammation and visceral pain. The aim of this investigation was to immunohistochemically localize the distribution of canonical cannabinoid receptor 1 (CB1) and 2 (CB2) and the cannabinoid-related receptors transient potential vanilloid receptor 1 (TRPV1), transient potential ankyrin receptor 1 (TRPA1), and serotonin receptor 5-HT1a (5-HT1aR) in the myenteric plexus (MP) of the pig ileum. CB1, TRPV1, TRPA1, and 5-HT1aR were expressed, with different intensities, in the cytoplasm of MP neurons. For each receptor, the proportions of the immunoreactive neurons were evaluated using the anti-HuC/HuD antibody. Moreover, these receptors were also localized on nerve fibers (CB1, TRPA1), smooth muscle cells of *tunica muscularis* (CB1, 5-HT1aR), and endothelial cells of blood vessels (TRPV1, TRPA1, 5-HT1aR). Nerve varicosities were also found to to be immunoreactive for both for TRPV1 and 5-HT1aR. No immunoreactivity was documented for CB2. Cannabinoid and cannabinoid-related receptors herein investigated showed a wide distribution in the enteric neurons and nerve fibers of the pig

MP. These results could provide an anatomical basis for additional research, supporting the therapeutic use of cannabinoid receptor agonists in relieving motility disorders in porcine enteropathies.

Introduction

The endocannabinoid system (ECS) is constituted of three fundamental components: receptors, signaling molecules, and the enzymes responsible for ligand biosynthesis and degradation. It typically comprises the prototypical cannabinoid receptors 1 and 2 (CB1 and CB2), endocannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG), and the enzymes involved in their biosynthesis and degradation (Iannotti et al., 2016; Ligresti et al., 2016; Lu and Anderson, 2017; Stella, 2004). The ECS is typically localized at the central nervous system (CNS) level (Maccarrone et al., 2015). CB1 was proven to be the most widely expressed receptor protein from the G protein-coupled receptors (GPCRs) family in the brain, mainly in basal ganglia, hippocampus, olfactory bulb, and cerebellum (Mackie, 2005). In contrast, CB2 is mainly expressed in immune tissues such as microglia, leukocytes, and cells of macrophage lineage (Klegeris et al., 2003; Turcotte et al., 2016). The broad localization of the CB1 in the CNS represents a limit to its potential as a pharmacological target for CNS pathologies due to the undesired psychoactive side effects related to its activation from agonist and antagonist (Moreira et al., 2009). On the other hand, CB2 may constitute a promising pharmacological target for inflammatory disorder, thanks to its anti-inflammatory properties (Parlar et al., 2018). It has recently been clarified that the localization of the ECS is not limited only to the CNS since it was found ubiquitously expressed throughout the body, serving a multiplicity of physiological roles, including the regulation of gastrointestinal functions (DiPatrizio, 2016; Izzo and Sharkey, 2010). In particular, the ECS is supposed to regulate gastrointestinal secretion and motility via the enteric nervous system (ENS) (Vera et al., 2017). Various studies have suggested a possible implication of CB1 and CB2 in inflammatory bowel disease (IBD),

exerting a protective effect, thus suggesting the potential of pharmacological agents capable of targeting and modulating these pathways (Di Marzo and Izzo, 2006; DiPatrizio, 2016; Izzo, 2004). Moreover, additional cannabinoid-related receptors and endocannabinoid-like molecules may also be involved. In particular, among the secondary receptors belonging to the ECS are found GPCRs, transient receptors potential (TRPs) channel, serotonin (5-HT) receptors, and nuclear peroxisome proliferator-activated receptors (PPARs) (Morales et al., 2017). In particular, TRPs channels are sensitive to harmful stimuli, pungent compounds, acid, temperature, and inflammation mediators, qualifying these receptors as being suitable candidates and novel targets for gastrointestinal pain (Yu et al., 2016).

Concerning the endocannabinoid-like mediators, growing interest is driven by palmitoylethanolamide (PEA) and cannabidiol (CBD) (Iannotti et al., 2016; Kreitzer and Stella, 2009). Growing research regarding this topic indicates that activation of cannabinoid and cannabinoid-related receptors, mediated by endogenous or plant-derived cannabinoids, may influence gastrointestinal tract (GIT) motility and secretion, with a reduction of inflammation and visceral pain (Carlo and Izzo, 2003; Duncan et al., 2008, 2005; Hornby and Prouty, 2004; Izzo, 2004; Storr and Sharkey, 2007; Wright et al., 2008).

To the authors' knowledge, only a few studies have described the presence of the ECS in the porcine GIT, limited to the mucosa (Toschi et al., 2020) or, in the ENS, only to CB1 (Kulkarni-Narla and Brown, 2000). For this reason, the aim of this study was to immunohistochemically characterize the distribution of the canonical cannabinoid receptors CB1 and CB2, and the cannabinoid-related receptors TRP vanilloid 1 (TRPV1), TRP ankyrin 1 (TRPA1), and 5-HT1a serotonin receptor (5-HT1aR) in the myenteric plexus (MP) of the pig ileum.

Materials and Methods

Animals

Intestinal tissues were collected from six pigs at the slaughterhouse. All animals were 7-month-old genetic hybrids (Landrace × Large White). The animals did not have a history of gastrointestinal disorders and did not show gross alterations of the gastrointestinal wall. Italian legislation (D. Lgs. n. 26/2014), according to the Directive 2010/63/EU of the European Parliament and of the Council of 22/09/2010 regarding the protection of animals used for scientific purposes, does not require any approval by the appropriate authorities or ethics committees since this research did not influence any therapeutic decisions.

Tissue collection

The ileum was harvested within 30 minutes from the animals' deaths and was longitudinally opened along the mesenteric border. The tissues were then washed in phosphate-buffered saline (PBS), fixed, and processed to obtain longitudinal (2.0 cm x 0.5 cm) and tangential cryosections (2.0 cm x 1.0 cm), which were later processed for immunohistochemistry, as previously described (Chiocchetti et al., 2015).

Immunofluorescence

After hydration in PBS, the cryosections were processed for immunostaining. To prevent non-specific bindings, the cryosections were incubated in a solution containing 20% normal donkey serum (Colorado Serum Co., Denver, CO, USA), 0.5% Triton X-100 (Sigma Aldrich, Milan, Italy, Europe), and bovine serum albumin (1%) in PBS for 1 h at room temperature (RT). The cryosections were then incubated in a humid chamber overnight at RT, and single or double immunostaining was carried out. In the single immunostaining, the cryosections were incubated with only one of the primary antibodies (Table 1) directed against the cannabinoid and cannabinoid-related receptors. For double immunostaining, the cryosections were incubated

with a cocktail of primary antibodies (Table 1). Since double immunostaining was carried out to identify the enteric neurons, the cryosections were co-incubated with one of the anti-cannabinoid receptors or anti-cannabinoid-related antibodies and the anti-HuC/HuD antibody. All the primary antibodies were diluted in 1.8% NaCl in 0.01 M PBS containing 0.1% sodium azide. After washing the sections in PBS (3 x 10 min), they were incubated for 1 h at RT in a humid chamber with the secondary antibodies (Table 2) diluted in PBS. The cryosections were then washed in PBS (3 x 10 min) and mounted in buffered glycerol at pH 8.6 with 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, CA, USA).

Table 1. Primary antibodies used in the study.

Primary antibodies	Host	Code	Diluition	Source
CB1	Rabbit	ab23703	1:100	Abcam
CB2	Rabbit	ab45942	1:200	Abcam
CB2	Mouse	sc-293188	1:50	Santa Cruz
TRPV1	Rabbit	ACC-030	1:200	Alomone
TRPA1	Rabbit	ab58844	1:100	Abcam
5-HT1a	Rabbit	ab85615	1:100	Abcam
HuC/HuD	Mouse	A21271	1:200	Life Technologies

Table 2. Secondary antibodies used in the study.

Primary antibodies	Host	Code	Dilution	Source
Anti-rabbit 488	Donkey	A21206	1:1000	Thermofisher
Anti-rabbit 594	Donkey	ab150076	1:1000	Abcam
Anti-mouse 594	Donkey	A21203	1:500	Thermofisher
Anti-mouse 488	Donkey	A21202	1:500	Thermofisher

The proportion of neurons that were HuC/HuD immunoreactive and that were also immunoreactive for CB1R, CB2R, TRPV1, TRPA1, and 5-HT1aR was determined by examining fluorescently labelled, double-stained preparations. The neurons were first identified by the presence of a fluorophore labeling one antigen (HuC/HuD), and the microscope filter was subsequently switched to determine whether or not the neuron expressed a second antigen

(CB1R, CB2R, TRPV1, TRPA1, and 5-HT1aR), identified with a different-colored fluorophore. In doing so, the proportion of neurons labeled for pairs of antigens was determined. A minimum of one hundred HuC/HuD immunoreactive MP neurons were counted for each marker expressed by nerve cell bodies. Data were collected from preparations obtained from at least three animals ($n \geq 3$). The percentages of immunopositive neurons were expressed as mean ± standard deviation.

Specificity of the primary antibodies

CB1, the synthetic peptide MSVSTDTSAEAL, corresponding to carboxy-terminal amino acids 461-472 of human CB1, was used as immunogen to obtain the anti-CB1 antibody. The homology between the full amino acid sequences of pig (F1S0E6) and human (P21554) CB1 was 97.9% (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the correspondence with the specific sequence of the immunogen was 100%. Therefore, the antibody anti-CB1 should also recognize the same receptor in pig. Since this antibody is human specific, it was applied on a submucosal wholemount preparation of human descending colon as a positive control, having previously obtained donor consent. The wholemount preparation was prepared and analyzed using pre-validated immunohistochemical protocols (Giancola et al., 2017).

CB2, the synthetic peptide conjugated to keyhole limpet hemocyanin (KLH) derived from within residues 200–300 of rat CB2, was used as an immunogen to obtain the rabbit ant-CB2 antibody (ab45942). The homology between the full amino acid sequences of pig (I3LUS5) and rat CB2 (Q9QZN9) was 76.3%; the correspondence with the specific sequence of the immunogen was 76%. The amino acid sequence 302–360 of CB2 of human origin (P34972) was used as an immunogen to obtain the mouse anti-CB2 antibody (sc-293188). The homology between the full amino acid sequences of pig and human CB2 was 81.9%.

TRPV1, the peptide (C)EDAEVFK DSMVPGEK, corresponding to residues 824–838 of rat TRPV1, was used as an immunogen to obtain the anti-TRPV1 antibody. The homology between the full amino acid sequences of pig (A0A4X1UCR0) and rat (O35433) TRPV1 was 84.52% (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the correspondence with the specific sequence of the immunogen was 93%. However, we tested this antibody on porcine nervous system (dorsal root ganglia) by Western blot (Wb) analysis (Russo et al., 2013).

TRPA1, the synthetic peptide CEKQHELIKLIIQKME, corresponding to amino acids 1060-1075 of rat TRPA1, was used as an immugen to obtain anti-TRPV1 antibody. The alignment of the immunogen sequence with the target protein in the pig was 93% (https://blast.ncbi.nlm.nih.gov/Blast.cgi). It is plausible that the commercially available antibody anti-rat TRPA1 should also recognize the same receptor in the pig.

5-HT1aR, the synthetic peptide corresponding to amino acids 100-200 (conjugated to keyhole limpet hemocyanin) of rat 5-HT1aR, was used as an immunogen to obtain the anti-5-HT1aR antibody. The alignment of the immunogen with the target protein sequence in the pig is 100% (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Therefore, the antibody anti-rat 5-HT1aR should also recognize the same receptor in pig.

The suppliers of the anti-CB2R, -TRPV1, -TRPA1, and -5-HT1aR antibodies employed in the present study stated that they were rat specific; thus, for comparison purposes, the anti-CB2R and -TRPV1 antibodies were applied on the positive control tissues, in particular on wholemount preparations of rat ileum (authorization no. 112/2018-PR of 12 February 2018). Data related to the anti-TRPA1 and 5-HT1aR antibodies have recently been published (Stanzani et al., 2020).

Specificity of the secondary antibodies

The specificity of the secondary antibodies (Table 2) was tested by the absence of signal after the exclusion of the primary antibody on pig intestinal tissues.

Fluorescence microscopy

Cryosections and wholemount preparations were examined by the same observer (Dr. R. Chiocchetti) using a Nikon Eclipse Ni microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) equipped with the appropriate filter cubes to differentiate the fluorochromes used. The images were recorded using a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Enteric neuron counts were carried out at 40× magnification. Slight adjustments to contrast and brightness were made using Corel Photo Paint whereas the figure panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, ON, Canada).

Results

CB1 immunoreactivity

Weak-to-moderate granular and diffuse CB1 immunoreactivity (CB1-IR) was expressed by the cytoplasm of the MP neurons; the brightest CB1-IR neurons showed large dimensions and smooth outline (Figure 1 a-c). The percentages of HuC/HuD immunoreactive neurons co-expressing CB1-IR was 57±19% (377/713 cells counted, n = 5). Nerve fibers within the MP ganglia, distributed in the interganglionic strands and scattered within the muscular layers, showed weak CB1-IR. Weak CB1-IR was also observed in smooth muscle cells of the *tunica muscularis* (longitudinal muscle layer, LML>circular muscle layer, CML) (data not shown).

Moderate CB1-IR was expressed by the cytoplasm of submucosal plexus neurons of the human colon (Figure S1).

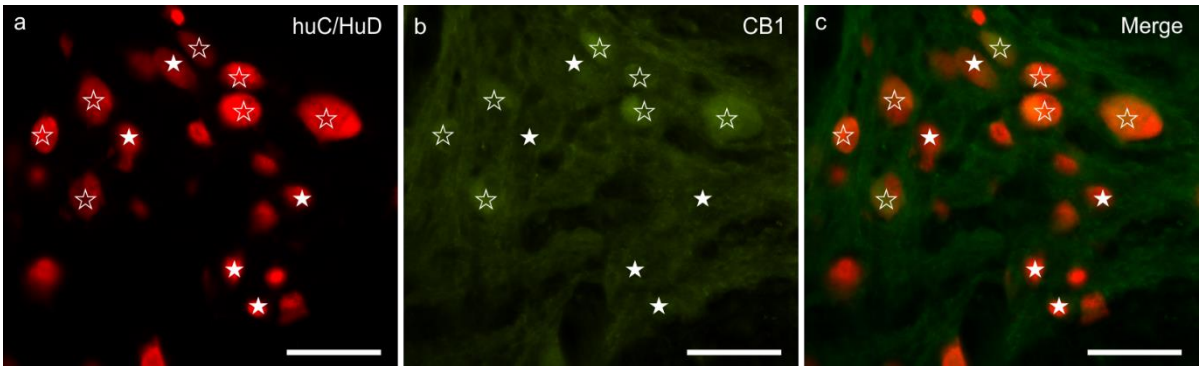

Figure 1. CB1 receptor immunoreactivity in the myenteric plexus of pig ileum: (**a)** HuC/HuD immunoreactive neurons, (**b**) CB1 receptor immunoreactivity, (**c**) merge image. The open stars indicate HuC/HuD immunoreactive neurons co-expressing weak-to-moderate CB1 immunoreactivity. The white stars indicate HuC/HuD immunoreactive neurons, which were CB1 negative. Scale bar: 50 µm.

CB2 immunoreactivity

No immunolabelings were observed in the porcine MP with either of the anti-CB2 antibodies. In the rat ileum, MP neurons expressed weak-to-moderate CB2-IR (Figure S2).

TRPV1 immunoreactivity

Moderate-to-bright granular TRPV1-IR was expressed by the cytoplasm of the majority of MP neurons (71±14%; 462/602 cells counted, n = 5). The TRPV1 immunolabelling, which was mainly confined to the cell bodies of neurons showing smooth outline, was more intense in neurons of large dimensions (Figure 2 a-c). However, TRPV1 immunoreactive nerve processes arising from large neurons were also visible (Figure 2d-f). Few TRPV1 immunoreactive nerve fibers were seen in the MP ganglia, in the interganglionic nerve strands and within the muscular layers; nevertheless, bright and small TRPV1 immunoreactive varicosities were seen in the neuropil of the ganglia and around some MP neurons. In one subject, TRPV1 was also brightly expressed by the enteric glial cells (Figure 2g–i). Moderate TRPV1-IR was expressed by the endothelial cells of thin blood vessels (capillaries) distributed in the tunica muscularis (data not shown). In the rat ileum, MP and enteric glial cells (EGCs) expressed TRPV1-IR (EGCs > neurons) (Figure S3).

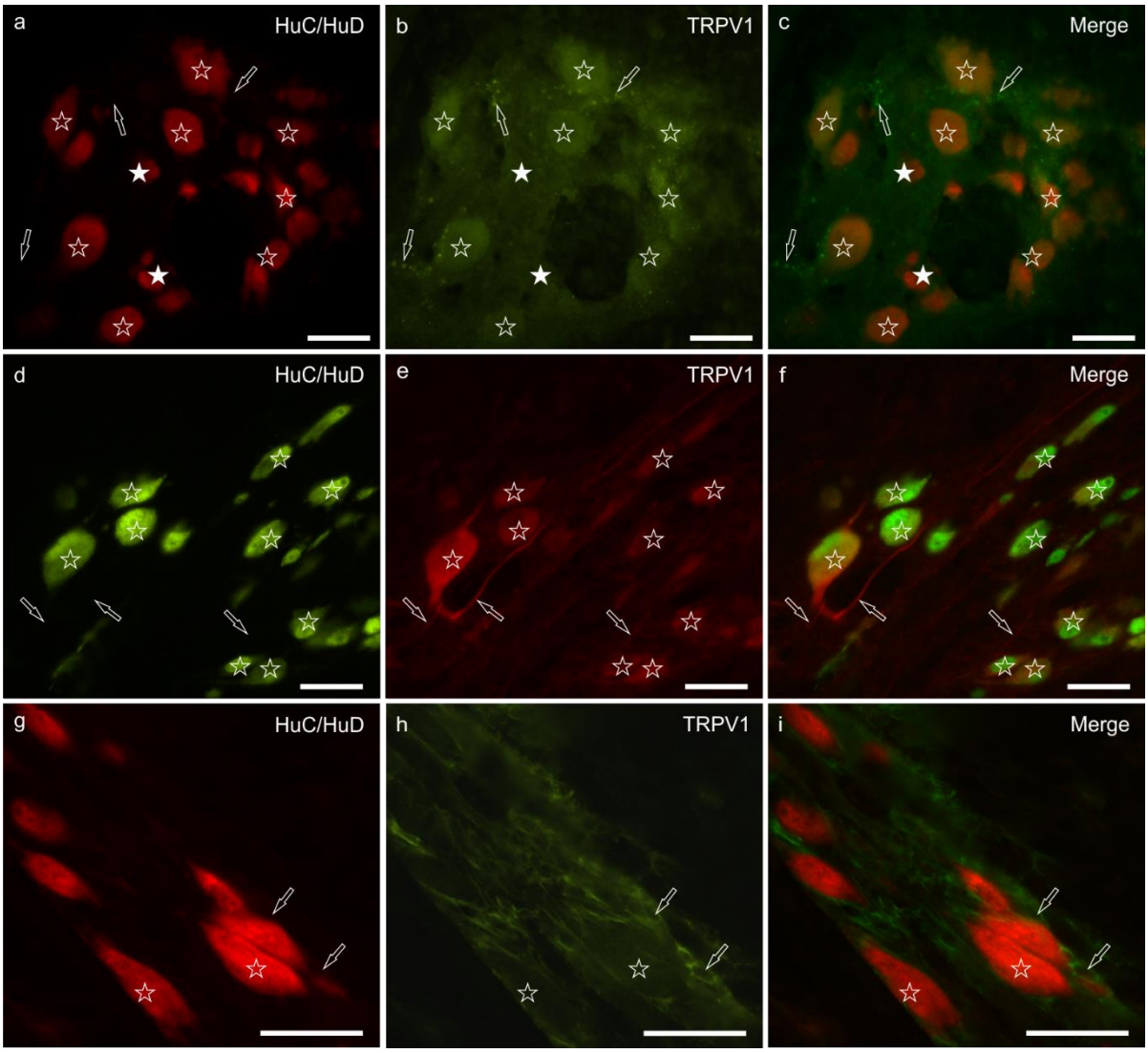

Figure 2. TRPV1 immunoreactivity in the myenteric plexus of the pig ileum: (**a**) HuC/HuD immunoreactive neurons, (**b**) TRPV1 immunoreactivity, (**c**) merge image. The open stars indicate the HuC/HuD immunoreactive neurons co-expressing moderate TRPV1 immunoreactivity. The white stars indicate HuC/HuD immunoreactive neurons, which were TRPV1 negative. The arrows indicate the TRPV1 immunoreactive varicosities encircling the neuronal cell bodies. (**d**) HuC/HuD immunoreactive neurons, (**e**) TRPV1 immunoreactivity, (**f**) merge image. The stars indicate the HuC/HuD immunoreactive neurons co-expressing moderate-to-bright TRPV1 immunoreactivity. The arrows indicate TRPV1 immunoreactive neuronal processes. (**g**) HuC/HuD immunoreactive neurons, (**h**) TRPV1 immunoreactivity, (**i**) merge image. The stars indicate two HuC/HuD immunoreactive myenteric plexus neurons co-expressing weak and diffuse TRPV1 immunoreactivity; the arrows indicate two perineuronal enteric glial cells expressing bright TRPV1 immunoreactivity. Scale bar: 50 µm.

TRPA1 immunoreactivity

Diffuse and moderate cytoplasmic TRPA1-IR was showed by a large percentage of MP neurons (66±23%; 336/527 cells counted, n = 5) and was brighter in the cytoplasm of large neurons

(Figure 3 a-c). Nerve fibers within the ganglia and those distributed along the nerve strands and musculature showed moderate TRPA1-IR (data not shown). Bright TRPA1-IR was displayed by the endothelial cells of blood vessels (Figure 3 d-f).

TRPA1 immunoreactivity was also expressed by MP neurons in the ileum of a control rat (Stanzani et al., 2020).

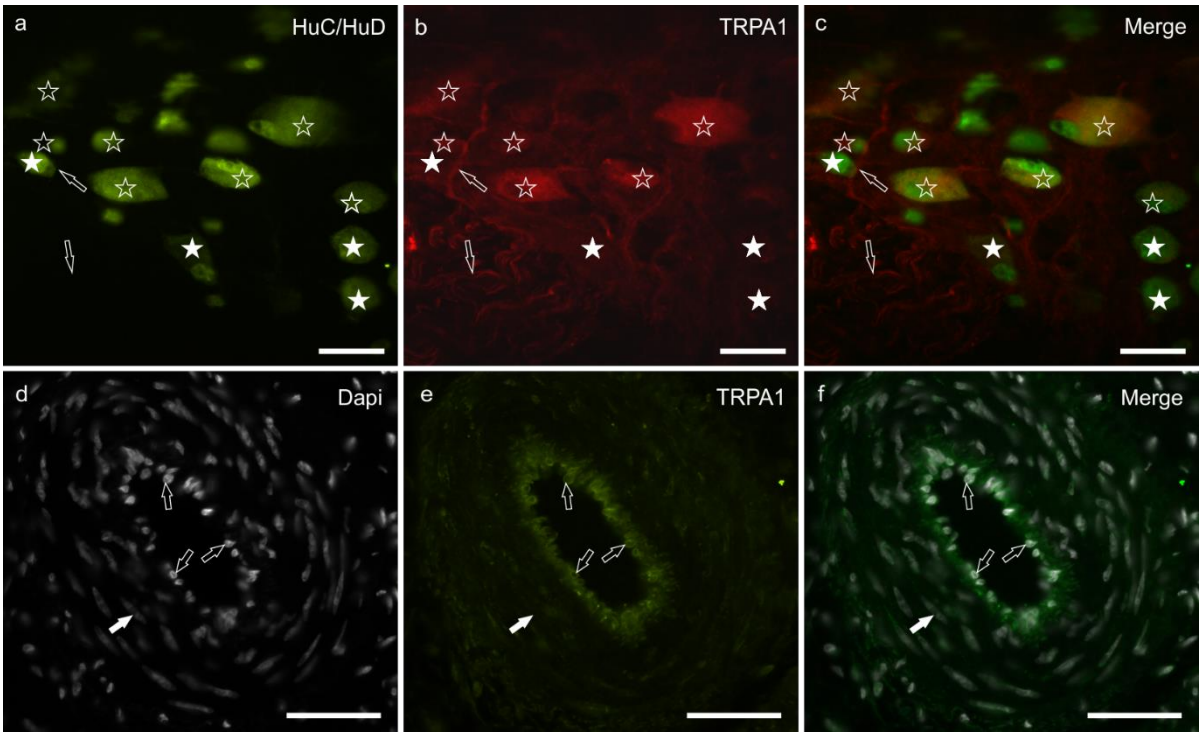

Figure 3. TRPA1 immunoreactivity in the myenteric plexus and blood vessel of *tunica muscularis* of pig ileum: (**a**) HuC/HuD immunoreactive neurons, (**b**) TRPA1 immunoreactivity, (**c**) merge image. The open stars indicate HuC/HuD immunoreactive neurons co-expresseing moderate-to-bright TRPA1 immunoreactivity. The white stars indicate HuC(HuD immunoreactive neurons, which were TRPA1 negative. The arrows indicate a TRPA1 immunoreactive neuronal process. (**d)** Dapi stained nuclei of endothelial cells and vascular smooth muscle cells, (**e**) TRPA1 immunoreactivity, (**f**) merge image. The open arrows indicate the Dapi stained nuclei of endothelial cells expressing bright TRPA1 immunoreactivity. The white arrow indicates the elongated Dapi stained nucleus of one smooth muscle cell of the arterial *tunica media.* Scale bar: 50 µm.

5-HT1aR immunoreactivity

Weak and diffuse 5-HT1aR immunolabelling was expressed by approximately half the MP neurons (51±6%; 345/682 cells counted, n = 5) (Figure 4 a-c). In general, neurons of large dimensions showed brighter immunofluorescence. The 5-HT1aR-IR was expressed by nerve

varicosities. Weak 5-HT1aR-IR was displayed by the smooth muscle cells of the blood vessels and the tunica muscularis (data not shown).

5-HT1aR was expressed by MP neurons also in the rat ileum (Stanzani et al., 2020).

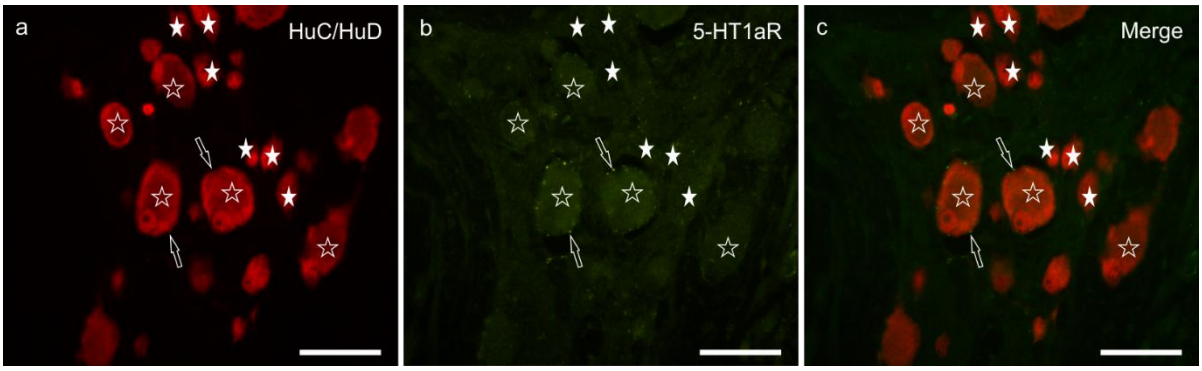

Figure 4. 5-HT1a receptor immunoreactivity in the myenteric plexus of pig ileum: (**a**) HuC/HuD immunoreactive neurons, (**b**) 5-HT1a receptor immunoreactivity, (**c**) merge image. The open stars indicate HuC/HuD immunoreactive neurons co-expressing weak 5-HT1a receptor immunoreactivity. The white stars indicate HuC/HuD immunoreactive neurons, which were 5-HT1a receptor negative. The arrows indicate 5-HT1a receptor immunoreactive varicosities encircling the neuronal cell bodies. Scale bar: 50 μm.

The results of the cellular distribution and intensity of the immunolabeling in the pig ileum are summarized in semiquantitative Table 3.

Table 3. Semiquantitative evaluation of the density of CB1, CB2, TRPV1, TRPA1, and 5-HT1aR immunoreactivity in different cellular elements (myenteric plexus neurons, nerve fibres, enteric glial cells, *tunica muscularis*, blood vessels) of the pig ileum.

Receptors	**MP neurons**	**Nerve fibres**	**EGCs**	***Tunica muscularis***	**Blood vessels**
CB1	C+/++	+	-	+	-
CB2	-	-	-	-	-
TRPV1	++/+++	+/++	-/+++	-	E++
TRPA1	C++	++	-	-	E+++
5-HT1aR	C+	-	-	+	SMC+

The immunoreactive cells were graded as: –, negative; +, weakly stained; ++, moderately stained; +++, brightly stained. C: cytoplasmic; E: endothelium; EGCs: enteric glial cells; M: membrane; MP: myenteric plexus; SMCs: vascular smooth muscle cells.

Discussion

In the GIT, cannabinoid receptors regulate motility, secretion, emesis, food intake, and inflammation (DiPatrizio, 2016; Duncan et al., 2005; Izzo, 2004; Storr and Sharkey, 2007). In this paper, the authors focused their attention on the presence of the ECS in the ileal MP of pigs, with particular emphasis on both the cannabinoid receptors, namely CB1R and CB2R, and the cannabinoid-related receptors TRPV1, TRPA1, and 5-HT1aR by carrying out immunohistochemical analysis.

The observation of CB1 in the MP neurons and nerve fibers in the porcine ileum is consistent with the findings of Kulkarni-Narla and Brown (2000). The expression of CB1-IR in enteric neurons has been observed in many other species, such as rodents, ferrets, dogs, cats, and humans (Coutts et al., 2002; Galiazzo et al., 2018; Stanzani et al., 2020; Van Sickle et al., 2001; Wright et al., 2005). Previous studies regarding various species, including pigs, have indicated that CB1 immunoreactive neurons show cholinergic phenotype, exerting an inhibitory effect on MP cholinergic neurotransmission (Coutts and Pertwee, 1997; Donnerer and Liebmann, 2018; Izzo et al., 2000; Kulkarni-Narla and Brown, 2000; Storr et al., 2010).

The evaluation of the proportion of myenteric CB1 immunoreactive neurons of the pig is not comparable with other data available in the literature. However, the percentage of CB1-IR neurons (57±19%) observed in the present study was similar to the percentage of cholinergic neurons observed in the guinea pig (approximately 80%; (Furness, 2006)), but was greater than the proportions of ChAT-IR neurons counted in other species such as horse (64%; (Chiocchetti et al., 2009)), sheep (62%; (Mazzuoli et al., 2007)), or pig (58%; (Brehmer et al., 2004)). In effect, Brehmer and colleagues (2004) observed that there was a subclass of enteric cholinergic neurons, which could be identified only by the use of the antibody directed against the peripheral form of ChAT (i.e. pChAT). Thus, the percentage of cholinergic neurons of the pig ileum should be greater than that identified with the only antibody anti-ChAT. However, the great percentage of CB1 immunoreactive neurons observed in the present study indicated that

this receptor might also be expressed by other neuronal subpopulations as well as by the cholinergic neurons. The influence on intestinal motility and contractility mediated by cannabinoids might be confirmed by the presence of CB1 on smooth muscle cells, suggesting a direct muscular mechanism of cannabinoids (Donnerer and Liebmann, 2018).

CB2 is mainly expressed in immune tissues and cells of macrophage lineage (Turcotte et al., 2016). The lack of immunoreactivity to the CB2R in enteric neurons is in line with the results obtained in the MP of dogs and cats (Galiazzo et al., 2018; Stanzani et al., 2020), in contrast with rats (present study) and mice (Duncan et al., 2008). In the pig ileum, the lack of results could also depend on the low homology between the full amino acid sequences of pig and rat CB2 (76%) and pig and human (81.9%), original immunogens of the antibodies employed in the present study.

TRPV1 is a non-selective cation channel expressed by peptidergic and non-peptidergic nociceptors in rodents and large mammals (Russo et al., 2013; Tominaga et al., 1998; Zwick et al., 2002). TRPV1-IR was observed in MP neurons of pig ileum, according to Poonyachoti et al. (2002), who indicated that the majority of TRPV1 immunoreactive neurons were cholinergic. The expression of TRPV1-IR in the intramural neurons is a matter of debate. However, there are publications supporting the expression of TRPV1 immunolabelling in intramural neurons of different mammalian species, as has been shown in the present study (Anavi-Goffer et al., 2002; Coutts et al., 2002; Filippova et al., 2018; Kulkarni-Narla and Brown, 2000;). In addition, functional studies have supported the existence of enteric TRPV1 immunoreactive neurons in mice (Penuelas et al., 2007).

The use of different anti-TRPV1 antibodies seems to be the reason for the discrepancy in the expression of TRPV1. In fact, Buckinx and colleagues (2013) found that different distribution patterns of TRPV1 in the ENS were due to the antibodies discriminating between different modulated forms of TRPV1, which influence the recognition of intracellular forms of TRPV1.

The large percentages of CB1 and TRPV1 immunoreactive neurons, which we found in the porcine MP ileum, allowed us to speculate that CB1 and TRPV1 may co-exist on the same subclass of cholinergic neurons as substantiated by functional and immunohistochemical studies (Bashashati et al., 2017; Coutts et al., 2002). Double labeling of CB1R with TRPV1 (and the other receptors) was not examined due to incompatibility of the species in which the antisera were raised.

The expression of TRPV1 by enteric neurons could constitute a target for the development of new therapies against nociceptive and inflammatory intestinal stimuli (Poonyachoti et al., 2002). Moreover, TRPV1 seems involved in the protection against pathogenic bacteria such as *Salmonella enterica*, releasing calcitonin gene related peptide (CGRP), which regulates the number of microfold (M) cells and the levels of segmented filamentous bacteria that fight pathogens colonization (Lai et al., 2020).

The expression of TRPV1-IR in EGCs might be involved in their differentiation/maturation as suggested by Yamamoto et al. (2016) or might be upregulated in different conditions of the gut homeostasis/physiology as observed in the porcine tissues. The localization of TRPV1 on endothelial cells of capillaries in the *tunica muscularis* suggested a modulation of vasoconstriction and vasorelaxation in an endothelium-dependent manner, supporting the therapeutic potential of TRPV1 as a target for improving vascular functionality (Ives et al., 2017).

TRPA1 has been successfully found in the GIT neuronal (Penuelas et al., 2007; Poole et al., 2011; Stanzani et al., 2020) and non-neuronal cells (Cho et al., 2014; Kaji et al., 2011; Stanzani et al., 2020), in which the receptor can detect specific food chemicals such as cinnamaldehyde, allyl isothiocyanate (AITC), allicin, and thymol (Kurganov et al., 2014; Lee et al., 2008). In the present study, TRPA1 was observed in enteric neurons as described in the MP neurons of rodents (Poole et al., 2011; Stanzani et al., 2020). Moreover, functional investigations have indicated that TRPA1 may regulate gastrointestinal motility through 5-HT release from

enterochromaffin cells (Nozawa et al., 2009). However, the localization of TRPA1 in enteric neurons indicated that it can directly modulate intestinal contraction/motility, as suggested by Sandor et al. (Sandor et al., 2019). In addition, localization on endothelial cells of blood vessels suggests a role in the controls of vasodilatation and vasoconstriction, as for TRPV1. The ability of botanicals to act on TRPA1 in GIT seemed to modulate the majority of its functionality. In fact, AITC modulated gastrointestinal contractions in mice (Capasso et al., 2012; Penuelas et al., 2007), guinea pig (Nozawa et al., 2009), and dogs (Doihara et al., 2009), and inhibits colonic transit (Poole et al., 2011) via TRPA1 activation. Moreover, AITC was also capable of inducing blood vessel dilatation due to the activation of TRPA1, as reported by Earley et al. (2012; 2009) and Sullivan et al. (2015). It is possible to assume that the use of botanicals capable of modulating TRPA1 and/or TRPV1 could also play a role in reducing or controlling also inflammatory stimuli. In fact, Blackshaw et al. (2011) suggested that TRPA1-IR expressed by intramural neurons might not contribute to normal ENS functions, exerting its role only during inflammation or injury, or in responses to exogenous agonism.

In the porcine ENS, 5-HT has been found in MP neurons of the pig colon (Barbiers et al., 1995) and perineuronal varicosities of the ileum (Cornelissen et al., 2001). Given the variety and the complexity of the effects that 5-HT exerts in the gut, it is not surprising that there is more than one type of enteric neuronal 5-HT receptor (Gershon, 2004). Many of the effects of CBD are mediated through 5-HT receptor activation in the CNS and peripheral nervous system, which regulate neuronal excitability and neurotransmitter release. Of the 5-HT receptors, CBD acts as an agonist on the 5-HT1aR, as a partial agonist on the 5-HT2aR, and as an antagonist on the 5-HT3R (Ligresti et al., 2016).

In the present study, 5-HT1aR-IR was observed in MP neurons and smooth muscle cells of the *tunica muscularis*, in line with Youn et al. (2015) and Delesalle et al. (2008), who observed 5-HT1aR-IR in MP neurons and *tunica muscularis* of the guinea pig stomach and muscular smooth muscle cells of the equine jejunum, respectively. Electrophysiological studies have

indicated that 5-HT1aR is primarily involved in presynaptic inhibition of transmitter release (Johnson et al., 1980). The location of 5-HT1aR-IR in varicosities around MP neurons observed in the present study supported the idea that the receptor might be involved in 5-HT mediated inhibition of cholinergic neurotransmission. The expression of 5-HT1aR-IR in approximately 50% of MP neurons also suggested that some of the cells able to produce 5-HT1aR were cholinergic. The expression of 5-HT1aR in the vascular smooth muscle cells of pig ileum observed in the present study may support the direct action of 5-HT on vascular smooth muscle. In pigs, direct vascular smooth muscle relaxation may be the predominant mechanism involved in the vasodilatation action of serotonin (Mylecharane, 1990).

The localization of cannabinoid and cannabinoid-related receptors in the MP of pigs implied a possible role of phytocannabinoids and botanicals in the control and support of various gastrointestinal activity. For example, CBD has been found to act as an agonist on 5-HT1aR, exerting a neuroprotective effect by modulating oxidative stress and inflammation (Pazos et al., 2013). Thymol also seemed to be capable of modulating the expression of the ECS in the porcine GIT (Toschi et al., 2020), representing a therapeutic approach to several gastrointestinal diseases. Additional investigations are required to obtain a better understanding of the localization of the ECS receptors in the GIT of pigs including other tracts, receptors, and phenotype of enteric neurons in an attempt to overcome the limitations posed by the absence of specific antibodies for pigs.

Conclusions

In conclusion, the data in the present study highlighted the expression of cannabinoid (CB1R and CB2R) and cannabinoid-related receptors (TRPV1, TRPA1, and 5-HT1aR) not only in the MP neurons and enteric glial cells, but also on the smooth muscle cells and the blood vessels of the porcine ileum. These morphological findings could be of particular relevance for future functional, pre-clinical, and clinical studies assessing the effects of cannabinoids in pigs in order

to manage the hypermotility associated with gastrointestinal inflammatory diseases and pain. In fact, this could justify the use of phytocannabinoids or natural molecules capable of modulating the ECS in the diet of pigs. By modulating the activation of cannabinoid and cannabinoid-related receptors, it seems possible to regulate gastrointestinal functionality at different levels. Of particular interest, TRPV1 can interfere with pathogen proliferation and, together with TRPA1, could play a role in reducing the inflammation that occurs during weaning.

Supplementary materials

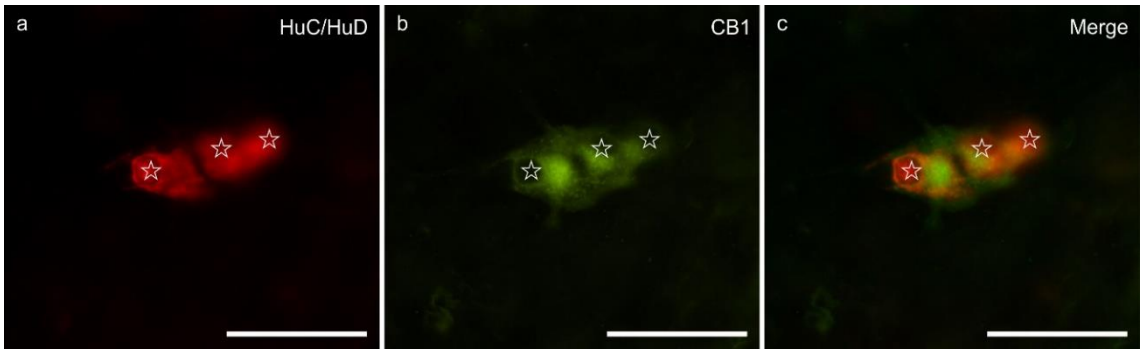

Figure S1. CB1 receptor immunoreactivity in the submucosal plexus of the human colon: (**a**) HuC/HuD immunoreactive neurons, (**b**) CB1 receptor immunoreactivity, (**c**) merge image. Stars indicate three HuC/HuD immunoreactive neurons, which co-expressed CB1 receptor immunoreactivity. Scale bar: 50 µm

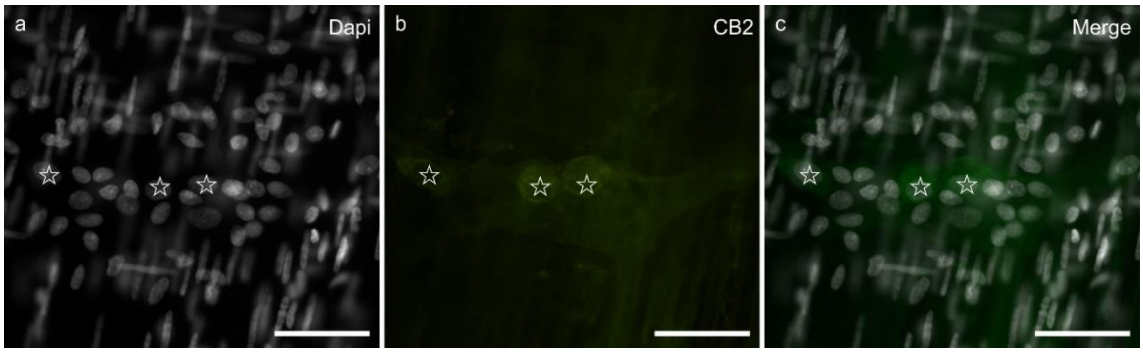

Figure S2. CB2 receptor immunoreactivity in the myenteric plexus neurons of the rat ileum: (a) Dapi stained neuronal nuclei of neurons, (**b**) CB2 receptor immunoreactivity, (**c**) merge image. Stars indicate the Dapi stained neuronal nuclei of three neurons, which co-expressed CB2 receptor immunoreactivity. Scale bar: 50 µm

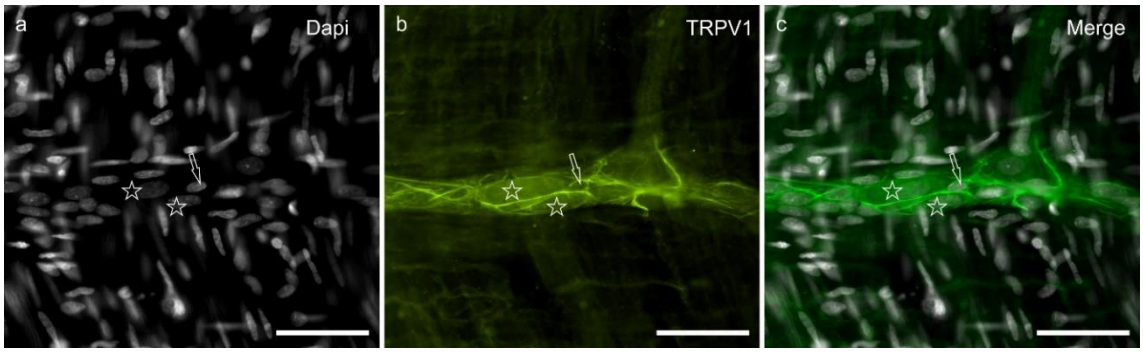

Figure S3. TRPV1 immunoreactivity in the myenteric plexus of the rat ileum: (a) Dapi stained neuronal nuclei of neurons, (**b**) TRPV1 immunoreactivity, (**c**) merge image. Stars indicate the Dapi stained neuronal nuclei of two neurons, which co-expressed weak TRPV1 immunoreactivity. The arrow indicates the nucleus of one enteric glial cell expressing bright TRPV1 immunoreactivity. Scale bar: 50 µm

ABSTRACTS

This project was presented as oral presentation at the Poultry Science Association 2018 Annual Meeting, San Antonio (TX), 23th–26th July 2018. Modified from:

Organic Acids and Nature-Identical Compounds increase the susceptibility of *E. coli* and *S. enteritidis* to broad spectrum antibiotics

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Abstract

Background: *Escherichia coli* and *Salmonella enteritidis* are gram-negative foodborne pathogens, both responsible for severe pathologies associated with poultry production and industry. Avian colibacillosis is responsible for a significant proportion of the mortality found in a poultry flock. On the other hand, *Salmonella enteritidis* is the causative agent of salmonellosis, which causes around 93.8 million illnesses and 155’000 deaths each year worldwide (Kabir, 2010). Both *Escherichia coli* and *Salmonella enteritidis* can penetrate the eggshell, with consequent food poisoning in humans caused by contaminated eggs or egg products. Nowadays, it is crucial to use alternatives to antibiotics (ABs) in the control of these pathogens.

The aim of this study was to assess the ability of nature-identical compounds (NICs) or organic acids (OAs) to increase the sensitivity of *Escherichia coli* and *Salmonella enteritidis* to broad spectrum ABs.

Materials and Methods: The ABs used in this study were amoxicillin and neomycin, the stock solutions were prepared in Brain Heart Infusion (BHI). The selected OAs and NICs were respectively sorbic acid and benzoic acid (stocks prepared in BHI), and thymol and carvacrol (stocks prepared in BHI supplemented with ethanol at a final concentration ≤ 3.5% to increase solubility). Each solution was buffered to ensure a final pH of 6.5, filter-sterilized, and diluted in sterile BHI to reach the final concentration tested. The chosen bacterial strains were one strain of *Escherichia coli* and one strain of *Salmonella enteritidis*, both isolated from broiler livers.

The minimal inhibitory concentration (MIC) of ABs, OAs, and NICs was determined using the microdilution method in 96-well microtiter plates with 10^5 cfu/mL. The plates were incubated for 24 h at 37 °C in aerobic conditions. Bacteria were tested against a wide range of concentrations of all the selected compounds: 0.5-128 mg/L of ABs, 1.56–100 mM for OAs, and 0.06–7.5 mM for NICs. Subsequently, bacteria were also tested against a combination of the same range of OAs and NICs with the lowest efficient dose of AB.

After incubation, the growth inhibition was evaluated by absorbance measurement at 630 nm. The MIC value was defined as the lowest concentration of each compound capable to zero the absorbance (i.e., the bacterial growth) after 24 h of incubation. Data were analyzed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Results: *Escherichia coli* was resistant to both ABs, whereas for both OAs the MIC was 50mM, and for carvacrol and thymol MIC was 1.87 and 3.75 mM, respectively (Figure 1). The addition of 50% of the MIC dose of both carvacrol and thymol to 32 mg/L of both ABs allowed to completely inhibit *Escherichia coli* growth ($p < 0.05$) (Figure 2). Again, the addition of 50% of the MIC dose of benzoic and sorbic acid to amoxicillin and neomycin allowed to reduce the growth of *Escherichia coli* by 92% and 76% and 82% and 56%, respectively ($p < 0.05$).

Salmonella enteritidis was sensitive to amoxicillin at 1 mg/L and resistant to neomycin up to 128 mg/L. The MIC of both NICs and OAs was 1.87 and 50 mM, respectively (Figure 3). Adding 0.94 mM (50% of the MIC) of either carvacrol or thymol to 0.5 mg/L of amoxicillin or 32 mg/L of neomycin allowed to completely inhibit the growth ($p < 0.05$). Similarly, adding 12.5 mM (25% of the MIC) of benzoic or sorbic acid to the lowest dose of amoxicillin and neomycin allowed to decrease the growth of Salmonella by 80% and 100%, respectively ($p < 0.05$) (Figure 4).

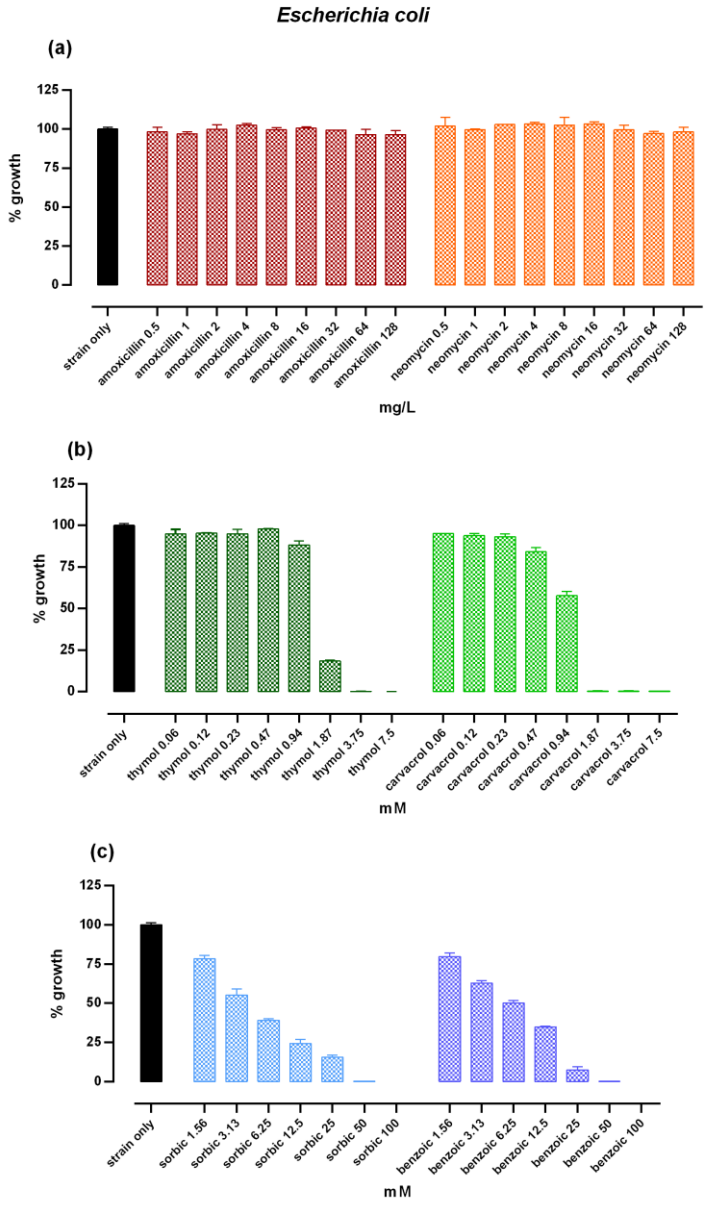

Figure 1. *Escherichia coli* growth after 24 h in the presence of antibiotics (**a**), nature identical compounds (**b**), or organic acids (**c**). Bacterial growth is expressed as a percentage relative to the control (strain only).

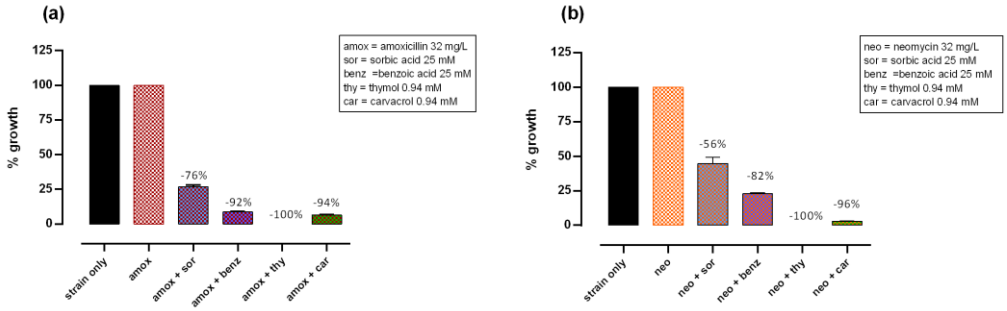

Figure 2. *Escherichia coli* growth after 24 h in the presence of a mixture of ABs and NICs or OAs. (**a**) Amoxicillin. (**b**) Neomycin. Bacterial growth is expressed as a percentage relative to the control (strain only).

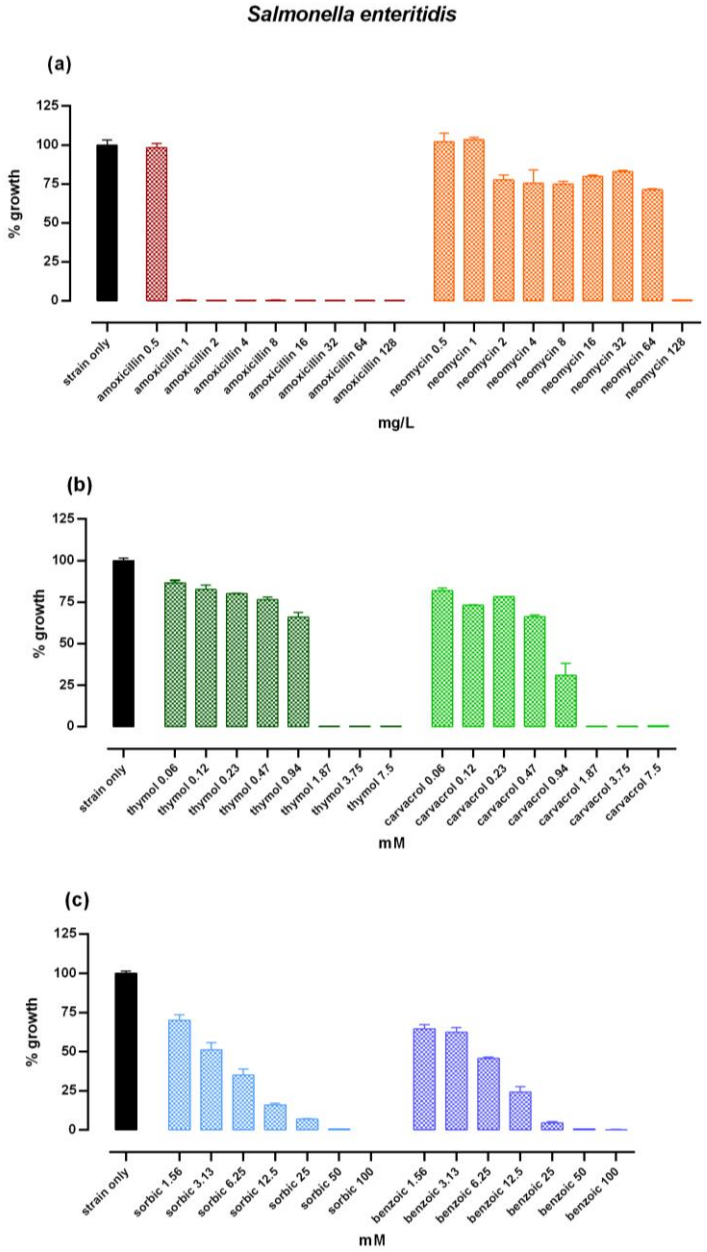

Figure 3. *Salmonella enteritidis* growth after 24 h in the presence of antibiotics (**a**), nature identical compounds (**b**), or organic acids (**c**). Bacterial growth is expressed as a percentage relative to the control (strain only).

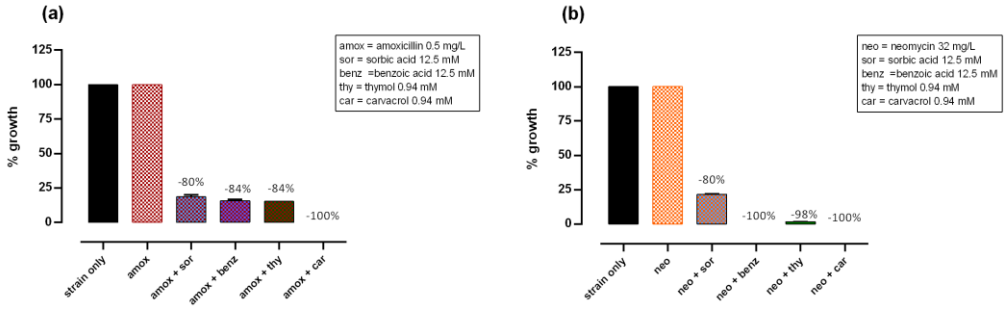

Figure 4. *Salmonella enteritidis* growth after 24 h in the presence of a mixture of ABs and NICs or OAs. (**A**) Amoxicillin. (**B**) Neomycin. Bacterial growth is expressed as a percentage relative to the control (strain only).

Conclusions: In conclusion, *Escherichia coli* and *Salmonella enteritidis* susceptibility to amoxicillin and neomycin was significantly increased by either OAs or NICs. As these substances are commonly used as feed additives, this opens new perspectives to the problem of the loss of efficacy of broad spectrum antibiotics and antibiotic resistance.

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Modified from:

Can feed additives increase antibiotics efficacy against *E. coli*?

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Abstract

Background: Colibacillosis is a common bacterial disease of great economic importance in poultry industry as it is associated with decreasing productivity, increasing mortality, and high cost of prophylaxis and treatments. Moreover, *Escherichia coli* infection is also associated with contamination of infected carcasses at slaughter. Antimicrobial resistant *Escherichia coli* strains create a severe problem for public health, as these strains might be passed to humans via the food chain. Furthermore, resistant bacteria may act as transporters for antimicrobial resistant genes to other pathogens (Ibrahim et al., 2019). Antibiotics (ABs) are one of the main prophylaxis approaches against bacterial infections in poultry industry. However, the target to fight antimicrobial resistance, together with the consumer demand for novel antibacterial compounds alternative to ABs, has supported the research towards alternative molecules, as organic acids (OAs) and nature-identical compounds (NICs) (Mehdi et al., 2018).

The aim of this study was to test whether non-antibiotic feed additives, i.e. OAs and NICs, can improve the efficacy of broad spectrum antibiotics against *Escherichia coli*.

Materials and Methods: The ABs used in this study were amoxicillin, neomycin, and colistin, the stock solutions were prepared in Brain Heart Infusion (BHI). The selected OA and NIC were respectively sorbic acid (stocks prepared in BHI) and thymol (stocks prepared in BHI supplemented with ethanol at a final concentration ≤ 3.5% to increase solubility). Each solution was buffered to ensure a final pH of 6.5, filter-sterilized, and diluted in sterile BHI to reach the final concentration tested. The chosen bacterial strains were eight strains of *Escherichia coli*, both isolated from broiler livers.

The minimal inhibitory concentration (MIC) of ABs, OA, and NIC, alone or combined, was determined using the microdilution method in 96-well microtiter plates with 10^5 cfu/mL. The plates were incubated for 24 h at 37 °C in aerobic conditions. Bacteria were tested against a wide range of concentrations for all the selected compounds: 1-128 mg/L of amoxicillin and neomycin, 0.25-32 mg/L of colistin, 0.10–50 mM for sorbic acid, and 0.05–6.24 mM for thymol. Subsequently, bacteria were also tested against a combination of sorbic acid and thymol, in a 2:1 ratio, starting from 6.25 and 3.12 mM to 0.10 and 0.05 mM. The same combination was also tested in combination with ABs.

After incubation, the growth inhibition was evaluated by absorbance measurement at 630 nm. The MIC value was defined as the lowest concentration of each compound capable to zero the absorbance (i.e., the bacterial growth) after 24 h of incubation. Data were analyzed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Results: All the strains were resistant to amoxicillin up to 128 mg/L. Concerning neomycin, two strains were inhibited at 64 mg/L and four strains at 32 mg/L, the remaining strains were resistant. All *Escherichia coli* isolates were sensitive to colistin at $0.5 \leq MIC \leq 1$ mg/L (Figure 1). MIC values for thymol and sorbic acid tested alone were 3.12 and 50 mM (Figure 1) respectively, but when in combination the MIC was reduced by 2 and 16-fold respectively (1.56 of thymol and 3.12 mM of sorbic acid) (Figure 2).

Sorbic acid at 1.56 mM plus thymol at 0.78 mM increased neomycin efficacy by 2 and 3-fold for six strains and by 10-fold for one strain ($p < 0.05$). Similarly, adding the same concentration of OA+NIC blend increased neomycin potency by 4-times for one strain. Again, 1.56 mM of sorbic acid with 0.78 mM of thymol to amoxicillin improved its efficacy up to 70% in one strain ($p < 0.05$) (Figure 3).

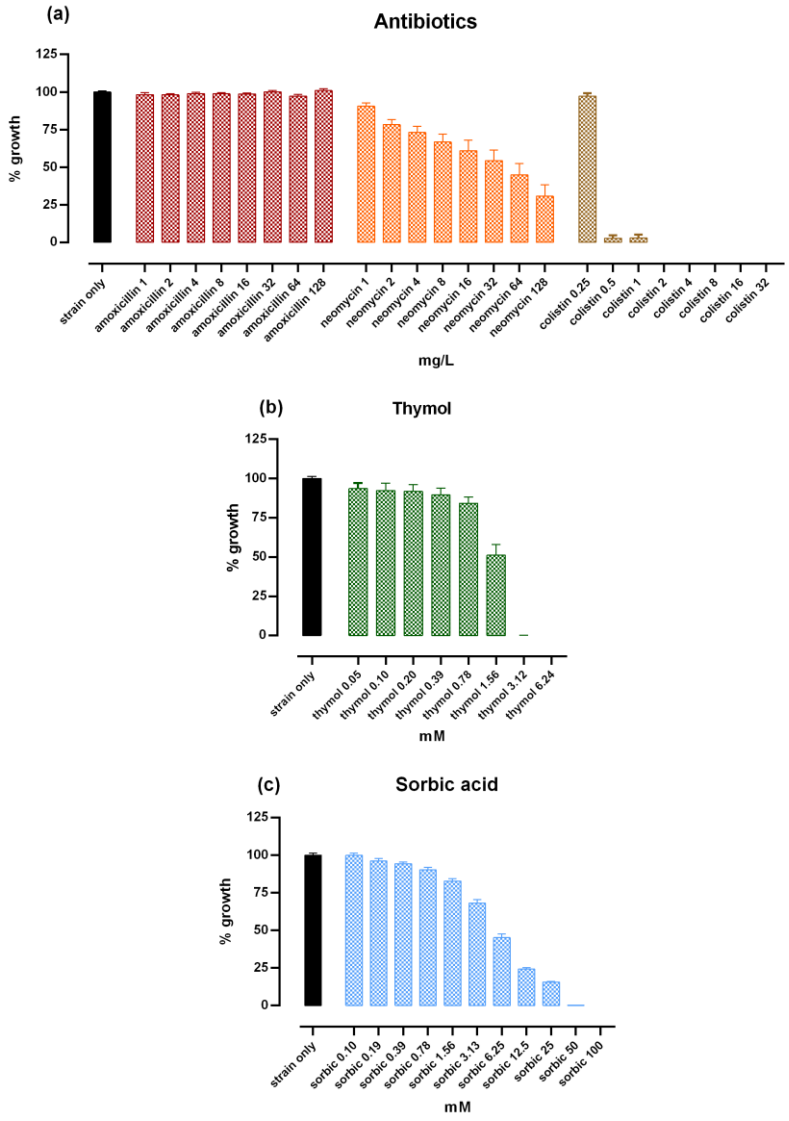

Figure 1. *Escherichia coli* growth after 24 h in the presence of different antibiotics (**a**), thymol (**b**), or sorbic acid (**c**). Bacterial growth is expressed as a percentage relative to the control (strain only).

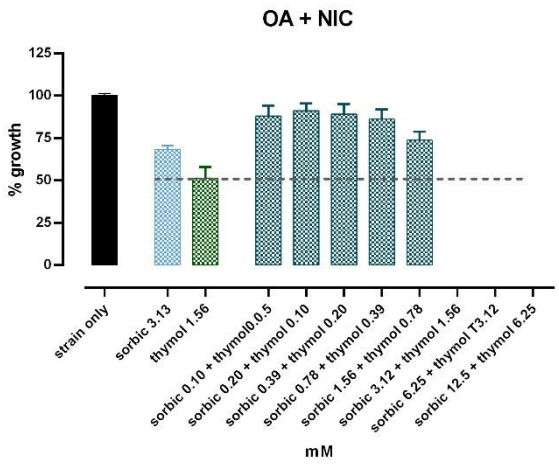

Figure 2. *Escherichia coli* growth after 24 h in the presence of combination of sorbic acid and thymol in 2:1 ratio. Bacterial growth is expressed as a percentage relative to the control (strain only). The dotted line indicates the minimum % of bacteria growth obtained with the same dose of a single molecule present in the blend.

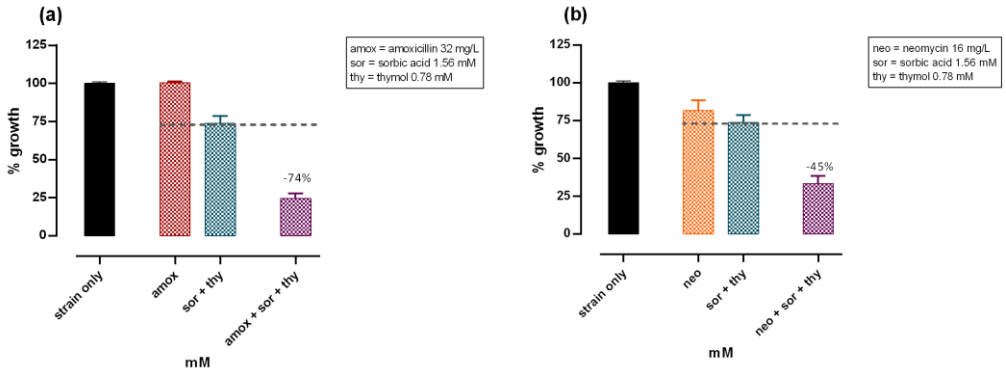

Figure 3. *Escherichia coli* growth after 24 h in the presence of a mixture of ABs and thymol + sorbic acid. (**a**) Amoxicillin. (**b**) Neomycin. Bacterial growth is expressed as a percentage relative to the control (strain only). The dotted line indicates the minimum % of bacteria growth obtained with the same dose of OA+NIC present in the blend with the antibiotic.

Conclusions: In conclusion, neomycin efficacy toward several strains of *Escherichia coli* was significantly increased by the inclusion of thymol and sorbic acid. Moreover, where amoxicillin and, more generally, wide spectrum antibiotics are ineffective, the combined use of sorbic acid and thymol can inhibit *Escherichia coli* growth. As these substances are commonly used as feed additives, this opens new perspectives to the problem of loss of efficacy of the broad spectrum antibiotics and antibiotic resistance.

DISCUSSION AND CONCLUSIONS

Intestinal health plays a vital role for the entire organism, as the GIT represents the largest fraction of the body that interfaces with the external environment. Intestinal health depends on proper epithelial integrity that leads to a correct barrier function, as the GIT is regulated and correlated with various systems of the body able to influence the barrier functionality. For example, endocannabinoids have been found to inhibit gap junction coupling, increasing barrier permeability when using pig brain microvascular endothelial cells (Nagasawa et al., 2006). Moreover, the ECS correlation with the GIT is not only related to the modulation of the intestinal permeability, but it is also related to an indirect control of intestinal motility and secretion, interaction with the microbiota, modulation of inflammation, and control of food intake (DiPatrizio, 2016). Both barrier function and ECS are potentially modulated by external factors, such as diet, stress, pathogens, and toxins. Components of the diet, as additives, can modulate and regulate the expression and localization of TJs on the enterocytes, playing a role in intestinal health and barrier functionality (Yang et al., 2017). In the same way, feed and additives can interact with the ECS, activating specific pathways to support the organism during stressful situations, inflammation, and infections (McPartland et al., 2014). The studies performed in this thesis aimed to examine how natural antimicrobial compounds could support and improve the function of the intestinal barrier. Moreover, the possible correlation between the use of NICs and the modulation of the ECS was assessed to evaluate the ECS as a new target to reduce inflammation and improve animal welfare in the livestock management cycle. OAs, NICs, and EOs are three categories of feed additives widely used in animal nutrition because of their beneficial effects on growth performance and intestinal morphology, as well as their antibacterial, antioxidant, and anti-inflammatory properties (Omonijo et al., 2018; Rossi et al., 2020; Tugnoli et al., 2020). In particular, the attention was focused on citric and sorbic acids for OAs, thymol and vanillin for NICs, and EOs or powder extracts from *Zingiber officinale*, *Melaleuca alternifolia*, and *Vitis vinifera*. The *experimental study 2* showed how the positive modulation of GEO, TTO, and GSE is not limited only in reducing the oxidative stress,

with a reduction of ROS and a re-establish of GSH levels, but is also effective in the stimulation of the intestinal integrity, with the enhancement of TEER and mRNA gene expression of TJs. Results obtained assumed a double meaning: ginger, tea tree, and grape seed are efficient not only in the stimulation of the intestinal barrier due to an increase of TJs, but also in the prevention of damages to the mucosa caused by oxidative stress. In fact, uncontrolled and persistent oxidative stress with overproduction of ROS, together with an inadequate removal of ROS by antioxidant systems, causes apoptosis and tissue injury (Aviello and Knaus, 2017), allowing a loss of the integrity of the intestinal barrier function and more susceptibility to inflammation and infections. In addition, in *experimental study 1*, the use of pure single botanicals and OAs were evaluated to establish their role in intestinal barrier function and anti-inflammatory potential. The most remarkable result is that thymol, vanillin, sorbic and citric acid, when tested individually, had little or no effect on Caco-2, whereas their combination was able to improve epithelial barrier by increasing TEER and gene expression of TJ components. This highlights how, when a single molecule fails or shows slight effects, its combination with other compounds allows for stronger results, or even empowers the outcomes by achieving newer startling effects. Finding the proper combination of different molecules and concentrations is the key point to extend the potentiality of these active principles from *in vitro* to feed additives formulation. If translated *in vivo*, the use of the blend of thymol, vanillin, citric and sorbic acid as feed additives could lead to a more rapid recovery of the intestinal mucosa after an inflammatory challenge, as well as a reduction of the negative effects of the same inflammatory challenge, while GEO, TTO, and GSE could reduce the risk of oxidative stress that normally leads to inflammation and intestinal damages.

An important limitation correlated with the use of EOs is the differences in the composition based on several factors, as plant families and genera, environmental and geographical conditions, and the part of the plant used for extraction (Fokou et al., 2020), triggering a variability in the response obtained (Dabague et al., 2011; El-Jalel et al., 2018). This could be

translated not only for the antimicrobial potential but also for the antioxidant and anti-inflammatory activity, making it difficult to standardize the results obtained from various research groups. For this reason, a valid alternative is represented by the NICs, the chemically synthesized counterpart of the active molecules of EOs, that allow a more standardized use. Other than EOs, the advantage of the use of a blend of OAs and NICs is the possibility to use the desired molecules in a precise and known concentration, without the problem of variation in the composition aforementioned for EOs. This does not mean that the use of EOs or natural extracts should be deserted, on the contrary, it should rather be implemented making the composition of the EOs more uniform. Clearly, by using pure molecules or their blends with a precise and known composition, the amount of inclusion in the animal diet is precise. Furthermore, it is possible to select the most effective compounds and combining them to obtain potential synergies, formulating a more performing product. The potential of natural molecules and EOs in reducing the problem triggered by antibiotic resistance is certainly undeniable (Craciun et al., 2015; Mittal et al., 2019; Yap et al., 2014). In these studies, we demonstrated the added value of phytochemicals acting against inflammation and oxidation and supporting the health of the intestinal mucosa. Besides anti-inflammatory and antimicrobial properties, NICs, and in particular thymol, are also potentially targeting TRP channels, which exhibit variable permeability ratios for Ca^{++} versus Na^{+}, regulating the cation flux (Premkumar, 2014). In detail, the modulation of TRPV1 and TRPV3 could partially explain the anti-inflammatory potential of the mixture of OAs and NICs, as they are known for anti-inflammatory (TRPV1) and pro-inflammatory (TRPV3) properties (Bujak et al., 2019; De Petrocellis et al., 2012). Moreover, TRP channels are recognized as secondary receptors of the ECS (Muller et al., 2019), suggesting a possible modulation of this system potentially performing by NICs. Indeed, it was evaluated more in depth the role of thymol on pathway indirectly but strictly correlated with the intestinal function and health, as gut chemosensory system and ECS.

The activity of the ECS as an inflammatory modulator has been widely investigated in human medicine, due to the pharmacological potential of cannabinoids as a therapy to clinical conditions (Ambrose and Simmons, 2019). Also, the gut chemosensory system shows a similar potential as a target for gluten sensitivity, obesity, and inflammatory bowel disease (IBD) (Depoortere, 2014). Considering that, to our knowledge, there is a lack of information about the same effects in livestock animals, the aim was to study the effects of thymol as modulator of these 2 systems. In *experimental study 3*, performed *in vivo* in piglets, thymol appeared able to modulate the expression of both endocannabinoid and chemosensory systems. Considering the amount of literature available about the therapeutic use of molecules able to modulate the ECS, as well as the dramatic increase of research articles about the role of ECS on various pathological conditions such as chronic inflammation (Barrie and Manolios, 2017), obesity or anorexia due to the appetite regulation (DiPatrizio et al., 2011; Soria-Gómez et al., 2014), and intestinal pathologies as IBD and ulcerative colitis (Leinwand et al., 2017; Sałaga et al., 2014), it is plausible to suppose that the use of thymol might support piglets during stressful situations, such as the weaning period. Interestingly, to our knowledge this is the first finding of the possible role of thymol as a modulator of cannabinoid receptors and enzymes. In fact, most of the interest gained for phytocannabinoid is driven by the component of *Cannabis sativa*. Phytocannabinoids comprise various constituents of *Cannabis sativa*, including the psychoactive component THC and the non-psychoactive constituent CBD (Fisar, 2009). A multitude of effects has been described including pain sensitivity, mood, appetite, and cognition implications (Hill et al., 2012). Other molecules recognized as ECS stimulators are the cannabimimetics CP 55,940 or WIN 55,212-2, the chemically synthetic cannabinoids that mimic the actions of phytocannabinoids (Karl et al., 2017). Finally, also a homolog of endocannabinoids, palmitoylethanolamide (PEA), is widely studied as a natural ligand of secondary endocannabinoid receptors, and probably TRPV1, without any affinity for CB1 and CB2 (Pesce et al., 2018). Thymol was reported as a natural agonist of TRPA1 channels

(Legrand et al., 2020), and as a strong modulator of TRPV3 and TRPM8 (Ortar et al., 2012), other receptors shared to ECS and chemosensing. Moreover, the activation of OR1G1 by luminal thymol plays a role in the alteration of the intestinal permeability (Kuwahara, 2015). All these findings solidly indicate that thymol, together with other botanical feed additives, besides phytocannabinoids, potentially represent a novel frontier for the treatment of intestinal inflammation and pathologies, due to the activation of ECS.

The ECS has been found to be widespread in several tissues in mammalian species. As mentioned before, compared to the information available about the benefits derived from cannabinoids in humans, there is still a lack of knowledge regarding the same benefits in animals, except for the laboratory animal species in which experimental studies have been performed (Silver, 2019). Data obtained from the immunohistochemistry assays in pigs (*experimental study 4*), accordingly with the gene expression analysis of the *experimental study 3*, highlighted the presence of main and secondary cannabinoid receptors in the GIT of pigs at various levels. CB1 has been observed not only in the myenteric plexus' neurons and nerve fibers, consistently with the opinion of Kulkarni-Narla and Brown (2000), but also on smooth muscle cells of *tunica muscularis* and on the mucosa. Similarly, TRPV1 has been found in myenteric neurons, EGCs, and endothelial cells. This expression is following the report of other species, like dogs, cats, and humans (Galiazzo et al., 2018; Stanzani et al., 2020; Wright et al., 2005). This localization suggests a large implication of the ECS in the control of a multitude of functions of the GIT at different levels, as regulation of the mucosal functionality, together with the intestinal contractility. In veterinary medicine, especially for dogs, the role of the ECS is emerging in numerous clinical implications, as modulation of anxiety and stress, inflammatory conditions as rheumatoid arthritis, colitis, hepatitis, suffering due to the reduction of the nociceptive neural signal of pain, and sense of satiety (Silver, 2019).

All these outcomes suggest that the ECS could represent an important target not only for its therapeutic potential in treating clinical conditions, but also in the management of the livestock

production cycles in stressful situations and to support animal productivity. Nevertheless, these results represent a first understanding of the mechanism of action of botanical feed additives at the intestinal level, more precisely in controlling intestinal inflammation, oxidation, motility, and health. Further investigations are required to obtain a better understanding of the potential and precise mode of action of these molecules.

In conclusion, natural alternative molecules, as NICs, OAs, and EOs, alone or combined in a blend, are well known to represent an important shield to replace the use of AGP and to support the classical pharmacological prophylaxis with the antibiotics. In fact, the strategic role of these alternative molecules in reinforcing the mucosa barrier function and preventing oxidative stress and inflammation is coming to light. Also, these alternative molecules act as antimicrobial agents, reducing the risk of antibiotic resistance and increasing the susceptibility of resistant bacteria to antibiotics. This opens alternative roads to solve the challenges posed by a new type of livestock industry requests, such as ABF and NAE productions. Furthermore, phytochemicals seem to play a role in the modulation of the ECS, partially explaining their various properties, which represent a novel frontier to support animal health and productivity. The upregulation of endocannabinoid and chemosensory markers throughout the GIT implicates a possible role for these feed additives as promoters of gut and animal health. Nevertheless, these results represent a first insight into the potential uses of NICs, OAs, and EOs to control intestinal health and inflammation. Further investigations are required to understand the role and connection of the different markers here analyzed, the mechanisms by which natural molecules modulate the expression of the endocannabinoid system, and how the activation of the endocannabinoid system in stress situations represent a crucial pathway for phytochemicals to exert their beneficial function as feed additives.

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