Archives of Biochemistry and Biophysics 694 (2020) 108591

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Efficacy of theobromine in preventing intestinal CaCo-2 cell damage induced by oxysterols

Noemi Iaia, Daniela Rossin, Barbara Sottero, Ivana Venezia, Giuseppe Poli, Fiorella Biasi *

Department of Clinical and Biological Sciences, University of Turin, 10043, Orbassano (Turin), Italy

ARTICLE INFO

Keywords:
Apoptosis
Dietary oxysterols
Intestinal inflammation
Tight junctions
Matrix metalloproteinases
Theobromine

ABSTRACT

The alteration of the intestinal barrier function is currently believed to be involved in the pathogenesis of gut diseases mainly associated with the activation of inflammation processes.

Diet plays an important role in the control of human gut integrity. Theobromine is a natural methylxanthine present in dark chocolate particularly abundant in cocoa bean shell. This is a polyphenol rich by-product generated in cocoa industrial processing, which is gaining value as a functional ingredient. This study aims to highlight for the first time the capability of theobromine in protecting the intestinal cell monolayer from a mixture of dietary oxysterols showing an inflammatory action in terms of IL-8 and MCP-1 overproduction. Differentiated CaCo-2 cells were treated with 60 μM oxysterol mixture and pre-incubated with 10 μM theobromine. Intestinal barrier damage was investigated in terms of tight junction claudin 1, occludin and JAM-A protein levels, matrix metalloproteinase (MMP) −2 and −9 activation and anti/pro-apoptotic protein changes.

The observed cell monolayer permeability protection by theobromine may be due to its ability to inhibit the production of cytokines and MMPs that can be responsible for tight junction loss and apoptosis in intestinal cells. Our findings provide additional mechanistic hints on the healthy effect of theobromine cocoa component as an attractive natural molecule in the prevention of inflammatory gut diseases.

1. Introduction

The intestinal mucosa is a key element in the maintenance of the human health thanks to its double physical and functional activities. It acts as a semi-permeable barrier allowing the absorption of different molecules, such as water or nutrients [1], and at the same time it plays a leading role in regulating the immune system through the recognition of potentially dangerous dietary antigens and microorganisms [2].

Diet plays an important role in the regulation of the human gut integrity. The dietary changes observed in those countries where Western nutritional lifestyle is progressively being adopted, the introduction of lower fiber intake, refined carbohydrates and higher amounts of animal fats have caused a rise in inflammatory intestinal diseases [3].

Animal fat rich foods make an excess of cholesterol and its oxidized compounds (i.e. oxysterols) available to intestinal mucosa, where they can modulate enterocyte signals associated to inflammatory and oxidative processes [4].

Oxysterols can be generated either exogenously (food) by non-enzymatic cholesterol auto-oxidation or endogenously by both enzymatic activity (through cytochrome P450-dependent/independent

Abbreviations: **α-epox**, 5α,6α-epoxycholesterol; **β-epox**, 5β,6β-epoxycholesterol; **7α-HC**, 7α-hydroxycholesterol; **7β-HC**, 7β-hydroxycholesterol; **7K**, 7-ketocholesterol; **Bax**, Bcl-2-associated x; **Bcl-xL**, B-cell lymphoma-extra-large; **cAMP**, cyclic adenosine monophosphate; **CBS**, cocoa bean shell; **DMEM**, Dulbecco's modified Eagle's medium; **DTT**, dithiothreitol; **ECL**, enhanced chemiluminescence; **ELISA**, enzyme-linked immunosorbent assay; **ERK**, extracellular signal-regulated kinase; **FBS**, fetal bovine serum; **HRP**, horseradish peroxidase; **IBD**, inflammatory bowel diseases; **IL**, interleukin; **IFN-β**, interferon β; **LDH**, lactate dehydrogenase; **LDS**, lithium dodecyl sulfate; **mTOR**, mammalian target of rapamycin; **MMP**, matrix metalloproteinase; **MCP-1**, monocyte chemoattractant protein; **NADH**, nicotinamide adenine dinucleotide; **NF-kB**, nuclear factor-kappa B; **Oxy-mix**, oxysterol mixture; **PBS**, phosphate buffer saline; **PDE**, phosphodiesterase; **PI3K**, phosphatidylinositol 3-kinase; **RT**, room temperature; **SDS**, sodium dodecyl sulfate; **ST**, standard deviation; **TBS**, tris-buffered saline; **TLR**, toll-like receptor; **TNF-α**, tumor necrosis factor-α; **TTBS**, TBS-Tween 20.

* Corresponding author. Department of Clinical and Biological Sciences, University of Turin, San Luigi Hospital, Regione Gonzole 10, 10043, Orbassano (Turin), Italy.

E-mail addresses: noemi.iaia@unito.it (N. Iaia), d.rossin@unito.it (D. Rossin), barbara.sottero@unito.it (B. Sottero), ivana.venezia@edu.unito.it (I. Venezia), giuseppe.poli@unito.it (G. Poli), fiorella.biasi@unito.it (F. Biasi).

https://doi.org/10.1016/j.abb.2020.108591
Received 2 July 2020; Received in revised form 17 September 2020; Accepted 18 September 2020
Available online 19 September 2020
0003-9861/

hydroxylases) and non-enzymatic reactions. Auto-oxidation primarily occurs on the sterol nucleus at position 7, or at 5,6 double bond of cholesterol structure [5]. Oxysterols are produced in mixture in processed foods high in cholesterol, mainly during their exposure to heat treatments and long-term storage. The most abundant dietary oxysterols found in mixture after cholesterol heating are 7-ketocholesterol (7 K), 5α,6α-epoxycholesterol (α-epox), 7β-hydroxycholesterol (7β-HC), and in less concentration 5β,6β-epoxycholesterol (β-epox) and 7α-hydroxycholesterol (7α-HC) [6,7]. The excess of dietary oxysterols added *in vitro* to intestinal cells in mixture, mimicking high cholesterol consumption, was demonstrated to affect mucosal barrier integrity. This mixture displayed pro-apoptotic and pro-inflammatory actions [8–10]. The same mixture was able to activate the immune system-related pattern recognition receptors Toll-like Receptor (TLR) 2 and 4 [11] and impair intestinal barrier by activating MMP-2 and -9, as well as altering tight junction cell distribution and production in the human enterocyte-like CaCo-2 cell monolayer [12].

Notably, dietary guidance from International Organization for the Study of Inflammatory Bowel Diseases (IBD) recommended the importance of a balanced nutritional support aimed at maintaining energy requirement and reducing pro-inflammatory foods [13]. The Mediterranean diet is gaining new consideration because of its high consumption of vegetables and fruits, and low consumption of saturated and *trans*-fats. It has been associated with a lower risk of IBD onset, disease activity improvement and inflammatory marker decrease [14,15].

The majority of scientific literature aimed at elucidating the Mediterranean diet health benefits is focused on polyphenol-rich plant foods, which are well-known for their strong antioxidant and anti-inflammatory properties concurring in the prevention of intestinal barrier damage [16–19].

Previous studies reported the ability of phenolic extracts from wine and olive oil in negatively modulating inflammatory-related signaling pathways activated by oxysterols in differentiated enterocyte-like CaCo-2 cells [10,20,21].

Extracts from cocoa bean shell (CBS) - the main by-product generated in cocoa industrial processing that has been raising attention to develop circular economy strategies - have recently shown different analytical recovery of phenolic and methylxanthine compounds depending on their fractionation. Their antioxidant and anti-inflammatory properties were tested. CBS fractions rich in (−)-epicatechin and tannins were highlighted for their antioxidant capacity and ability to prevent TLR activation and interleukin (IL)-8 increase upon CaCo-2 cell treatment with dietary oxysterols added in mixture. However, also that CBS fraction containing high amounts of theobromine (3,7-dimethylxanthine) showed significant anti-inflammatory effects. This finding suggested that theobromine could concur with polyphenol CBS compounds in preserving intestinal damage [11].

Theobromine is a natural compound belonging to the family of purine-based natural heterocyclic alkaloids generated from xanthine methylation. Theophylline, caffeine and theobromine are the most known methylxanthines present in plants such as *Camellia sinensis L.*, *Coffea sp.* and *Theobroma cacao L.* used for the production of tea, coffee, and chocolate [22]. They are known for their action as adenosine receptor antagonists and non-selective phosphodiesterase (PDE) inhibitors on the central nervous system. They have been widely used for their bronchodilator - in particular theophylline - neurostimulatory and psychoactive actions - in particular caffeine [23,24].

The main source of theobromine is dark chocolate and is also particularly abundant in CBS. Together with caffeine, it accumulates in the cotyledons of cocoa beans and crosses from the seed into the shell during cocoa fermentation. Theobromine is present in this fraction in a concentration 5-7-fold higher than caffeine [25].

Compared with other methylxanthines, theobromine effects have been less studied. Similarly to other xanthine derivatives, theobromine possesses smooth-muscle relaxant, diuretic and coronary vasodilator properties. Theobromine is used as a natural therapeutic compound in asthma and in other respiratory tract problems [26]. In particular, the antitussive effect showed by theobromine suggests it as a safety alternative natural compound to the use of codeine. Experiments performed in guinea-pigs showed that the preventing action against persistent cough by theobromine was related to its ability to inhibit improper excitation of the vagus nerve. In the same article, a beneficial antitussive effect in humans was also reported [27].

Methylxanthines have a narrow therapeutic range depending on the specific drugs and animal species involved with high incidence of adverse effects when their serum concentration in humans exceeds 20 mg/ml [28].

Theobromine normal intake ranges as for a moderate cocoa consumption was associated with its beneficial effects. Compared with its chemical homologous caffeine, theobromine appears to have differential and weaker action, avoiding the unpleasant consequences observed for caffeine-associated effects like anxiety and insomnia. Theobromine lower affinity for adenosine receptors compared to caffeine may be responsible for the observed different psychoactive effects of theobromine [29].

Studies describing theobromine toxicity in humans are very scarce. However, acute ingestion of a high dose of theobromine such as 700 mg showed blood pressure increase and self-report calmness decrease in randomized healthy subjects [30]. Theobromine negative mood effects and increased heart rate were also observed at a highest theobromine dose intake (1000 mg) in a double-blind placebo-controlled study involving 80 healthy participants [31]. Similarly, to caffeine, precautions are necessary in case of high chocolate consumption during pregnancy because theobromine goes through the placenta and increases its concentration in the maternal serum. Its high serum concentration before 20-week gestation was associated with increased risk of preeclampsia in late pregnancy [32]. Notably, theobromine can become very toxic in other mammals, the most known example being dogs. Chocolate ingestion is very dangerous for these animals, which metabolize theobromine more slowly than humans [24].

Noteworthy, theobromine anti-inflammatory potential has also been suggested. As a non-selective PDE inhibitor, it could be responsible for intracellular cyclic adenosine monophosphate (cAMP) level increase, protein kinase A activation and tumor necrosis factor (TNF)-α inhibition [33].

This molecule also displays anti-tumor effects in human glioblastoma cells by attenuating extracellular signal-regulated kinase (ERK) activity, phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) and nuclear factor-kappa B (NF-kB) inflammatory signaling axis [34].

Theobromine antioxidant and anti-inflammatory action independently from its ability to block adenosine receptor pathway has also been suggested [26].

To date, the possible theobromine beneficial effects on the intestine are not yet known.

The aim of our current investigation is to give *in vitro* evidence of theobromine ability in preventing the intestinal mucosal layer derangement induced by a mixture of oxysterols present in a hypercholesterol diet. In particular, this cocoa bean compound can decrease inflammatory cytokines, IL-8 and monocyte chemoattractant protein (MCP)-1, and MMPs activated by oxysterols at cellular level. One of the mechanisms by which theobromine maintains epithelial barrier integrity could be associated to its ability to suppress the apoptotic program triggered by oxysterols. Our findings provide additional mechanistic hints on the healthy effect of theobromine as an attractive natural molecule in the prevention of inflammatory gut diseases.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all reagents and chemicals were obtained

from Sigma Aldrich Srl (Milan, Italy).

Human IL-8 and MCP-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from.

PeProtech (DBA Italia Srl, Segrate, Milan, Italy).

Rabbit anti-JAM-A (SC-25629), mouse *anti*-occludin (SC-133256), mouse *anti*-claudin 1 (SC- 166338), mouse anti- B-cell lymphoma (Bcl)-xL (SC-8392) and mouse *anti*-Bcl-2-associated x (Bax) (SC-7480) polyclonal primary antibodies were from Santa Cruz Biotechnology (DBA Italia Srl, Segrate, Milan, Italy). Anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (7074S) and anti-mouse IgG HRP- conjugated secondary antibody (7076S) were from Cell Signaling Technology (Euroclone SpA, Milan, Italy). Lithium dodecyl sulfate (LDS) sample buffer 4X and dithiothreitol (DTT) Sample Reducer 10X were purchased from Thermo Fisher Scientific (Life Technologies Italia, Monza, Italy). Proteases inhibitors cocktail "cOmplete ULTRA Tablets Mini EASYpack" and the nicotinamide adenine dinucleotide (NADH) were obtained from Roche SpA (Monza, Italy). Bio-Rad protein assay dye reagent and enhanced chemiluminescence (ECL)® Western Blotting System were from Bio-Rad Srl (SIAL, Rome, Italy). Hybond ECL nitrocellulose membrane was from GE Healthcare Srl (Milan, Italy).

Dulbecco's modified Eagle's medium (DMEM) with high glucose content, fetal bovine serum (FBS) and trypsin solution (trypsin 5 g/L) were obtained from Euroclone SpA (Milan, Italy).

Oxysterols (7 K, α-epox, 7β-HC, β-epox, 7α-HC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Theobromine (≥98.0%) was from Fluka (Milan, Italy).

2.2. Cell culture and treatments

Human colorectal adenocarcinoma CaCo-2 cells were provided by the Cell Bank Interlab Cell Line Collection (Genoa, Italy). Cells were plated at 1×10^6/ml density and were cultured in DMEM supplemented with 10% heat inactivated FBS, 1% antibiotic/antimycotic solution (100 U/ml penicillin, 0.1 mg/ml streptomycin, 250 ng/ml amphotericin B and 0.04 mg/ml gentamicin) at 37 °C under 5% CO_2 humidified atmosphere. After reaching 100% confluence, cells were grown for additional 18 days in order to allow their spontaneous differentiation into enterocyte-like phenotype.

Before each treatment, differentiated CaCo-2 cells were maintained in serum-free medium overnight to make them quiescent, then placed in 1% FBS DMEM. Cells were pre-incubated or not with theobromine for 1 h (concentrations are reported below in the analysis sections) and treated with a mixture of dietary oxysterols (Oxy-mix) at 60 μM final concentration at 37 °C for 24 h.

The percentage composition of oxysterols used in the Oxy-mix was: 42.96% 7 K, 32.3% α-epox, 5.76% β-epox, 4.26% 7α-HC, and 14.71% 7β-HC. The molarity of each oxysterol was calculated as 25.8 μM 7 K, 19.4 μM α-epox, 3.4 μM β-epox, 2.6 μM 7α-HC, 8.8 μM 7β-HC by considering their average molecular weight of 403 g/mol [20].

2.3. Cell death evaluation

The extracellular release of lactate dehydrogenase (LDH), which was considered as a parameter of cytolysis, was estimated spectrophotometrically at 340 nm wavelength by recording NADH production/min. Cell death was evaluated in cells treated with 60 μM Oxy-mix and pretreated or not with theobromine increasing concentrations (10–30 μM).

LDH release was expressed as enzyme release percentage into cell culture medium (100% cell lysis was obtained by 0.5% Triton X-100 addition to cell plate containing the same cell density as treated cells). Untreated cells were considered as control.

2.4. Evaluation of cytokine protein levels by ELISA

Cells placed in 1% FBS DMEM were pre-incubated or not with 10 μM theobromine for 1 h and treated or not with 60 μM Oxy-mix for 24 h. Untreated cells were incubated in 1% FBS DMEM alone for the same period of time as treated cells and were considered as control.

Collected cell culture medium from each sample was used for ELISA detection. IL-8 and MCP-1 extracellular protein levels were quantified by using commercial ELISA kits according to the manufacturer's instructions. Sample absorbance values were read in a 96-multiwell plate reader (Model 680 Microplate Reader, Bio-Rad laboratories Srl, Milan, Italy) at 450 nm with wavelength correction at 655 nm as reference value.

Data were elaborated by SlideWrite Plus software (Advanced Graphics Software, Rancho Santa Fe, CA, USA). Cytokine levels were normalized for total proteins present in the corresponding cell culture medium. Total protein concentration was evaluated in each cell culture medium sample by using Bio-Rad protein assay dye reagent [35].

The analyses were performed in triplicate and values expressed as pg cytokines/mg cell culture medium proteins.

2.5. Immunoblotting

At the end of each treatment, cells were scraped and washed with one ml of ice-cold phosphate buffer (PBS). For protein extraction 150 μl of lysis buffer [PBS supplemented with 1% Triton X-100 (v/v), 1% sodium dodecyl sulfate (w/v) (final volume)] were added to each sample. Lysates were incubated for 30 min on ice and centrifuged at 12,000 g for 15 min. Total cell extract protein concentration was evaluated with Bio-Rad protein assay dye reagent.

All samples, containing 50 μg total proteins, were boiled in the sample buffer at 100 °C for 5 min (LDS sample buffer 4X and DTT Reducer 100X). Boiled samples were subjected to electrophoretic migration on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and proteins were transferred to Hybond ECL nitrocellulose membranes.

After protein transfer, the membranes were incubated in TTBS blocking buffer [TTBS: tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20] plus 5% (w/v) skimmed milk powder (final volume) at room temperature (RT) for 1 h.

For tight junction protein detection, membranes were then incubated at 4 °C overnight with either mouse *anti*-claudin 1 (1:800 dilution), mouse *anti*-occludin (1:500 dilution), or rabbit anti-JAM-A (1:200 dilution) polyclonal antibodies in TBS containing 0.1% Tween-20 (v/v) and 5% skimmed milk powder (w/v).

Immunoblotting technique was also used to analyze apoptotic protein levels. In this case, the membranes were incubated at 4 °C overnight with mouse *anti*-Bcl-xL (1:600 dilution) or mouse *anti*-Bax (1:200 dilutions) polyclonal antibodies in TBS containing 0.1% Tween-20 (v/v) and 5% skimmed milk powder (w/v).

All the blots were washed twice in TTBS and incubated with anti-rabbit or anti-mouse HRP-conjugated IgG (1:1000 dilutions) in TBS with 0.1% Tween-20 (v/v) and 5% skimmed milk powder (w/v) for 1 h. At the end of this incubation time the membranes were washed as previously described.

The kit Clarity Western ECL and the ChemiDoc™ Touch Imaging System machine (Bio-Rad laboratories Srl, Segrate, Italy) were used to detect chemiluminescence. Densitometric measurements were conducted using software ImageJ data processing (Bethesda, Maryland, USA).

Protein levels were expressed as percentage of the control (untreated cells). Apoptotic proteins were also shown as Bax/Bcl-xL ratio expressed as fold increase versus control.

2.6. Gelatin zymography

MMP-2 and MMP-9 activities were evaluated in the cell medium by gel zymography. For this analysis, 8 μl of LDS sample buffer 4X were added to each protein sample (20 μg proteins). Samples were used in non-reducing conditions, i.e. in absence of heating and reducing agents, then subjected to electrophoretic migration on 8% SDS-PAGE gel

containing gelatin (0.8 mg/ml) with a constant voltage of 110 V. Gelatin is one of the most frequently used substrate for MMPs. After electrophoretic migration, the gel was washed with Tris buffer (2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5, final solution) at RT for 1 h and incubated at 37 °C overnight in proteolysis buffer (40 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl2, 0.02% NaN3, pH 7.5, final solution). The gel was then stained for 3 h with Coomassie Blue solution (0.05% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid, final solution) and finally destained with 5% methanol and 7% acetic acid (final solution). The resulting destained bands corresponding to MMP proteolytic activities look clear over a deep blue background. The activity of the two MMPs was estimated by densitometric analysis using ImageJ software and expressed as fold increase versus control.

2.7. Statistical analyses

Results were expressed as mean ± Standard Deviation (SD) and data were analysed with GraphPad InStat software (San Diego, CA, USA). Statistical differences among experimental data were evaluated by using the one-way ANOVA test associated with Bonferroni's multiple comparison post-test.

3. Results

3.1. Effects of different theobromine concentrations on cell death

In order to investigate whether theobromine exerts cytotoxic effect on differentiated CaCo-2 cells in the presence or absence of Oxy-mix, the percentage of lactate dehydrogenase cell release was detected. Cells were incubated with increasing concentrations of theobromine (10, 15, 20, 25 or 30 μM) for 24 h. LDH % increase up to 10% was not considered as cytotoxic. All theobromine concentrations did not exert any necrogenic effect whether the compound was added alone or with Oxy-mix. Notably, the enzyme percentage release was found higher in 60 μM Oxy-mix alone than in theobromine + Oxy-mix-treated cells (in particular, both 10 and 15 μM theobromine concentrations) (Table 1). Based on this observation, all subsequent experiments were performed by using 10 μM theobromine.

Cell death was evaluated in terms of lactate dehydrogenase (LDH) release in the culture media of differentiated CaCo-2 cells pre-treated or not with different concentrations of theobromine, and incubated or not with 60 μM oxysterol mixture (Oxy-mix) for 24 h.

LDH was calculated as a percentage referring to 100% cell enzyme release into the medium by adding 0.5% Triton X-100 to cultured cells grown at the other samples' density.

Data are reported as means ± SD of three independent experiments. Statistical differences within experimental groups were calculated using ANOVA associated with the Bonferroni post test: significantly different vs. untreated cells: *$p < 0.05$; **$p < 0.01$; significantly different vs. Oxy-mix: #$p < 0.05$, ##$p < 0.01$.

Table 1
Effect of theobromine increasing concentrations on cell death induced in differentiated CaCo-2 cells by a dietary oxysterol mixture.

LDH (% cell release)		
	Untreated cells	60 μM Oxy-mix-treated cells
	2.0 ± 0.05$^{\#\#}$	8.3 ± 0.5 **
	Theobromine	*Theobromine + Oxy-mix*
10 μM Theobromine	2.4 ± 0.05$^{\#\#}$	5.2 ± 0.1 $^{*,\#}$
15 μM Theobromine	2.3 ± 0.2$^{\#\#}$	5.1 ± 0.1 $^{*,\#}$
20 μM Theobromine	2.8 ± 0.1$^{\#\#}$	5.7 ± 0.2 $^{*,\#}$
25 μM Theobromine	2.9 ± 0.03$^{\#\#}$	5.8 ± 0.02 $^{*,\#}$
30 μM Theobromine	3.5 ± 0.1$^{\#\#}$	6.3 ± 0.1 $^{*,\#}$

3.2. Oxy-mix-dependent IL-8 and MCP-1 increased levels observed in differentiated CaCo-2 cell medium were reduced by theobromine pre-treatment

Theobromine anti-inflammatory property was evaluated in terms of IL-8 and MCP-1 release in the incubation medium of differentiated CaCo-2 cells (Fig. 1).

The oxysterol mixture induced a strong increase in the production of both pro-inflammatory cytokines. Cell pre-treatment with 10 μM theobromine was able to decrease the cytokine overproduction induced by the Oxy-mix treatment. IL-8 and MCP-1 medium protein levels in the presence of theobromine alone were similar to those evaluated in untreated cells (control).

3.3. Theobromine counteracts tight junction impairment dependent from Oxy-mix cell treatment

Claudin 1, JAM-A and occludin were considered as tight junction proteins involved in the regulation of the intestinal layer permeability.

Cell protein levels of these three tight junctions were evaluated in order to clarify the potential beneficial effect of theobromine in preserving the integrity of the intestinal epithelial barrier.

Western blotting analyses performed in differentiated CaCo-2 cell monolayers treated with Oxy-mix for 24 h showed a significant decrease in all tight junction protein levels. On the contrary, cell pre-incubation with 10 μM theobromine for 1 h attenuated the Oxy-mix-dependent decrease in tight junction protein levels. In particular, Claudin 1 and JAM-A amounts similar to controls were observed (Fig. 2).

3.4. Cell release of active MMP-2 and MMP-9 is prevented by theobromine

MMPs are zinc-dependent enzymes, which play a key role in modifying cellular adhesion and motility thus concurring to epithelial barrier destabilization. MMP-2 and MMP-9 were evaluated by direct gelatin zymography in the cell medium, where their activated form is present.

The treatment of differentiated CaCo-2 cells with 60 μM Oxy-mix allowed to observe the induction of both MMP-2 and -9 activities compared to controls (Fig. 3).

Cell pre-incubation with theobromine brought back MMPs activities to control values, thus strengthening its protective role against Oxy-mix-dependent damage on cell monolayer.

3.5. Theobromine may play a role in the maintenance of the epithelial barrier integrity by regulating apoptosis

Excessive proteolysis together with tight junction protein damage, as well as the activation of intestinal epithelial cell apoptosis can concur in affecting barrier function. We focused our attention on the apoptosis induced by the mixture of dietary oxysterols and the possible protective effect of theobromine by evaluating cell protein level changes in pro-apoptotic Bcl-2 associated X (Bax) and anti-apoptotic B-cell lymphoma-extra-large (Bcl-xL).

Differentiated CaCo-2 cells were pre-treated or not with theobromine for 1 h and treated with Oxy-mix for 24 h. Cell incubation with 60 μM Oxy-mix significantly increased pro-apoptotic Bax protein levels (Fig. 4A), while markedly reduced anti-apoptotic Bcl-xL protein levels (Fig. 4B). The significant increased Bax/Bcl-xL protein level ratio versus control (considered as 1) confirmed the activation of the apoptotic pathway by the Oxy-mix (Fig. 4C).

On the other hand, oxysterol-dependent apoptotic protein changes were reversed when cells were pre-incubated with 10 μM theobromine, thus supporting a further beneficial role of this molecule in exerting anti-apoptotic activity in intestinal cell monolayer.

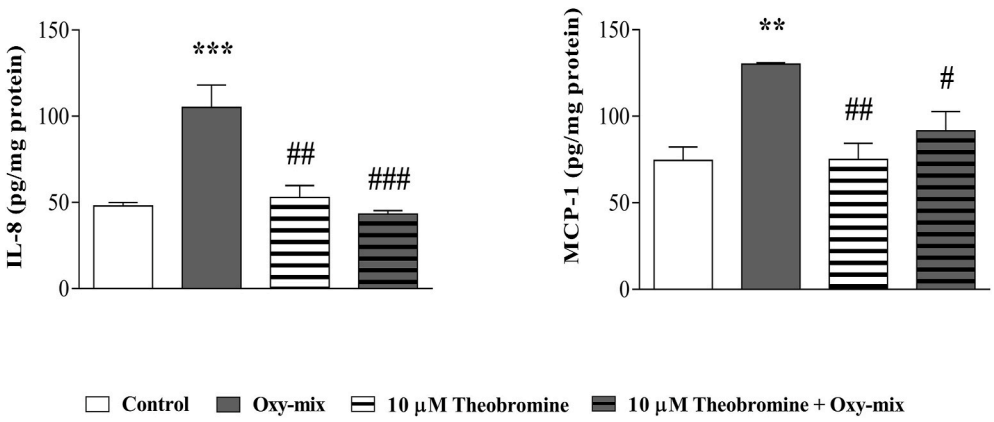

Fig. 1. Theobromine preserves increased levels of inflammatory markers in the incubation medium of differentiated CaCo-2 cells treated with the oxysterol mixture.
Differentiated CaCo-2
cells were pre-treated or not with theobromine (10 μM) for 1 h and incubated with 60 μM oxysterol mixture (Oxy-mix) for 24 h. IL-8 and MCP-1 protein levels were quantified by ELISA in the cell culture medium. Control: untreated cells. Values are shown as pg chemokines normalized for cell incubation medium mg proteins, and are referred as means ± SD of three independent experiments. Significantly different vs. control: **p < 0.01, ***p < 0.001; significantly different vs. Oxy-mix: #p < 0.05, ##p < 0.01, ###p < 0.001.

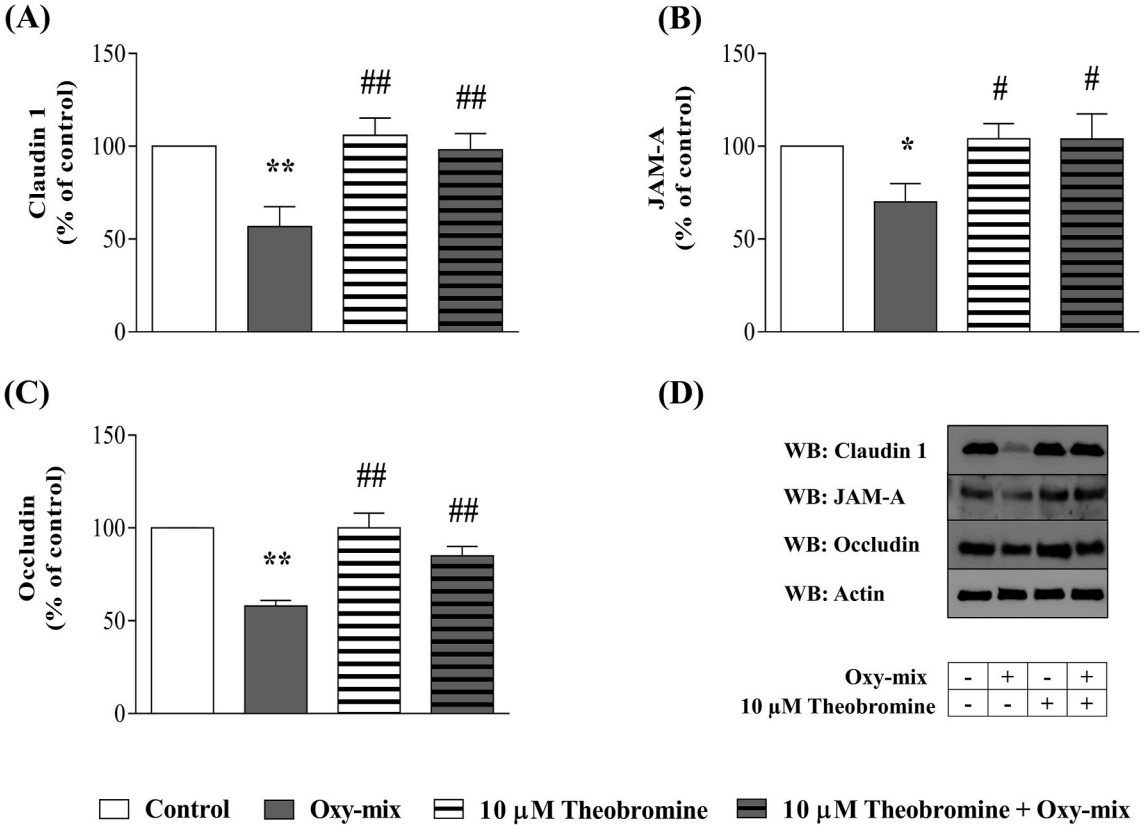

Fig. 2. Tight junction protein levels in differentiated CaCo-2 cells treated with the oxysterol mixture in the presence or absence of theobromine.
Decreased protein levels of claudin 1, JAM-A and occludin were evaluated in lysates from CaCo-2
cells incubated with 60 μM oxysterol mixture (Oxy-mix) for 24 h; 10-μM theobromine-pre-treated cells for 1 h protected oxysterol-mediated tight junction loss. Control: untreated cells. Data are expressed as percentage of control (considered as 100%). Values are means ± SD of three independent experiments. Significantly different vs. control: *p < 0.05, **p < 0.01; significantly different vs. Oxy-mix: #p < 0.05, ##p < 0.01.

4. Discussion

The alteration of the intestinal barrier function is currently believed to play an important role in the pathogenesis of gut diseases mainly associated with the activation of inflammation processes. The mucosa layer integrity loss could allow dietary/bacteria-derived molecules triggering uncontrollable inflammatory signaling cascade. The maintenance of barrier integrity is both negatively and positively influenced by eating habits. Nowadays, a rise in Western diet-associated diseases represents a public health problem. Over the last decades, worldwide health organizations adopted many dietary strategies to reduce disease risk or outcomes. In this context, many studies aim to explore and utilize natural compounds providing antioxidant and anti-inflammatory benefits. They represent a possible strategy in attenuating and/or preventing inflammatory diseases, including the intestinal ones.

Cocoa and its related products like chocolate are widely consumed. Several studies demonstrated its beneficial effects as functional food, especially related to the high content in antioxidant flavanols - particularly epicatechin and procyanidins - and methylxanthines.

Theobromine is the main methylxanthine found in cocoa and cocoa-containing foods. Theobromine is often associated with chocolate positive effect on cognitive performance and mood. In addition to that, its

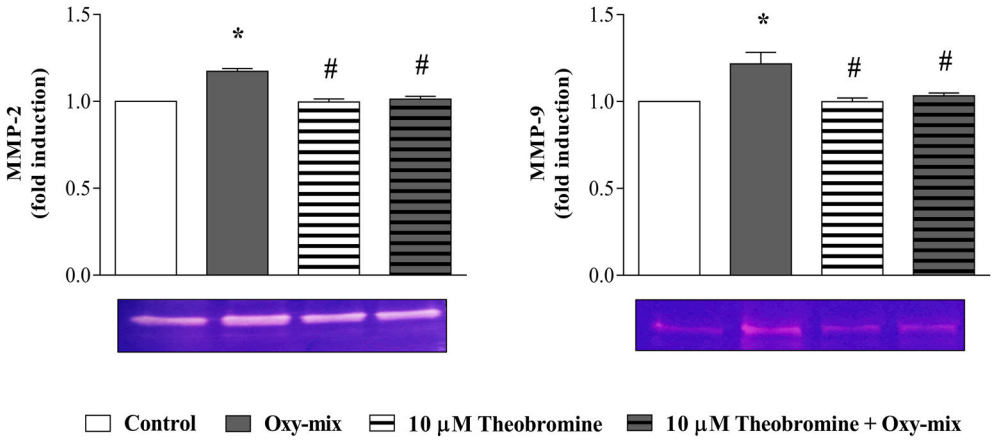

Fig. 3. MMP-2 and MMP-9 induction in differentiated CaCo-2 cells treated with the oxysterol-mixture. Effect of theobromine.
The induction of MMP-2 and MMP-9 was evaluated by gel-zymography in differentiated CaCo-2
cells after 60 μM oxysterol mixture (Oxy-mix)-treatment for 24 h. Control: untreated cells. Theobromine pre-treatment reduces MMPs' activation to normal values. All data represent means ± SD of three independent experiments. Significantly different vs. control: *p < 0.05; significantly different vs. Oxy-mix: #p < 0.05.

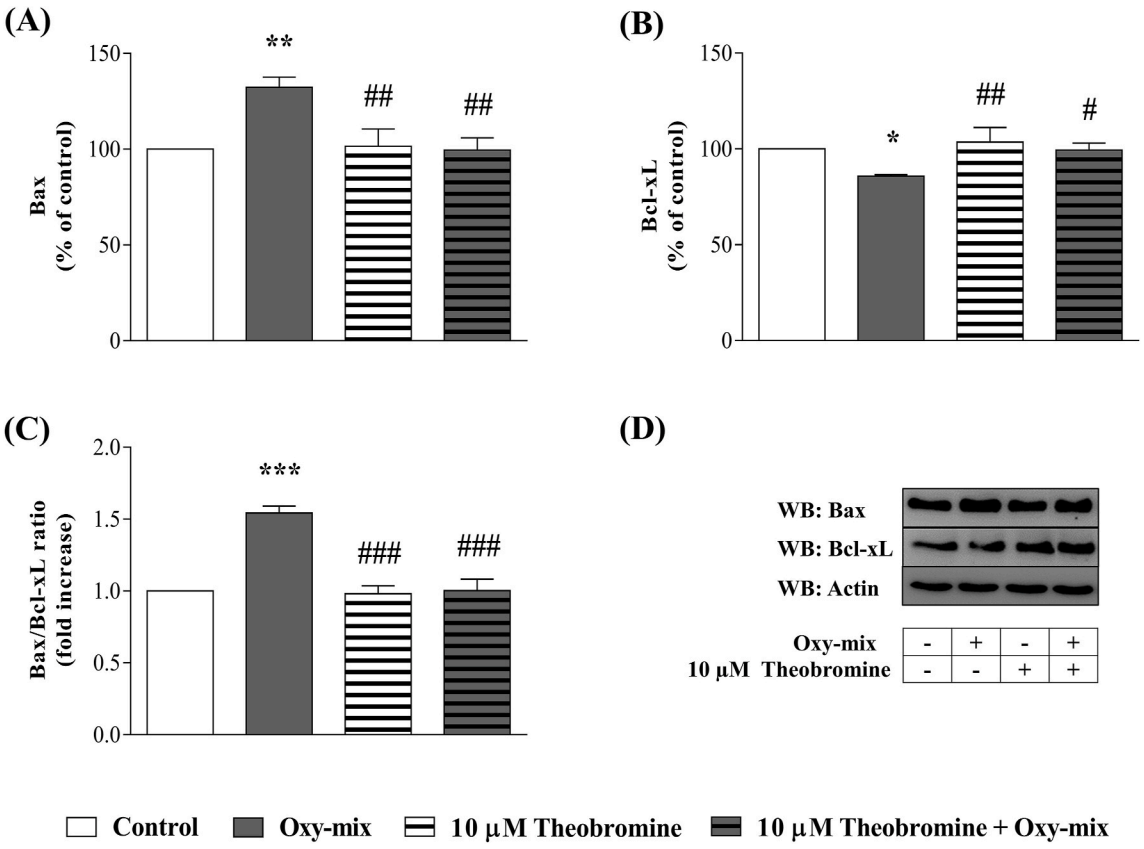

Fig. 4. Theobromine restores apoptotic protein levels impaired by the oxysterol mixture-cell treatment.
60 μM oxysterol mixture (Oxy-mix)-cell incubation reduced anti-apoptotic Bcl-xL, while markedly increased pro-apoptotic Bax proteins. One-hour cell pre-treatment with theobromine (10 μM) prevented changes of the two above-mentioned proteins involved in the apoptosis modulation. Bax (A) and Bcl-xL (B) cell protein levels were determined by Western Blotting in differentiated CaCo-2 cells treated with Oxy-mix for 24 h pre-treated or not with theobromine. Control: untreated cells. Bax/Bcl-xL ratio is expressed as fold increases versus control (C). Representative immunoblots of Bax, Bcl-xL, and actin as reference protein are shown (D). All values represent means ± SD of three independent experiments. Significantly different vs. control: *p < 0.05, **p < 0.01, ***p < 0.001; significantly different vs. Oxy-mix: #p < 0.05, ##p < 0.01, ###p < 0.001.

anti-inflammatory and anti-tumoral properties, as well as cardiovascular disease risk protection have been observed [26,36].

In the present study we demonstrated theobromine ability to prevent intestinal damage induced by dietary oxysterols in differentiated CaCo-2 cell monolayer, which represents an established model of intestinal barrier. The oxysterols used in our experiments were added to the cells in mixture and concentrations as they can be detectable in cholesterol-rich foods.

Oxysterol constituents of the mixture used for our experiments are widely known for their ability to induce cell damage. The effect of these oxysterols is strongly dependent from cell types involved and if they are added individually or in combination. For instance, 7 K, the most abundant component of the oxysterol mixture, can activate NADPH oxidase rapidly increasing oxidative stress and apoptotic cell death in macrophages [37]. However, concomitant addition of 7β-OH inhibited the 7-ketocholesterol-induced ROS production and apoptosis in the

same cells [38]. The administration of α-epox and β-epox has been recently found able to increase TNF-α, IL-1β and IL-6 in Wistar rats blood serum [39]. 7β-OH was proven to strongly increase intestinal cell release of IL-1α, IL-6, IL-8, MCP-1 and IL-23. However, its inflammatory action was strongly quenched when used with other dietary oxysterols added in mixture in differentiated CaCo-2 cells [9]. The concept that a simultaneous cell treatment with different oxysterols can have different effects than oxysterols added singularly, and that oxysterols are always present in food as a mixture must be taken into account.

In agreement with other studies on differentiated CaCo-2 cells, oxysterols used in mixture, at the same concentration used in our experiments, showed remarkable pro-inflammatory effects in terms of IL-8 and MCP-1 production, and MMP induction. Deiana and colleagues demonstrated that MMP activation by the dietary oxysterol mixture was closely associated with decreased levels of tight junctions and increased cell monolayer permeability in differentiated CaCo-2 cells [12]. Notably, a marked increase of MMP-9 was observed in the intestinal tissue of patients with IBD closely associated with increasing intestinal tight junction permeability, as also proved in CaCo-2 cells [40].

The anti-inflammatory property of theobromine against Oxy-mix action was demonstrated in our experimental model in terms of IL-8 and MCP-1 decrease in cell culture medium. These two cytokines have been shown to be involved in the induction of inflammatory and immune response when intestinal mucosa is damaged by bacteria toxin exposure [41].

Consistently, theobromine was also able to avoid tight junction protein level loss induced by the oxysterol mixture. Occludin, claudins and JAM-A are transmembrane proteins, which cooperate in the regulation of paracellular permeability of the barrier [42]. Proteinase deregulation was suggested to cause increased intestinal barrier damage, which on the other hand, contributes to fueling inflammatory and immune response, a typical feature of chronic diseases such as IBD [43]. Therefore, our observed cell monolayer permeability protection by theobromine could be due to its ability to inhibit the production of cytokines and MMPs in CaCo-2 cells.

Most of the pharmacological effects reported for methylxanthines are referred to their chemical structure and the sites where methyl group substitutions occur or not in basic xanthine.

For instance, caffeine was demonstrated to be a potent adenosine receptor inhibitor because of CH_3 substitution at N1position, while theobromine, which is not methylated in N1, showed lower affinity than caffeine for A1 and A2A adenosine receptor subtypes [22]. Methylxanthines was demonstrated to inhibit PDE4 by inserting them in the hydrophobic pocket of the enzyme catalytic site. However, their action is often non-selective and the exact chemical way by which they exert different potencies in blocking different PDE isoforms and induce cAMP accumulation is still unclear [44].

Despite caffeine is considered an efficient antioxidant [45], theobromine was found exerting a stronger scavenger effect in a L-dihydroxyphenylalanine-Cu(II) producing hydroxyl radical system because of methyl group absence at N1 position, which allows additional oxygen-centered radical formation to the one observed in caffeine reaction with $OH^{\cdot}$ at C-6 carbonyl oxygen [46]. Furthermore, the formation of a spontaneous chemical complex of theobromine (as well as theophylline) with copper-zinc superoxide dismutase was suggested to increase superoxide dismutase lifetime, thus prolonging antioxidant activity of this enzyme [47]. Theobromine was found to posses anti-inflammatory and antioxidant effects in chondrocytes treated with IL-1β, by suppressing cyclooxygenase-2, prostaglandin E2, TNF-α, MCP-1, MMP-3 and MMP-13, as well as reactive oxygen species, nitric oxide production and inducible nitric oxide synthase expression [48].

Therefore, theobromine could exert cell beneficial action by decreasing oxysterol pro-oxidant effects. Different studies showed that dietary oxysterols increased intracellular levels of oxygen species and triggered inflammatory damage in differentiated CaCo-2 cells. The principal cell signaling axis involved in oxygen species production appeared to be the up-regulation of colonic NADPH-oxidase followed by p38 and JNK MAPKs' activation, and NF-kB induction [10,21].

Theobromine anti-inflammatory action was proposed to be associated with its ability to down-regulate NF-kB activity [48]. Similarly, theobromine was suggested as a possible therapeutic approach in treating obesity for its capacity to reduce MCP-1 and IL-1β associated with the inhibition of pre-adipocyte differentiation into mature adipocytes in an *in vitro* model of fat tissue [36]. Pentoxifylline, a synthetic methylxanthine widely used as a drug, was observed to decrease MMP-2 activity via TNF-α inhibition in hypertensive rats' aorta [49].

Pentoxifylline action was also attributed to its ability to inhibit TLR pro-inflammatory pathway [50]. In our recent study using CaCo-2 cells treated with the dietary oxysterol mixture, we observed that not only CBS fractions with high quantities of (−)-epicatechin and tannins, but also the CBS fraction mainly containing theobromine were able to prevent TLR2 and 4 activation and TLR downstream effectors' production, i.e. IL-8, interferon (IFN)-β and TNF-α [11].

Theobromine inhibitory effect on PDE4 dependent-regulatory pathway was suggested as a mechanism of action to reduce obesity and related adipose tissue inflammation in mice [51,52]. The switch off of inflammation through the inhibition of PDE4 is actually considered as a promising therapeutic target for many inflammatory diseases [53,54], and could represent a possible mechanism involved in counteracting oxysterols-mediated cell damage.

An interesting study recently described the involvement of an endogenous enzymatically-induced oxysterol, 24-hydroxycholesterol, in the regulation of β-adrenoreceptor-dependent contraction in mice cardiomyocytes by activating PDE4 and transiently enhancing cell calcium. Inhibition of PDE, with unspecific or specific inhibitor, isobutylmethylxanthine or rolipram respectively, was found to suppress the effect of 24-hydroxycholesterol on β2-adrenoreceptor-dependent contractility [55].

PDE4 inhibition was found to attenuate the production of TNF-α and its apoptotic effect in neuronal cells by suppressing the activation of JNK and NF-kB [56]. Increased apoptosis can also play a role in barrier derangement; the excessive lumen cell shedding is associated to inflammatory intestinal disease activity [57,58]. We demonstrated that Oxy-mix induced apoptosis by enhancing pro-apoptotic Bax and decreasing anti-apoptotic Bcl-xL protein levels in differentiated CaCo-2 cells. In addition to that, theobromine pre-treatment reduced apoptotis in the intestinal monolayer by reversing cell protein levels, thus decreasing Bax/Bcl-xL ratio. Pro-inflammatory TNF-α overproduction is considered as one of the factors responsible for excessive epithelial death in IBD patient ileum and colon [59]. We would like to highlight the hypothesis that MMPs, which are responsible for increased intestinal permeability in IBD and experimental colitis [40,60], may also participate in apoptosis induction. MMP involvement in apoptosis was already shown in hepatic stellate and human cardiac stem cells [61,62]. Intestinal epithelium integrity requires highly dynamic interactions between cell-cell and cell-matrix. In fact, when normal intestinal epithelial cells lose their contact with the neighboring cells and extracellular matrix, a particular form of apoptosis generally called anoikis is triggered [63], and cell-cell contacts in primary colon cells have been shown to prevent apoptosis [64]. In a previous study, we demonstrated that increased intestinal permeability associated with tight junction level decrease was closely dependent by MMP-2 and -9 activations in CaCo-2 cell monolayer treated with the dietary oxysterol mixture [12].

Therefore, theobromine anti-apoptotic effect could be due to its ability to decrease the production of molecules directly involved in the apoptotic fate such as TNF-α, but also to avoid cell barrier derangement due to MMP activation and tight junction loss in an inflammatory environment.

5. Conclusions

The here reported data underline theobromine ability in preserving

intestinal cell monolayer from the damaging action of a mixture of oxysterols detectable in cholesterol rich foods. The main theobromine action mechanism consists in decreasing specific inflammatory mediators, which destabilize membrane structure integrity. CBS is gaining value as a polyphenol-rich cocoa by-product to be reused as a functional ingredient or cocoa substitute. Theobromine concentration levels are very high in CBS. Therefore, it is reasonable to hypothesize that this molecule can be partly responsible for the benefits associated with cocoa consumption.

Funding

This work was supported by the Università degli Studi di Torino (IT) [Award number BIAF_RILO_17_01]; [Award number BIAF_RILO_18_01], by the Ministero dell'Istruzione, dell'Università e della Ricerca (IT) [Award number BIAF_FFABR_17_01] grant recipient Fiorella Biasi.

Author Contributions

Conceptualization, F.B., N.I.; Funding acquisition, F.B.; Investigation, D.R., N.I., I.V.; Supervision, F.B., G.P.; Validation, B.S.; Writing –original draft, F.B., N.I., I.V.; Writing - review & editing, F.B., N.I.

Declarations of competing interest

None.

References

[1] M. Vancamelbeke, S. Vermeire, The intestinal barrier: a fundamental role in health and disease, Expet Rev. Gastroenterol. Hepatol. 11 (9) (2017) 821–834. https://doi:10.1080/17474124.2017.1343143.

[2] F. Scaldaferri, M. Pizzoferrato, V. Gerardi, L. Lopetuso, A. Gasbarrini, The gut barrier: new acquisitions and therapeutic approaches, J. Clin. Gastroenterol. 46 (Suppl) (2012) S12–S17. http://doi:10.1097/MCG.0b013e31826ae849.

[3] F. Rizzello, E. Spisni, E. Giovanardi, V. Imbesi, M. Salice, P. Alvisi, M.C. Valerii, P. Gionchetti, Implications of the westernized diet in the onset and progression of IBD, Nutrients 11 (5) (2019). http://doi:10.3390/nu11051033.

[4] B. Sottero, D. Rossin, G. Poli, F. Biasi, Lipid oxidation products in the pathogenesis of inflammation-related gut diseases, Curr. Med. Chem. 25 (11) (2018) 1311–1326. http://doi:10.2174/0929867324666170619104105.

[5] A. Otaegui-Arrazola, M. Menéndez-Carreño, D. Ansorena, I. Astiasarán, Oxysterols: a world to explore, Food Chem. Toxicol. 48 (12) (2010) 3289–3303. http://doi:10.1016/j.fct.2010.09.023.

[6] J. Plat, J.A. Nichols, R.P. Mensink, Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation, J. Lipid Res. 46 (11) (2005) 2468–2476. http://doi:10.1194/jlr.M500272-JLR200.

[7] M.I. Khan, J.S. Min, S.O. Lee, D.G. Yim, K.H. Seol, M. Lee, C. Jo, Cooking, storage, and reheating effect on the formation of cholesterol oxidation products in processed meat products, Lipids Health Dis. 14 (2015) 89. http://doi:10.1186/s12944-015-0091-5.

[8] F. Biasi, C. Mascia, M. Astegiano, E. Chiarpotto, M. Nano, B. Vizio, G. Leonarduzzi, G. Poli, Pro-oxidant and proapoptotic effects of cholesterol oxidation products on human colonic epithelial cells: a potential mechanism of inflammatory bowel disease progression, Free Radic. Biol. Med. 47 (12) (2009) 1731–1741. http://doi:10.1016/j.freeradbiomed.2009.09.020.

[9] C. Mascia, M. Maina, E. Chiarpotto, G. Leonarduzzi, G. Poli, F. Biasi, Proinflammatory effect of cholesterol and its oxidation products on CaCo-2 human enterocyte-like cells: effective protection by epigallocatechin-3-gallate, Free Radic. Biol. Med. 49 (12) (2010) 2049–2057. http://doi:10.1016/j.freeradbiomed.2010.09.033.

[10] T. Guina, M. Deiana, S. Calfapietra, B. Cabboi, M. Maina, C.I. Tuberoso, G. Leonarduzzi, P. Gamba, S. Gargiulo, G. Testa, G. Poli, F. Biasi, The role of p38 MAPK in the induction of intestinal inflammation by dietary oxysterols: modulation by wine phenolics, Food Funct 6 (4) (2015) 1218–1228. http://doi:10.1039/c4fo01116c.

[11] D. Rossin, L. Barbosa-Pereira, N. Iaia, G. Testa, B. Sottero, G. Poli, G. Zeppa, F. Biasi, A dietary mixture of oxysterols induces in vitro intestinal inflammation through TLR2/4 activation: the protective effect of cocoa bean shells, Antioxidants 8 (6) (2019). http://doi:10.3390/antiox8060151.

[12] M. Deiana, S. Calfapietra, A. Incani, A. Atzeri, D. Rossin, R. Loi, B. Sottero, N. Iaia, G. Poli, F. Biasi, Derangement of intestinal epithelial cell monolayer by dietary cholesterol oxidation products, Free Radic. Biol. Med. 113 (2017) 539–550. http://doi:10.1016/j.freeradbiomed.2017.10.390.

[13] A. Levine, J.M. Rhodes, J.O. Lindsay, M.T. Abreu, M.A. Kamm, P.R. Gibson, C. Gasche, M.S. Silverberg, U. Mahadevan, R.S. Boneh, E. Wine, O.M. Damas, G. Syme, G.L. Trakman, C.K. Yao, S. Stockhamer, M.B. Hammami, L.C. Garces, G. Rogler, I.E. Koutroubakis, A.N. Ananthakrishnan, L. McKeever, J.D. Lewis, Dietary guidance from the international organization for the study of inflammatory bowel diseases, Clin. Gastroenterol. Hepatol. 18 (6) (2020) 1381–1392. http://doi:10.1016/j.cgh.2020.01.046.

[14] H. Khalili, N. Håkansson, S.S. Chan, Y. Chen, P. Lochhead, J.F. Ludvigsson, A. T. Chan, A.R. Hart, O. Olén, A. Wolk, Adherence to a Mediterranean diet is associated with a lower risk of later-onset Crohn's disease: results from two large prospective cohort studies, Gut 69 (9) (2020) 1637–1644. http://doi:10.1136/gutjnl-2019-319505.

[15] F. Chicco, S. Magrì, A. Cingolani, D. Paduano, M. Pesenti, F. Zara, F. Tumbarello, E. Urru, A. Melis, L. Casula, M.C. Fantini, P. Usai, Multidimensional impact of mediterranean diet on IBD patients, Inflamm. Bowel Dis. izaa097 (2020) 1–9. In press, http://doi:10.1093/ibd/izaa097.

[16] F. Biasi, M. Astegiano, M. Maina, G. Leonarduzzi, G. Poli, Polyphenol supplementation as a complementary medicinal approach to treating inflammatory bowel disease, Curr. Med. Chem. 18 (31) (2011) 4851–4865. http://doi:10.2174/092986711797535263.

[17] T.C. Contreras, E. Ricciardi, E. Cremonini, P.I. Oteiza, (-)-Epicatechin in the prevention of tumor necrosis alpha-induced loss of Caco-2 cell barrier integrity, Arch. Biochem. Biophys. 573 (2015) 84–91. http://doi:10.1016/j.abb.2015.01.024.

[18] E. Cremonini, E. Daveri, A. Mastaloudis, A.M. Adamo, D. Mills, K. Kalanetra, S. N. Hester, S.M. Wood, C.G. Fraga, P.I. Oteiza, Anthocyanins protect the gastrointestinal tract from high fat diet-induced alterations in redox signaling, barrier integrity and dysbiosis, Redox. Biol. 26 (2019), 101269. http://doi:10.1016/j.redox.2019.101269.

[19] R. Nallathambi, A. Poulev, J.B. Zuk, I. Raskin, Proanthocyanidin-rich grape seed extract reduces inflammation and oxidative stress and restores tight junction barrier function in caco-2 colon cells, Nutrients 12 (6) (2020). http://doi:10.3390/nu12061623.

[20] F. Biasi, T. Guina, M. Maina, B. Cabboi, M. Deiana, C.I. Tuberoso, S. Calfapietra, E. Chiarpotto, B. Sottero, P. Gamba, S. Gargiulo, V. Brunetto, G. Testa, M.A. Dessì, G. Poli, G. Leonarduzzi, Phenolic compounds present in Sardinian wine extracts protect against the production of inflammatory cytokines induced by oxysterols in CaCo-2 human enterocyte-like cells, Biochem. Pharmacol. 86 (1) (2013) 138–145. http://doi:10.1016/j.bcp.2013.03.024.

[21] G. Serra, A. Incani, G. Serreli, L. Porru, M.P. Melis, C.I.G. Tuberoso, D. Rossin, F. Biasi, M. Deiana, Olive oil polyphenols reduce oxysterols -induced redox imbalance and pro-inflammatory response in intestinal cells, Redox. Biol. 17 (2018) 348–354. http://doi:10.1016/j.redox.2018.05.006.

[22] J.P. Monteiro, M.G. Alves, P.F. Oliveira, B.M. Silva, Structure-bioactivity relationships of methylxanthines: trying to make sense of all the promises and the drawbacks, Molecules 21 (8) (2016). http://doi:10.3390/molecules21080974.

[23] N. Singh, A.K. Shreshtha, M.S. Thakur, S. Patra, Xanthine scaffold: scope and potential in drug development, Heliyon 4 (10) (2018), e00829. http://doi:10.1016/j.heliyon.2018.e00829.

[24] J. Monteiro, M.G. Alves, P.F. Oliveira, B.M. Silva, Pharmacological potential of methylxanthines: retrospective analysis and future expectations, Crit. Rev. Food Sci. Nutr. 59 (16) (2019) 2597–2625. http://doi:10.1080/10408398.2018.1461607.

[25] O. Rojo-Poveda, L. Barbosa-Pereira, G. Zeppa, C. Stévigny, Cocoa bean shell-A by-product with nutritional properties and biofunctional potential, Nutrients 12 (4) (2020). http://doi:10.3390/nu12041123.

[26] E. Martínez-Pinilla, A. Oñatibia-Astibia, R. Franco, The relevance of theobromine for the beneficial effects of cocoa consumption, Front. Pharmacol. 6 (2015) 30. http://doi:10.3389/fphar.2015.00030.

[27] O.S. Usmani, M.G. Belvisi, H.J. Patel, N. Crispino, M.A. Birrell, M. Korbonits, D. Korbonits, P.J. Barnes, Theobromine inhibits sensory nerve activation and cough, Faseb. J. 19 (2) (2005) 231–233. http://doi:10.1096/fj.04-1990fje.

[28] B. Gottwalt, P. Tadi, Methlxanthines, StatPearls Publishing, Treasure Island (FL), 2020. http://www.ncbi.nlm.nih.gov/books/NBK559165/.

[29] I. Cova, V. Leta, C. Mariani, L. Pantoni, S. Pomati, Exploring cocoa properties: is theobromine a cognitive modulator? Psychopharmacology (Berlin) 236 (2) (2019) 561–572. http://doi:10.1007/s00213-019-5172-0.

[30] E.S. Mitchell, M. Slettenaar, N. vd Meer, C. Transler, L. Jans, F. Quadt, M. Berry, Differential contributions of theobromine and caffeine on mood, psychomotor performance and blood pressure, Physiol. Behav. 104 (5) (2011) 816–822. http://doi:10.1016/j.physbeh.2011.07.027.

[31] M.J. Baggott, E. Childs, A.B. Hart, E. de Bruin, A.A. Palmer, J.E. Wilkinson, H. de Wit, Psychopharmacology of theobromine in healthy volunteers, Psychopharmacology (Berlin) 228 (1) (2013) 109–118. http://doi:10.1007/s00213-013-3021-0.

[32] M.A. Klebanoff, J. Zhang, C. Zhang, R.J. Levine, Maternal serum theobromine and the development of preeclampsia, Epidemiology 20 (5) (2009) 727–732. http://doi:10.1097/EDE.0b013e3181aba664.

[33] J. Deree, J.O. Martins, H. Melbostad, W.H. Loomis, R. Coimbra, Insights into the regulation of TNF-alpha production in human mononuclear cells: the effects of non-specific phosphodiesterase inhibition, Clinics 63 (3) (2008) 321–328. http://doi:10.1590/s1807-59322008000300006.

[34] N. Sugimoto, S. Miwa, Y. Hitomi, H. Nakamura, H. Tsuchiya, A. Yachie, Theobromine, the primary methylxanthine found in Theobroma cacao, prevents malignant glioblastoma proliferation by negatively regulating phosphodiesterase-4, extracellular signal-regulated kinase, Akt/mammalian target of rapamycin kinase, and nuclear factor-kappa B, Nutr. Canc. 66 (3) (2014) 419–423. http://doi:10.1080/01635581.2013.877497.

[35] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254. http://doi:10.1006/abio.1976.9999.
[36] M.P. Fuggetta, M. Zonfrillo, C. Villivà, E. Bonmassar, G. Ravagnan, Inflammatory Microenvironment and adipogenic differentiation in obesity: the inhibitory effect of theobromine in a model of human obesity in vitro, Mediat. Inflamm. 2019 (2019) 1–10. ID:1515621, http://doi:10.1155/2019/1515621.
[37] G. Leonarduzzi, B. Vizio, B. Sottero, V. Verde, P. Gamba, C. Mascia, E. Chiarpotto, G. Poli, F. Biasi, Early involvement of ROS overproduction in apoptosis induced by 7-ketocholesterol, Antioxidants Redox Signal. 8 (3–4) (2006) 375–380. http://doi:10.1089/ars.2006.8.375.
[38] F. Biasi, G. Leonarduzzi, B. Vizio, D. Zanetti, A. Sevanian, B. Sottero, V. Verde, B. Zingaro, E. Chiarpotto, G. Poli, Oxysterol mixtures prevent proapoptotic effects of 7-ketocholesterol in macrophages: implications for proatherogenic gene modulation, Faseb. J. 18 (6) (2004) 693–695. http://doi:10.1096/fj.03-0401fje.
[39] T. Wielkoszyński, J. Zalejska-Fiolka, J.K. Strzelczyk, A.J. Owczarek, A. Cholewka, K. Kokoszczyk, A. Stanek, 5α,6α-Epoxyphytosterols and 5α,6α-epoxycholesterol increase nitrosative stress and inflammatory cytokine production in rats on low-cholesterol diet, Oxid. Med. Cell. Longev. 2020 (2020), 4751803. http://doi:10.1155/2020/4751803.
[40] R. Al-Sadi, M. Youssef, M. Rawat, S. Guo, K. Dokladny, M. Haque, M.D. Watterson, T.Y. Ma, MMP-9-induced increase in intestinal epithelial tight permeability is mediated by p38 kinase signaling pathway activation of MLCK gene, Am. J. Physiol. Gastrointest. Liver Physiol. 316 (2) (2019) G278–G290. http://doi:10.1152/ajpgi.00126.2018.
[41] C. Andrews, M.H. McLean, S.K. Durum, Cytokine tuning of intestinal epithelial function, Front. Immunol. 9 (2018) 1270. http://doi:10.3389/fimmu.2018.01270.
[42] A. Hartsock, W.J. Nelson, Adherens and tight junctions: structure, function and connections to the actin cytoskeleton, Biochim. Biophys. Acta 1778 (3) (2008) 660–669. http://doi:10.1016/j.bbamem.2007.07.012.
[43] H. Van Spaendonk, H. Ceuleers, L. Witters, E. Patteet, J. Joossens, K. Augustyns, A. M. Lambeir, I. De Meester, J.G. De Man, B.Y. De Winter, Regulation of intestinal permeability: the role of proteases, World J. Gastroenterol. 23 (12) (2017) 2106–2123. http://doi:10.3748/wjg.v23.i12.2106.
[44] S.H. Francis, K.R. Sekhar, H. Ke, J.D. Corbin, Inhibition of cyclic nucleotide phosphodiesterases by methylxanthines and related compounds, Handb. Exp. Pharmacol. (200) (2011) 93–133. http://doi:10.1007/978-3-642-13443-2_4.
[45] J.R. León-Carmona, A. Galano, Is caffeine a good scavenger of oxygenated free radicals? J. Phys. Chem. B 115 (15) (2011) 4538–4546. http://doi:10.1021/jp201383y.
[46] S. Azam, N. Hadi, N.U. Khan, S.M. Hadi, Antioxidant and prooxidant properties of caffeine, theobromine and xanthine, Med. Sci. Mon. Int. Med. J. Exp. Clin. Res. 9 (9) (2003) BR325–330.
[47] F. Wu, R. Liu, X. Shen, H. Xu, L. Sheng, Study on the interaction and antioxidant activity of theophylline and theobromine with SOD by spectra and calculation, Spectrochim. Acta Mol. Biomol. Spectrosc. 215 (2019) 354–362. http://doi:10.1016/j.saa.2019.03.001.
[48] R. Gu, Y. Shi, W. Huang, C. Lao, Z. Zou, S. Pan, Z. Huang, Theobromine mitigates IL-1β-induced oxidative stress, inflammatory response, and degradation of type II collagen in human chondrocytes, Int. Immunopharm. 82 (2020), 106226. http://doi:10.1016/j.intimp.2020.106226.
[49] B.R. Mattos, G.F. Bonacio, T.R. Vitorino, V.T. Garcia, J.H. Amaral, R. Dellalibera-Jovilliano, S.C. Franca, J.E. Tanus-Santos, E. Rizzi, TNF-α inhibition decreases MMP-2 activity, reactive oxygen species formation and improves hypertensive vascular hypertrophy independent of its effects on blood pressure, Biochem. Pharmacol. (2020), 114121. http://doi:10.1016/j.bcp.2020.114121.
[50] E.M. Speer, D.J. Dowling, L.S. Ozog, J. Xu, J. Yang, G. Kennady, O. Levy, Pentoxifylline inhibits TLR- and inflammasome-mediated in vitro inflammatory cytokine production in human blood with greater efficacy and potency in newborns, Pediatr. Res. 81 (5) (2017) 806–816. http://doi:10.1038/pr.2017.6.
[51] M.H. Jang, S. Mukherjee, M.J. Choi, N.H. Kang, H.G. Pham, J.W. Yun, Theobromine alleviates diet-induced obesity in mice via phosphodiesterase-4 inhibition, Eur. J. Nutr. (2020) 1–14. In press, http://doi:10.1007/s00394-020-02184-6.
[52] R. Zhang, E. Maratos-Flier, J.S. Flier, Reduced adiposity and high-fat diet-induced adipose inflammation in mice deficient for phosphodiesterase 4B, Endocrinology 150 (7) (2009) 3076–3082. http://doi:10.1210/en.2009-0108.
[53] M. Spadaccini, S. D'Alessio, L. Peyrin-Biroulet, S. Danese, PDE4 inhibition and inflammatory bowel disease: a novel therapeutic avenue, Int. J. Mol. Sci. 18 (6) (2017). http://doi:10.3390/ijms18061276.
[54] H. Li, J. Zuo, W. Tang, Phosphodiesterase-4 inhibitors for the treatment of inflammatory diseases, Front. Pharmacol. 9 (2018) 1048. http://doi:10.3389/fphar.2018.01048.
[55] U.G. Odnoshivkina, V.I. Sytchev, O. Starostin, A.M. Petrov, Brain cholesterol metabolite 24-hydroxycholesterol modulates inotropic responses to β-adrenoceptor stimulation: the role of NO and phosphodiesterase, Life Sci. 220 (2019) 117–126. http://doi:10.1016/j.lfs.2019.01.054.
[56] J. Xiao, R. Yao, B. Xu, H. Wen, J. Zhong, D. Li, Z. Zhou, J. Xu, H. Wang, Inhibition of PDE4 attenuates TNF-α-triggered cell death through suppressing NF-κB and JNK activation in HT-22 neuronal cells, Cell. Mol. Neurobiol. 40 (3) (2020) 421–435. http://doi:10.1007/s10571-019-00745-w.
[57] A. Negroni, S. Cucchiara, L. Stronati, Apoptosis, necrosis, and necroptosis in the gut and intestinal homeostasis, Mediat. Inflamm. 2015 (2015), 250762. http://doi:10.1155/2015/250762.
[58] M.E. Delgado, T. Grabinger, T. Brunner, Cell death at the intestinal epithelial front line, FEBS J. 283 (14) (2016) 2701–2719. http://doi:10.1111/febs.13575.
[59] A. Parker, L. Vaux, A.M. Patterson, A. Modasia, D. Muraro, A.G. Fletcher, H. M. Byrne, P.K. Maini, A.J.M. Watson, C. Pin, Elevated apoptosis impairs epithelial cell turnover and shortens villi in TNF-driven intestinal inflammation, Cell Death Dis. 10 (2) (2019) 108. http://doi:10.1038/s41419-018-1275-5.
[60] P. Nighot, R. Al-Sadi, M. Rawat, S. Guo, D.M. Watterson, T. Ma, Matrix metalloproteinase 9-induced increase in intestinal epithelial tight junction permeability contributes to the severity of experimental DSS colitis, Am. J. Physiol. Gastrointest. Liver Physiol. 309 (12) (2015) G988–G997. http://doi:10.1152/ajpgi.00256.2015.
[61] M. Roderfeld, R. Weiskirchen, S. Wagner, M.L. Berres, C. Henkel, J. Grötzinger, A. M. Gressner, S. Matern, E. Roeb, Inhibition of hepatic fibrogenesis by matrix metalloproteinase-9 mutants in mice, Faseb. J. 20 (3) (2006) 444–454. http://doi:10.1096/fj.05-4828com.
[62] S.K. Yadav, T.N. Kambis, S. Kar, S.Y. Park, P.K. Mishra, MMP9 mediates acute hyperglycemia-induced human cardiac stem cell death by upregulating apoptosis and pyroptosis in vitro, Cell Death Dis. 11 (3) (2020) 186. http://doi:10.1038/s41419-020-2367-6.
[63] M. Shanmugathasan, S. Jothy, Apoptosis, anoikis and their relevance to the pathobiology of colon cancer, Pathol. Int. 50 (4) (2000) 273–279. http://doi:10.1046/j.1440-1827.2000.01047.x.
[64] C. Hofmann, F. Obermeier, M. Artinger, M. Hausmann, W. Falk, J. Schoelmerich, G. Rogler, J. Grossmann, Cell-cell contacts prevent anoikis in primary human colonic epithelial cells, Gastroenterology 132 (2) (2007) 587–600. http://doi:10.1053/j.gastro.2006.11.017.