

## Effect of beta- and alpha-glucans on immune modulating factors expression in enterocyte-like Caco-2 and goblet-like LS 174T cells



Bruna De Felice<sup>a,\*,1</sup>, Simona Damiano<sup>b,1</sup>, Concetta Montanino<sup>b</sup>, Andrea Del Buono<sup>c</sup>, Giuliana La Rosa<sup>b</sup>, Bruna Guida<sup>b</sup>, Mariarosaria Santillo<sup>b</sup>

<sup>a</sup> Department of Environmental, Biological and Pharmaceutical Sciences and Technologies (DISTABIF), University of Campania "Luigi Vanvitelli", Via Vivaldi 43, 81100 Caserta, Italy

<sup>b</sup> Department of Clinical Medicine and Surgery, University of Naples "Federico II, Italy

<sup>c</sup> DD Research Clinich, Caserta, Italy

### ARTICLE INFO

#### Article history:

Received 1 December 2019

Received in revised form 6 March 2020

Accepted 7 March 2020

Available online 10 March 2020

#### Keywords:

$\alpha$  and  $\beta$ -glucans

ROS

Tumor necrosis factor (TNF- $\alpha$ )

Interleukin 1  $\beta$  (IL-1 $\beta$ )

Cyclooxygenase-2 (COX-2)

Oxidative stress

### ABSTRACT

Glucans are complex polysaccharides consisting of repeated units of D-glucose linked by glycosidic bonds. The nutritional contribution in  $\alpha$ -glucans is mainly given by starch and glycogen while in  $\beta$ -glucans by mushrooms, yeasts and whole grains, such as barley and spelt well represented in the Mediterranean Diet. Numerous and extensive studies performed on glucans highlighted their marked anti-tumor, antioxidant and immunomodulatory activity. It has recently been shown that rather than merely being a passive barrier, the intestinal epithelium is an essential modulator of immunity. Indeed, epithelial absorptive enterocytes and mucin secreting goblet cells can produce specific immune modulating factors, driving innate immunity to pathogens as well as preventing auto-immunity. Despite the clear evidence of the effects of glucans on immune system cells, there are only limited data about their effects on immune activity of mucosal intestinal cells strictly related to intestinal barrier integrity. The aim of the study was to evaluate the effects of  $\alpha$  and  $\beta$  glucans, alone or in combination with other substances with antioxidant properties, on reactive oxygen species (ROS) levels, on the expression of ROS-generating enzyme DUOX-2 and of the immune modulating factors Tumor Necrosis Factor (TNF- $\alpha$ ), Interleukin 1  $\beta$  (IL-1 $\beta$ ) and cyclooxygenase-2 (COX-2) in two intestinal epithelial cells, the enterocyte-like Caco-2 cells and goblet cell-like LS174T.

In our research, the experiments were carried out incubating the cells with glucans for 18 h in culture medium containing 0.2% FBS and measuring ROS levels fluorimetrically as dihydrodichlorofluoresce diacetate (DCF-DA) fluorescence, protein levels of DUOX-2 by Western blotting and mRNA levels of, TNF- $\alpha$ , IL-1 $\beta$  and COX-2 by qRT-PCR.  $\alpha$  and  $\beta$  glucans decreased ROS levels in Caco-2 and LS 174T cells. The expression levels of COX-2, TNF- $\alpha$ , and IL-1 $\beta$  were also reduced by  $\alpha$ - and  $\beta$ -glucans. Additive effects on the expression of these immune modulating factors were exerted by vitamin C. In Caco-2 cells, the dual oxidase DUOX-2 expression is positively modulated by ROS. Accordingly, in Caco-2 or LS174T cells treated with  $\alpha$  and  $\beta$ -glucans alone or in combination with Vitamin C, the decrease of ROS levels was associated with a reduced expression of DUOX-2. The treatment of cells with the NADPH oxidase (NOX) inhibitor apocynin decrease ROS, DUOX-2, COX-2, TNF- $\alpha$  and IL-1 $\beta$  levels indicating that NOX dependent ROS regulate the expression of immune modulating factors of intestinal cells. However, the combination of vitamin C,  $\alpha$  and  $\beta$ -glucans with apocynin did not exert an additive effect on COX-2, TNF- $\alpha$  and IL-1 $\beta$  levels when compared with  $\alpha$ -,  $\beta$ -glucans and Vitamin C alone. The present study showing a modulatory effect of  $\alpha$  and  $\beta$ -glucans on ROS and on the expression of immune modulating factors in intestinal epithelial cells suggests that the assumption of food containing high levels of these substances or dietary supplementation can contribute to normal immunomodulatory function of intestinal barrier.

© 2020 Published by Elsevier B.V.

\* Corresponding author at: Department of Environmental, Biological and Pharmaceutical Sciences and Technologies (DISTABIF), University of Campania "Luigi Vanvitelli", Via Vivaldi 43, 81100 Caserta, Italy.

E-mail address: [bruna.defelice@unina2.it](mailto:bruna.defelice@unina2.it) (B. De Felice).

<sup>1</sup> Equally contributed.

### 1. Introduction

Glucans belong to the class of "functional foods", food products that bring benefits to human health. Chemically, glucans are non-starchy, non-digestible and highly viscous polysaccharides, including various D-glucose polymers that differ in the position of glycosidic bonds, and can be short or long, branched or unbranched,  $\alpha$  or  $\beta$  isomers [1].  $\beta$ -

glucans from different sources share some common structures as they are all composed of  $\beta$ -linked glucose monomer. The health benefits of  $\beta$ -glucans have been extensively documented over the past two decades.  $\beta$ -glucans are allowed in several countries, (including the United States of America, Canada, Finland, Sweden, China, Japan, and Korea) as potent immunological activators and as disease-preventing agents for their strong anti-insulin resistance, anti-hypertension and anti-obesity effects as well as a part of anticancer or anti-inflammatory therapy.  $\beta$ -glucans are believed to stimulate the immune system, modulating humoral and cellular immunity, and thereby they have also beneficial effects in fighting infectious diseases, such as bacterial, viral, fungal, and parasitic diseases [2].

$\beta$ -glucans act on several immune receptors including Dectin-1, complement receptor (CR3) and TLR-2/6 and trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells. As a consequence, both innate and adaptive response can be modulated by beta-glucans and they can also enhance phagocytosis. The small beta-glucans fragments are eventually released by the macrophages and taken up by other immune cells leading to various immune responses [3].

Active Hexose-Related Compound (AHCC) is a mushroom extract derived from several species of Basidiomycetes mushrooms including Shiitake (*Lentinus edodes*) and Shimeji (*Lyophyllum shimeji*). This natural product is composed of a mixture of amino acids, minerals, polysaccharides and lipids enriched in  $\alpha$ -1,4-linked glucans. AHCC is used as a nutritional supplement in Japan and has been shown to be effective against hyperlipidemia, obesity and cancer. AHCC is an immunostimulatory agent and has improved the prognosis and quality of life of patients with liver, lung, and head and neck cancer [2]. AHCC has the potential to be used as an immunoenhancer in cases in which the immune system is compromised [4]. AHCC could induce high levels of IL-1 $\beta$  production from human monocytes [5] and a high production of various cytokines by macrophages and T lymphocytes, such as interferon- $\gamma$  (IFN- $\gamma$ ), interleukin (IL)-8, IL-1 $\beta$ , and tumor necrosis factor (TNF- $\alpha$ , IL-2, and IL-12) [6]. These findings provide new insight into how AHCC supplements could enhance human immunity by modulating monocytes and T cells response.

The gut mucosa is continuously exposed to food and microbial antigens. Both intestinal epithelial cells and gut-associated lymphoid tissue (GALT) have a pivotal role in maintaining the integrity of intestinal mucosa, as these cells guarantee a first line of defense against pathogens and toxic molecules. It has recently been shown that rather than merely being a non-immune passive barrier, the intestinal epithelium is itself an essential modulator of immunity. Indeed, epithelial cells are both a source as well as a target for numerous cytokines tuning mucosal functions and alterations of the intestinal microenvironment defined by both immune and epithelial cells, might actively contribute to the development of intestinal immune disorders, as inflammatory bowel disease (IBD) and celiac disease (CD). [7–9]

Despite the numerous and extensive studies highlighting the marked anti-tumor, antioxidant and immunomodulatory activity of glucans at systemic and intestinal level, the cellular and molecular mechanisms behind the reported effects remain unsolved. In particular, the effects of AHCC ( $\alpha$ -glucans) or  $\beta$ -glucans on the levels of immune modulating factors derived by epithelial intestinal cells has not been investigated yet.

The aim of the study was to evaluate the effects of  $\alpha$  and  $\beta$  glucans, alone or in combination with other substances with antioxidant properties, on reactive oxygen species (ROS) levels, on the expression of ROS-generating enzyme DUOX-2 involved in gut immunity. We also assessed the expression of the immune modulating factors Tumor Necrosis Factor (TNF- $\alpha$ ), Interleukin 1  $\beta$  (IL-1 $\beta$ ) and cyclooxygenase-2 (COX-2) in two intestinal epithelial cells, the enterocyte-like Caco-2 cells and goblet cell-like LS174T.

## 2. Materials and methods

### 2.1. Materials

Active Hexose-Related Compound (AHCC-Standard-FG) (extracted by *Lenticula Edodes*, mushroom),  $\beta$ -glucans 85 (by *Saccharomyces cerevisiae*) and ascorbic acid or vitamin C were purchased from Amino up chemical CO. LTD (Japan). 1-(4-Hydroxy-3-methoxyphenyl) ethanone, also known as Apocynin was purchased from Sigma-Aldrich (USA). The  $\alpha$ -glucans (AHCC),  $\beta$ -glucans and vitamin C was solubilized in sterile water and subsequently filtered with Sterile Millex® Filters for Cell Culture with 0.2  $\mu$ m pore size.

### 2.2. Cells cultures

LS174T and Caco-2 cells were obtained from the ATCC (American Type Culture Collection, USA) and routinely grown in 75 cm<sup>2</sup> flask. LS174T were grown in Advanced modified Eagle medium (A-MEM, GIBCO) containing 4.5 g/l glucose, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin (Sigma-Aldrich). Caco-2 cells were grown in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, USA), containing 4.5 g/l glucose, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin (Sigma-Aldrich, USA). The cells were kept in a 5% CO<sub>2</sub> and 95% air atmosphere at 37 °C.

### 2.3. Fluorimetric determination of reactive oxygen species (ROS)

Fluorimetric determination of ROS levels were determined by the membrane-permeant ROS sensitive fluorogenic probe 5,6-carboxy-2,7-dichlorofluorescein diacetate, DCHF-DA (Molecular Probes, Leiden, The Netherlands). LS174T and Caco-2 cells were grown to semi-confluence in 24 multiwell plates and then incubated for 18 h before the experiments in medium complete in presence and in absence of 100  $\mu$ M of  $\alpha$ -glucans (AHCC),  $\beta$ -glucans and 25  $\mu$ M vitamin C alone or in combination (mix). The cells were washed twice with PBS and incubated with 10  $\mu$ M DCHFDA in the culture medium without serum for 10 min at 37 °C. The cells were washed three times with PBS containing 10 mM glucose, 1.2 mM MgCl<sub>2</sub> and 1.2 mM CaCl<sub>2</sub>. Dichlorofluorescein (DCF) fluorescence was measured at different time intervals using the plate reader Fluoroskan Ascent FL fluorometer (Thermo Electron Oy, Vantaa, Finland) and data were analysed by Ascent software.

### 2.4. Western blotting analysis

LS174T and Caco-2 cells lysates were obtained in RIPA buffer containing : 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% deoxycholate 0.1% sodium dodecyl sulphate (SDS), 2.5 mM Napyrophosphate, 1mM  $\beta$ -glycerophosphate, 1mM NaVO<sub>4</sub>, 1mM NaF, 0.5 mM phenyl-methyl-sulfonyl-fluoride (PMSF), and a cocktail of protease inhibitors (Roche Applied Bioscience). The cells were kept for 15 min at 4 °C and disrupted by repeated aspiration through a 21-gauge needle. Cell lysates were centrifuged for 10 min at 11,600  $\times$  g and the pellets were discarded. Fifty micrograms of total proteins were subjected to SDS – 7.5% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After electrophoresis, the proteins were transferred onto a nitrocellulose filter membrane (GE-Healthcare) in presence of transfer Buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. Then the membranes were incubated with 5% non-fat milk in tris buffered saline, 0.1% Tween 20 (TBST, Bio-Rad Laboratories) at room temperature. Filters were probed with specific rabbit polyclonal antibodies against Duox-2 (Upstate), and then incubated with a peroxidase conjugated anti-rabbit secondary antibody (GE-Healthcare, UK). Protein bands were revealed by enhanced chemiluminescence (ECL) system (GE-Healthcare). and when specified,

quantified by densitometry using Scion Image software. Densitometric values were normalized to anti  $\alpha$ -tubulin antibody (Sigma–Aldrich).

### 2.5. RNA extraction from LS174T and Caco-2 cell line

Ten micrograms of total RNA from LS174T and Caco-2 cells were obtained. Trizol (Invitrogen, no. 15596-026) method has been used for isolation and purification of RNA [10]. RNA was isolated including a DNase digestion step. These standardized RNA isolation procedures guarantee high-quality RNA. Using the Agilent 2100 Bioanalyzer platform (Agilent Technologies) RNA samples were quality-checked documenting the identification of 18-S and 28-S ribosomal RNA (rRNA) peaks. The yields were 9–15  $\mu$ g, and the RNA Integrity Number (RIN) was between 8.2 and 10.

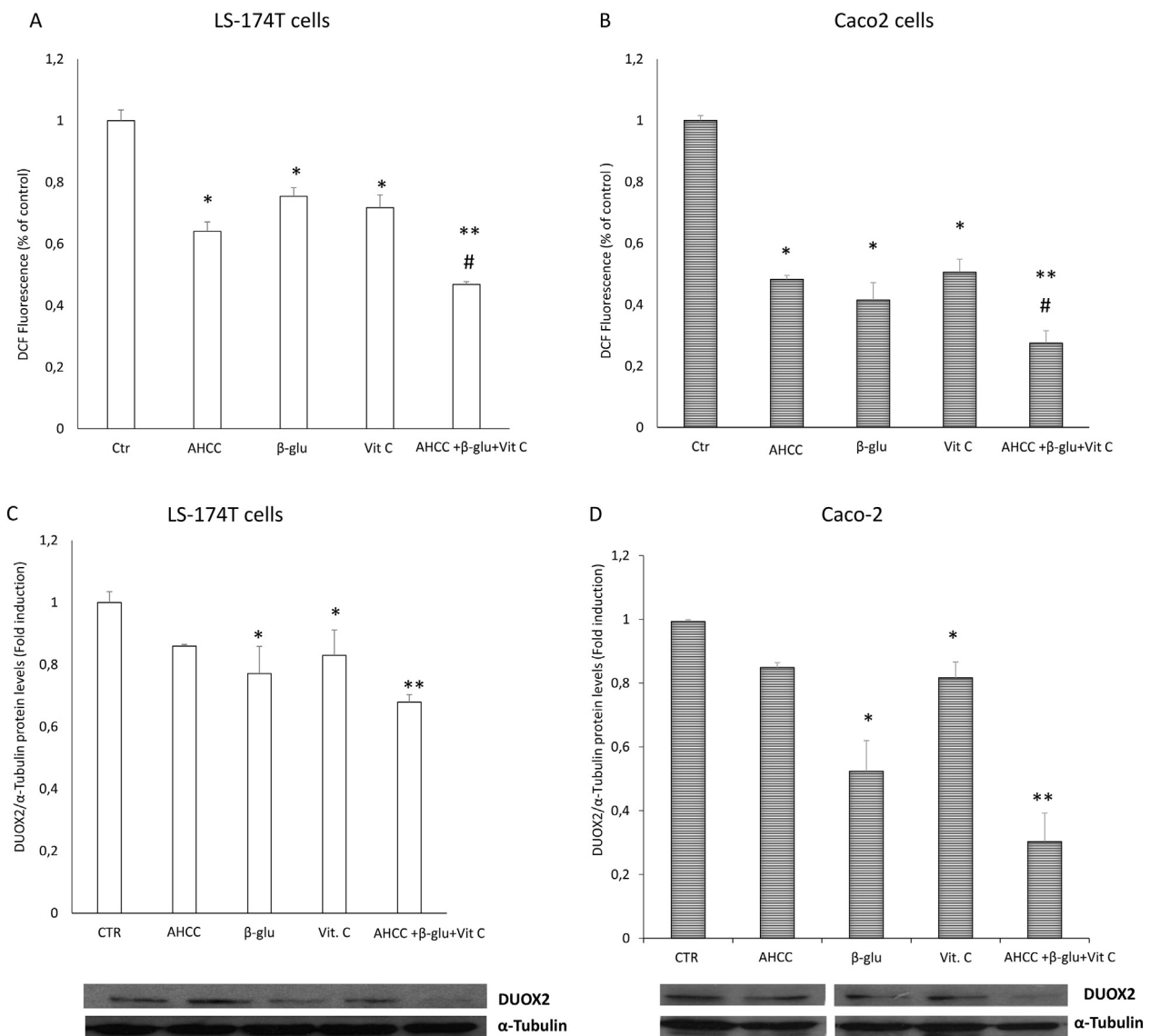
### 2.6. Quantitative reverse transcription-PCR (real-time RT-PCR)

To confirm the expression patterns of TNF- $\alpha$ , IL-1 $\beta$  and COX-2 genes, we performed a quantitative RT-PCR using the comparative Ct

method. Transcript levels of the target genes were normalized to G6PD (the internal control) after correcting for differences in amplification efficiencies. qRT-PCR reactions ( $n = 3$ ) were performed for each gene of interest using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). All genes investigated have previously been identified and sequences were available in GenBank. Primers for qRT-PCR analysis were designed using the Primer3 program (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>).

The final PCR reactions contained: 0.4  $\mu$ M of each primer; 0.25  $\times$  SYBR Green (Invitrogen); 4 mM MgCl<sub>2</sub> and as template 5  $\mu$ l of cDNA reverse transcribed from a standardized amount of total RNA (0.3  $\mu$ g). qRT-PCR was performed using Hotstart Taq polymerase (Qiagen) in a final volume of 20  $\mu$ l. All quantitative reactions were subjected to: 95  $^{\circ}$ C for 15 min followed by 45 cycles at 94  $^{\circ}$ C for 15 s, 59  $^{\circ}$ C 15 s and 72  $^{\circ}$ C 15 s. Melting curve analysis was applied to all reactions to ensure homogeneity of the reaction product.

Potential contamination was assessed by including non-reverse transcribed total RNA (genomic DNA contamination) and controls without template, observing no products in these reactions.



**Fig. 1.** Effects of  $\alpha$  and  $\beta$ -glucans on ROS levels in LS174 (A) and Caco2 (B) cells and on DUOX-2 protein levels on LS174 (C) and Caco2 (D) cells. LS174T and Caco-2 cells were grown to semi-confluence in 60-mm dishes the cells and stimulated for 18 h before the experiments in complete medium in presence and in absence of 100  $\mu$ M of  $\alpha$ -glucans (AHCC),  $\beta$ -glucans and 25  $\mu$ M vitamin C alone or in combination. The histograms, in A and B panel, show the mean  $\pm$  SEM of DCF fluorescence values relative to control of three independent experiments. The histograms, in C and D panels, show the DUOX 2 protein levels values (means  $\pm$  SEM) relative to control obtained by densitometric analysis of protein bands normalized to  $\alpha$ -Tubulin of three independent experiments. Under the histogram a representative experiment is shown \* $p < 0,05$  vs ctr; \*\* $p < 0,005$  vs ctr; # $p < 0,05$  vs AHCC,  $\beta$ -glu and Vit C.

### 3. Results

#### 3.1. $\alpha$ and $\beta$ glucans decreased ROS and DUOX-2 levels in LS-174TT and Caco-2 cells

We first evaluated whether glucans affect ROS levels in the enterocyte-like Caco-2 cells and goblet cell-like LS174T. The cells were incubated with 100  $\mu$ M of AHCC ( $\alpha$ -glucans),  $\beta$ -glucans or 25  $\mu$ M vit C alone or in combination, for 18 h in complete medium. ROS levels were measured fluorimetrically as dihydrodichlorofluorescein diacetate (DCF-DA) fluorescence. AHCC and  $\beta$ -glucans, as well as vitamin C, decreased ROS levels in LS 174T (Fig. 1A) and in Caco-2 (Fig. 1B) cells. Additive effects were observed when AHCC and  $\beta$ -glucans and vitamin C were administered in combination.

A key role in gut immunity is played by dual oxidases (DUOXs) [11]. DUOX 1 and 2 are NADPH oxidase enzymes that contain the membrane-bound flavocytochrome segment of NADPH oxidase (NOX), fused to an extra-cellular peroxidase domain [12]. Besides the targeted production of ROS essential for gut immunity, DUOX enzymes as well as the other NOX isoforms regulate different physiological functions, such as redox signaling, gene expression, cellular growth and differentiation [13–15]. DUOX enzymes are finely regulated by several signals at the intestinal level [16]. We previously demonstrated that, DUOX1 and 2 expression levels are positively modulated by NOX-derived ROS in SK-N-BE cells [17]. Accordingly, in Caco-2 cells expressing duox-2 isoform [18], treated with AHCC or  $\beta$ -glucans alone and in combination with Vit C, the decrease of ROS levels is associated with a reduced expression of DUOX-2 (Fig. 1D). DUOX-2 is expressed also in LS-174T cells and its protein levels are modulated by glucans and vitamin C as in Caco-2 cells (Fig. 1C).

#### 3.2. $\alpha$ and $\beta$ glucans decreased mRNA levels of COX-2, TNF- $\alpha$ , and IL-1 $\beta$

Then, we evaluated the effects of AHCC and  $\beta$ -glucans, alone or in association with vitamin C on mRNA levels of COX-2, TNF- $\alpha$ , and IL-1 $\beta$ , measured by qRT-PCR.

When administered alone, all the substances decrease the expression levels of the three immune modulatory markers in LS174T cells (Fig. 2A) and CaCo-2 (Fig. 2B) cells, and additive effects were observed when glucans and vitamin C were administered in combination.

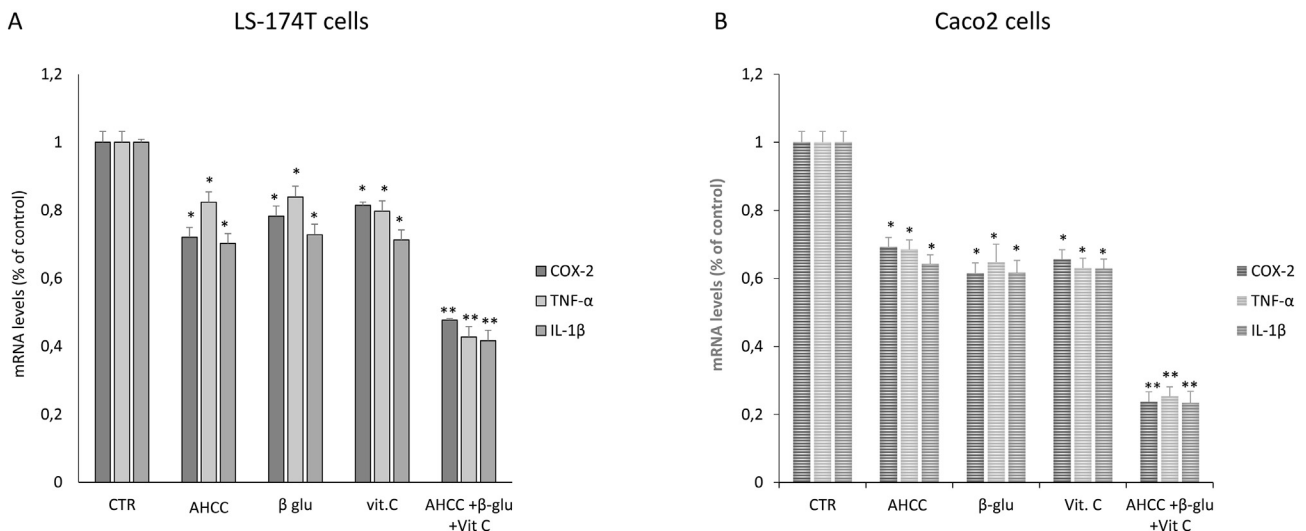
#### 3.3. NADPH oxidase inhibitor apocynin reduces ROS and DUOX-2 protein levels and COX-2, TNF- $\alpha$ , and IL-1 $\beta$ expression

The treatment of cells with the NADPH oxidase (NOX) inhibitor apocynin decrease ROS and DUOX-2 protein levels indicating that NOX-dependent ROS regulate the constitutive expression of immune modulating factors in intestinal cells (Fig. 3A–D). However, the combination of vitamin C, AHCC and  $\beta$ -glucans with apocynin did not exert an additive effect on ROS or DUOX-2 levels when compared with apocynin alone. Similar results on COX-2, TNF- $\alpha$  and IL-1 $\beta$  mRNA levels in both cell lines were observed when apocynin was administered alone or in association with glucans and vitamin C (Fig. 4A, B).

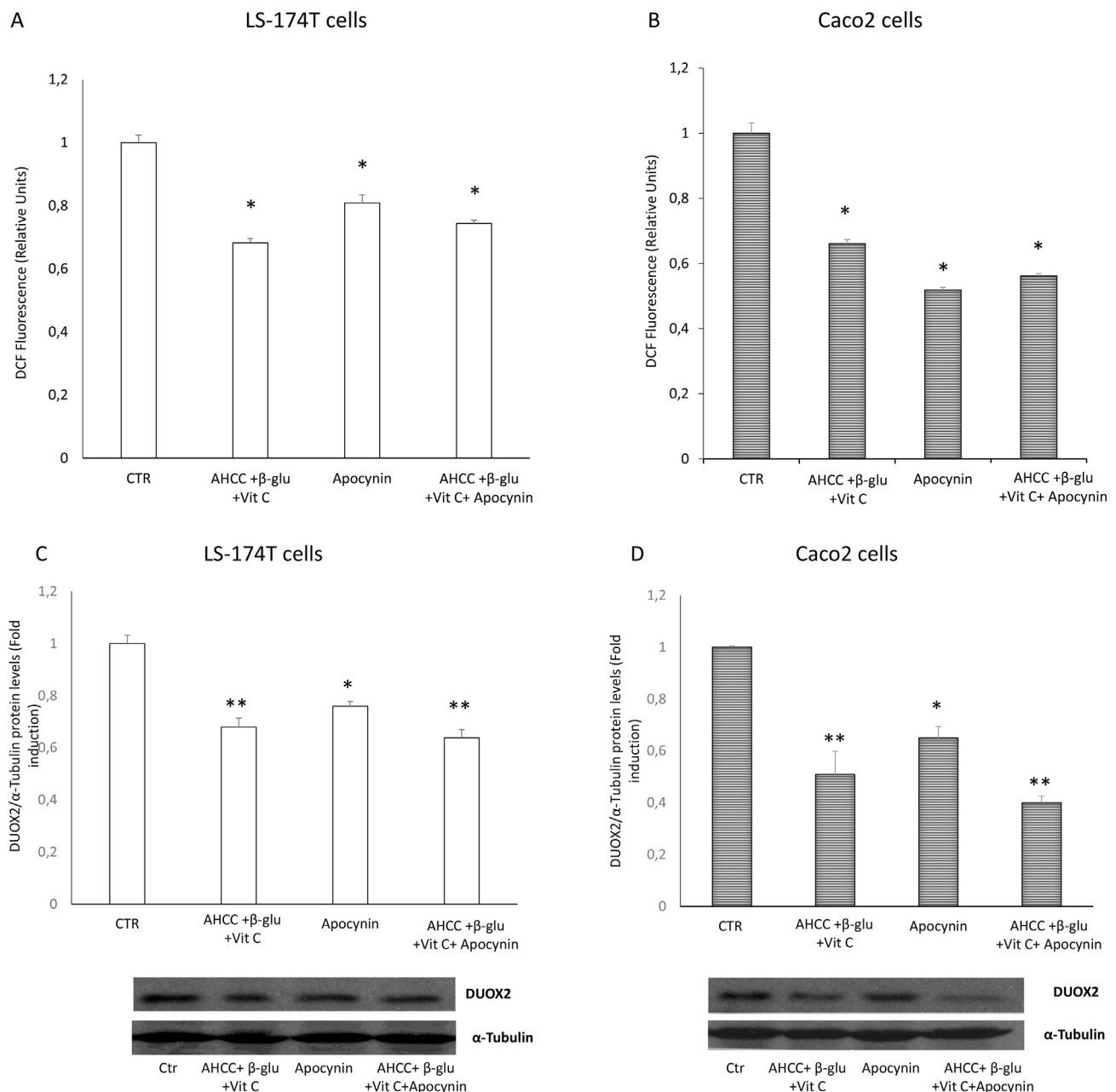
### 4. Discussion

Active hexose correlated compound has been established to have health benefits mediated by both immunomodulatory and antiproliferative effects [19,20]. Currently, AHCC has been shown to have an antiproliferative effect on ovarian cancer cell lines, via STAT3 phosphorylation, suggesting a potential alternative therapy for the treatment of ovarian cancer [20].

Several studies have reported a variety of beneficial effects, such as antioxidant, prevention of the onset of diabetes and enhancement of immune response. AHCC has been associated to immune functions modulation and playing a protective role against infection [21]. Intestinal epithelial cells provide a mucosal barrier protecting the intestine against environmental harmful substances and pathogenic microbial species [22]. Gut homeostasis can be positively or negatively affected by food components modulating mucus production [23] or barrier permeability [24]. The intestinal epithelium, rather than being only a passive physical barrier and a target of cytotoxic immune cells, is now considered as an active part of mucosal innate immunity. Indeed, intestinal epithelial cells are non-professional antigen-presenting cells and are both a source as well as a target for numerous cytokines [25]. This study was aimed at evaluating the combinatorial effect of natural compounds such as  $\alpha$ -glucans (AHCC) and  $\beta$ -glucans, with vitamin C, on immune functions of intestinal epithelial cells for their possible use as a nutritional supplement. We first evaluated glucans effect on ROS levels in the epithelial colorectal adenocarcinoma Caco-2 cells and goblet cell-like LS174T. Then, we measured protein levels of DUOX-2 and mRNA levels of TNF- $\alpha$ , IL-1 $\beta$  and COX-2.



**Fig. 2.** Effects of  $\alpha$  and  $\beta$ -glucans on mRNA levels of COX-2, TNF- $\alpha$  and IL-1 $\beta$  in LS174 (A) and CaCo2 (B) cells. LS174T and Caco-2 cells were grown to semi-confluence in 60-mm dishes the cells and stimulated for 18 h before the experiments in complete medium in presence and in absence of 100  $\mu$ M of  $\alpha$ -glucans (AHCC),  $\beta$ -glucans and 25  $\mu$ M vitamin C alone or in combination. The histograms show the mRNA values (means  $\pm$  SEM) relative to control of three independent experiments. \* $p < 0,05$  vs ctr; \*\* $p < 0,005$  vs ctr.



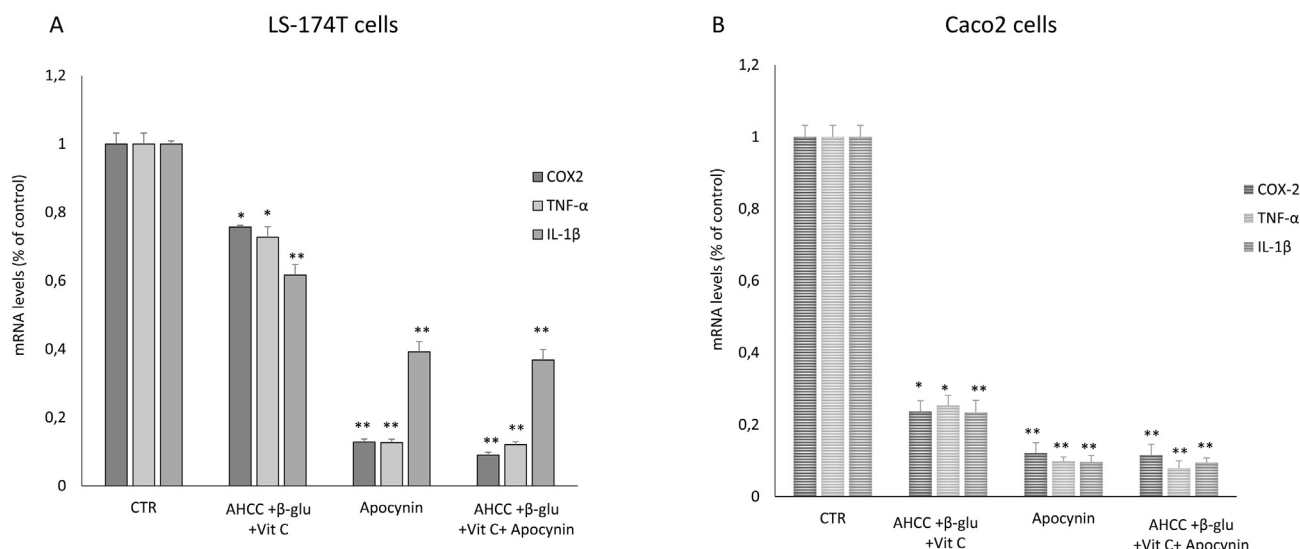
**Fig. 3.** Effects of apocynin (50  $\mu$ M) on ROS levels in LS174 (A) and CaCo2 (B) cells and on DUOX-2 protein levels on LS174 (C) and CaCo2 (D) cells. LS174T and Caco-2 cells were grown to semi-confluence in 60-mm dishes the cells were preincubated for 1 h with Apocynin (50  $\mu$ M) and then stimulated for 18 h before the experiments in complete medium in presence and in absence of 100  $\mu$ M of  $\alpha$ -glucans (AHCC),  $\beta$ -glucans and 25  $\mu$ M vitamin C alone or in combination. The histograms in A and B panel show the DCF fluorescence values (mean  $\pm$  SEM) relative to control of three independent experiments. \* $p < 0.05$  vs ctr. The histograms, in C and D panels, show the DUOX 2 protein values (means  $\pm$  SEM) relative to control obtained by densitometric analysis of protein bands normalized to  $\alpha$ -Tubulin of three independent experiments. Under the histogram a representative experiment is shown \* $p < 0,05$  vs ctr; \*\* $p < 0,005$  vs ctr.

$\beta$ -glucans have significant *in vitro* antioxidant properties [26]. In addition, in fish,  $\beta$ -glucans have been demonstrated to protect the intestine by oxidative stress through the activation, among others, of the transcription factor Nrf-2, which is a master modulator of ROS-inducible detoxifying enzymes, increasing resistance to oxidative stress [27–30]. Interestingly, among ROS-inducible enzyme, SOD1, in addition to antioxidant effects, has also neuromodulatory activity through the interaction with M1 receptor and activation of downstream signaling [31–33]. Accordingly, with data showing antioxidant effects of  $\beta$ -glucans, we found that ROS levels decreased both in LS174T and in Caco-2 cells when stimulated with  $\beta$ -glucans. Also AHCC, known for its immunomodulatory activity [34–36] and for the inhibitory effects on iNOS in hepatocytes [37] decrease ROS levels in intestinal cells.

When glucans have been administered in combination with vitamin C a stronger effect on ROS levels was observed.

Since oxidative stress is one of the primary causal factors for intestinal inflammatory diseases, scavenging of reactive oxygen species by glucans, in addition to their other biological activities, can contribute to the prevention of these diseases and even cancer.

Oxidative stress is an hallmark also for neurodegenerative and autoimmune diseases. An example is given by excessive glutamate release at the site of demyelination and axonal degeneration in multiple sclerosis plaques inducing oxidative stress [38,39]. Some disease-modifying drugs induce antioxidant enzyme activation ameliorating oxidative stress in multiple sclerosis patients [40]. Recently, it has been shown that patients affected by neurodegenerative or autoimmune diseases



**Fig. 4.** Effects of apocynin on mRNA levels of COX-2, TNF- $\alpha$  and IL-1 $\beta$  in LS174 (A) and Caco2 (B) cells. LS174T and Caco-2 cells were grown to semi-confluence in 60-mm dishes the cells were preincubated for 1 h with Apocynin (50  $\mu$ M) and then stimulated for 18 h before the experiments in complete medium in presence and in absence of 100  $\mu$ M of  $\alpha$ -glucans (AHCC),  $\beta$ -glucans and 25  $\mu$ M vitamin C alone or in combination. The histograms show the mRNA values (mean  $\pm$  SEM) relative to control in three independent experiments. \* $p < 0,05$  vs ctr; \*\* $p < 0,005$  vs ctr.

have an altered microbiome and changes in intestinal permeability that can lead to profound alterations of peripheral and central nervous system immune regulation [41]. Glucans, due to their antioxidant and immunomodulatory effects on intestinal cells can contribute to the maintenance of gut homeostasis suggesting its use also in other diseases in addition to that of gastrointestinal tract.

Previous data evidenced that DUOX enzymes are induced by PDGF in neuronal cells through NOX2-dependent ROS production [17]. In Caco-2 and LS174T cells, the decrease of ROS levels by glucans was paralleled by a decrease of DUOX-2 protein levels confirming the existence of a redox-dependent regulation of DUOX-2 expression also at intestinal level. DUOX enzyme have an essential role in oxidant-dependent antimicrobial response in epithelia as well as in maintaining intestinal barrier integrity through the modulation of gut permeability, in intracellular redox signaling, and others biological activities [42,43]. However, if low levels of DUOX-derived  $H_2O_2$  are essential for many gut functions, unwarranted, long-term DUOX activation can lead to chronic gut inflammation; considering this aspect, the inhibitory effects of glucans on DUOX-2 levels could be beneficial to counteract intestinal inflammation.

Enterocyte-like Caco-2 cells have been shown to produce cytokine and eicosanoids in response to pathogenic and non-pathogenic bacteria [44]. In addition, goblet cells, at the airway level, have been shown to secrete pro-inflammatory cytokines [45,46]. We found that intestinal goblet cells and Caco-2 cells constitutively produce proinflammatory cytokines; a reduction in the expression levels of COX-2, TNF- $\alpha$  and IL-1 $\beta$  has been observed when glucans and vitamin C were used alone and a drastic reduction when they were used in combination. Vitamin C has well known antioxidant, anti-inflammatory, and many other beneficial properties [47,48]. In rats, Vitamin C supplementation attenuated 5-fluorouracil-induced lipid peroxidation, myeloperoxidase (MPO) activity, activation of NF- $\kappa$ B and expression of COX-2, thus preventing gastrointestinal toxicity [49]. Our data confirm the antiinflammatory activity of vitamin C in intestinal cells evidencing a synergic effect of vitamin C and glucans.

In the attempt to evaluate the presence of a regulatory network linking NOX derived ROS with DUOX-2, COX-2, TNF- $\alpha$  and IL1- $\beta$  levels in cell stimulated with glucans we evaluated the effect of apocynin, a NOXs inhibitor and a bioactive phytochemical with prominent and anti-oxidant activities [50]. Apocynin alone exerted an inhibitory effect

on ROS/DUOX and inflammatory markers; however, the absence of additive effects of apocynin and glucans suggest that the pathway activated by these two substances converge: glucans and apocynin could reduce the levels of ROS and consequently of DUOX-2, COX-2 and cytokines by inhibiting NOX enzyme.

COX-2 is an enzyme expressed at low levels in normal cells in response to physical, biological, chemical, or UV light stimuli and it is released at the site of tissue injury to produce a hormone-like substance called prostaglandin E2 (PGE2) that stimulates pain and inflammation. Pro-inflammatory signals including cytokines, TNF- $\alpha$  and IL-1, stimulate COX-2 transcription via activation of different signaling pathways [6]. The effects of glucans and vitamin C on COX-2 expression levels could be mediated by their inhibitory effects on cytokine production.

It has been hypothesized that nonsteroidal anti-inflammatory drugs play an anti-inflammatory and anti-cancer role through the inhibition of COX-2. A valid preventive treatment could be the use of (NSAIDs), which however have an important side effect: increased cardiovascular risk or target therapies selectively against Cox-2, however, the latter approach is also counterproductive because inhibition of this enzyme would involve a reduction in prostanoids with antithrombotic activity [51]. Glucans represent future perspectives about the possibility of using a natural component to lower the activity of an enzyme currently lowered only with target therapy or NSAIDs.

The modulatory effect of  $\alpha$  and  $\beta$ -glucans on ROS and DUOX-2 protein levels and on the expression of immune modulating factors in intestinal epithelial cells suggest that the intake of food containing high levels of these substances or their dietary supplementation. Besides, maintaining low levels of oxidative stress and of pro-inflammatory molecules in the intestinal microenvironment, can contribute to the integrity and normal functions of intestinal mucosal barrier.

#### CRedit authorship contribution statement

**Bruna De Felice:** Conceptualization, Methodology, Writing - review & editing. **Simona Damiano:** Data curation, Software. **Concetta Montanino:** Visualization, Investigation. **Andrea Del Buono:** Supervision. **Giuliana La Rosa:** Software, Validation. **Bruna Guida:** Investigation. **Mariarosaria Santillo:** Writing - original draft.

## References

- [1] A. Choromanska, J. Kulbacka, J. Harasym, M. Dubinska-Magiera, J. Saczko, Anticancer activity of oat  $\beta$ -glucan in combination with electroporation on human cancer cells, *Acta Pol. Pharm.* 74 (2017) 616–623.
- [2] K.I. B., J.-S. Choi, Clinical and physiological perspectives of  $\beta$ -glucans: the past, present, and future, *Int. J. Mol. Sci.* 18 (2017) 1906.
- [3] G.C. Chan, W.K. Chan, D.M. Sze, The effects of beta-glucan on human immune and cancer cells, *J. Hematol. Oncol.* 2 (2009) 25, <https://doi.org/10.1186/1756-8722-2-25>.
- [4] Y. Zhinan, F. Hajime, T. Walshe, Effects of active hexose correlated compound on frequency of CD4+ and CD8+ T cells producing interferon- $\gamma$  and/or tumor necrosis factor- $\alpha$  in healthy adults, *Hum. Immunol.* 71 (2010) 1187–1190, <https://doi.org/10.1016/j.humimm.2010.08.006>.
- [5] Won-Woo Lee, Naeun Lee, Hajime Fujii, Insoo Kang, Active hexose correlated compound promotes T helper (Th) 17 and 1 cell responses via inducing IL-1 $\beta$  production from monocytes in humans, *Cell. Immunol.* 275 (2012) 19–23.
- [6] B. Corradetti, Bioactive immunomodulatory compounds: a novel combinatorial strategy for integrated medicine in oncology? BAIC Exposure in Cancer Cells, *Integr. Cancer Ther.* 18, 2019 <https://doi.org/10.1177/1534735419866908>.
- [7] C.S. Guerreiro, Á. Calado, J. Sousa, J.E. Fonseca, Diet, microbiota, and gut permeability –The unknown triad in rheumatoid arthritis, *Front. Med.* (14 December 2018) <https://doi.org/10.3389/fmed.2018.00349>.
- [8] D. Wang, R.N. DuBois, The role of COX-2 in intestinal inflammation and colorectal cancer, *Oncogene* 29 (2010) 781–788.
- [9] W.D. Rees, L.M. Sly, T.S. Steiner, How do immune and mesenchymal cells influence the intestinal epithelial cell compartment in inflammatory bowel disease? Let's crosstalk about it! *J. Leukoc. Biol.* (2020) <https://doi.org/10.1002/JLB.3MIR0120-567RFeb.14>.
- [10] P. Chomczynski, A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples, *Biotechniques* 15 (1993) 532–534.
- [11] E.M. Ha, K.A. Lee, S.H. Park, S.H. Kim, H.J. Nam, Regulation of DUOX by the G alpha q-phospholipase C beta-2a+ pathway in Drosophila gut immunity, *Dev. Cell* 16 (2009) 386–397.
- [12] A. Donko, Z. Peterfi, A. Sum, T. Leto, M. Geiszt, Dual oxidases, *Phil Trans R Soc* 360 (2005) 2301–2308.
- [13] K. Bedard, K.H. Krause, The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology, *Physiol. Rev.* 87 (2007) 245–313.
- [14] S. Damiano, E. Muscaricello, G. La Rosa, M. Di Maro, P. Mondola, M. Santillo, Dual role of reactive oxygen species in muscle function: can antioxidant dietary supplements counteract age-related sarcopenia?, *Int. J. Mol. Sci.* 20 (2019) art. no. 3815, DOI: <https://doi.org/10.3390/ijms20153815>.
- [15] R. Accetta, S. Damiano, A. Morano, P. Mondola, R. Paternò, E.V. Avvedimento, M. Santillo, Oxygen species derived from NOX3 and NOX5 drive differentiation of human oligodendrocytes, *Front. Cell. Neurosci.* 10 (2016) <https://doi.org/10.3389/fncel.2016.00146art. no. 146>.
- [16] F. Leulier, J. Royet, Maintaining immune homeostasis in fly gut, *Nat. Immunol.* 10 (2009) 936–938.
- [17] S. Damiano, R. Fusco, A. Morano, M. de Mizio, R. Paternò, A. de Rosa, R. Spinelli, S. Amente, R. Frunzio, P. Mondola, F. Miot, P. Laccetti, M. Santillo, E.V. Avvedimento, Reactive oxygen species regulate the levels of dual oxidase (duox1-2) in human neuroblastoma cells, *PLoS One* 7 (2012) art. no. e34405, DOI: <https://doi.org/10.1371/journal.pone.0034405>.
- [18] R.A. El Hassani, N. Benfares, B. Caillou, M. Talbot, J.C. Sabourin, V. Belotte, S. Morand, S. Gnidehou, D. Agnandji, R. Ohayon, J. Kaniewski, M.S. Noël-Hudson, J.M. Bidart, M. Schlumberger, A. Virion, C. Dupuy, Dual oxidase 2 is expressed all along the digestive tract, *Am. J. Physiol. Gastrointest. Liver Physiol.* 288 (2005) 933–942 (Epub 2004 Dec 9).
- [19] Lata Mathew, Anjali Gaikwad, Anneliese Gonzalez, Elizabeth K. Nugent, Judith A. Smith, Evaluation of active hexose correlated compound (AHCC) in combination with anticancer hormones in orthotopic breast cancer models, *Integr. Cancer Ther.* 16 (2017) 300–307, <https://doi.org/10.1177/1534735417704948>.
- [20] Jin Young Choi, Seul Lee, Sun-Mi Yun, Dong Hoon Suh, Kidong Kim, Jae Hong No, Eun-Hwan Jeong, Yong Beom Kim, Active hexose correlated compound (AHCC) inhibits the proliferation of ovarian cancer cells by suppressing signal transducer and activator of transcription 3 (STAT3) activation, *Nutr. Cancer* 70 (2018) 109–115, <https://doi.org/10.1080/01635581.2018.1380203>.
- [21] Y. Gao, D. Zhang, B. Sun, H. Fujii, K. Kosuna, Y. Zhinan, Active hexose correlated compound enhances tumor surveillance through regulating both innate and adaptive immune responses, *Cancer Immunol. Immunother.* 55 (2006) 1258–1266.
- [22] R. Sender, S. Fuchs, R. Milo, Revised estimates for the number of human and bacteria cells in the body, *PLoS Biol.* 14 (2016), e1002533. <https://doi.org/10.1371/journal.pbio.1002533>.
- [23] S. Damiano, A. Sasso, B. De Felice, I. Di Gregorio, G. La Rosa, G.A. Lupoli, A. Belfiore, P. Mondola, M. Santillo, Quercetin increases MUC2 and MUC5AC gene expression and secretion in intestinal goblet cell-like LS174T via PLC/PKC $\alpha$ /ERK1-2 pathway, *Front. Physiol.* 9 (2018) art. no. 357, DOI: <https://doi.org/10.3389/fphys.2018.00357>.
- [24] L. Adrian Lopresti, The problem of curcumin and its bioavailability: could its gastrointestinal influence contribute to its overall health-enhancing effects? *Adv. Nutr.* 9 (2018) 41–50, <https://doi.org/10.1093/advances/nmx011>.
- [25] S. Vitale, C. Strisciuglio, L. Pisapia, E. Miele, P. Barba, A. Vitale, S. Cenni, V. Bassi, M. Maglio, G. Del Pozzo, R. Troncone, A. Staiano, C. Gianfrani, Cytokine production profile in intestinal mucosa of paediatric inflammatory bowel disease, *PLoS One* 12 (2017), e0182313. <https://doi.org/10.1371/journal.pone.0182313> (eCollection 2017).
- [26] K. Kofuji, A. Aoki, K. Tsubaki, M. Konishi, T. Isobe, Y. Murata, Antioxidant activity of  $\beta$ -glucan, *ISRN Pharm.* (2012), 125864. <https://doi.org/10.5402/2012/125864> (Epub 2012 Feb 19).
- [27] R. Faraonio, P. Vergara, D. Di Marzo, M. Napolitano, T. Russo, F. Cimino, Transcription regulation in NIH3T3 cell clones resistant to diethylmaleate-induced oxidative stress and apoptosis, *Antioxid. Redox Signal.* 8 (2006) 365–374.
- [28] R. Caggiano, F. Cattaneo, O. Moltedo, G. Esposito, C. Perrino, B. Trimarco, R. Ammendola, R. Faraonio, miR-128 is implicated in stress responses by targeting MAFG in skeletal muscle cells, *Oxidative Med. Cell. Longev.* (2017), 9308310. <https://doi.org/10.1155/2017/9308310>.
- [29] S. Dhakshinamoorthy, D.J. Long 2nd, A.K. Jaiswal, Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens, *Curr. Top. Cell. Regul.* 36 (2000) 201–216.
- [30] S. Damiano, A. Sasso, R. Accetta, M. Monda, B. De Luca, L.M. Pavone, A. Belfiore, M. Santillo, P. Mondola, Effect of mutated Cu, Zn superoxide dismutase (SOD1G93A) on modulation of transductional pathway mediated by M1 muscarinic receptor in SK-N-BE and NSC-34 cells, *Front. Physiol.* 9 (2018), art. no. 611, DOI: <https://doi.org/10.3389/fphys.2018.00611>.
- [31] S. Damiano, T. Petrozziello, V. Ucci, S. Amente, M. Santillo, P. Mondola, Cu-Zn superoxide dismutase activates muscarinic acetylcholine M1 receptor pathway in neuroblastoma cells, *Mol. Cell. Neurosci.* 52 (2013) 31–37, <https://doi.org/10.1016/j.mcn.2012.11.001>.
- [32] A. Viggiano, R. Serù, S. Damiano, B. De Luca, M. Santillo, P. Mondola, Inhibition of long-term potentiation by CuZn superoxide dismutase injection in rat dentate gyrus: involvement of muscarinic M1 receptor, *J. Cell. Physiol.* 227 (2012) 3111–3115, <https://doi.org/10.1002/jcp.23062>.
- [33] A. Secondo, M. De Mizio, L. Zirpoli, M. Santillo, P. Mondola, The Cu-Zn superoxide dismutase (SOD1) inhibits ERK phosphorylation by muscarinic receptor modulation in rat pituitary GH3 cells, *Biochem. Biophys. Res. Commun.* 376 (2008) 143–147, <https://doi.org/10.1016/j.bbrc.2008.08.110>.
- [34] J.F. Mallet, E. Graham, B.W. Ritz, K. Homma, C. Matar, Active hexose correlated compound (AHCC) promotes an intestinal immune response in BALB/c mice and in primary intestinal epithelial cell culture involving toll-like receptors TLR-2 and TLR-4, *Eur. J. Nutr.* 55 (2016) 139–146, <https://doi.org/10.1007/s00394-015-0832-2>.
- [35] B.W. Ritz, S. Nogusa, E.A. Ackerman, E.M. Gardner, Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection, *J. Nutr.* 136 (2006) 2868–2873.
- [36] S. Nogusa, J. Gerbino, B.W. Ritz, Low-dose supplementation with active hexose correlated compound improves the immune response to acute influenza infection in C57BL/6 mice, *Nutr. Res.* 29 (2009) 139–143.
- [37] K. Matsui, Yusai Kawaguchi, Takashi Ozaki, Katsuji Tokuhara, Hironori Tanaka, Masaki Kaibori, Yoichi Matsui, Yasuo Kamiyama, Koji Wakame, Takehito Miura, Mikio Nishizawa, Tadayoshi Okumura, Effect of active hexose correlated compound on the production of nitric oxide in hepatocytes, *J. Parenter. Enter. Nutr.* 31 (2007) 373–381.
- [38] N. Potenza, N. Mosca, P. Mondola, S. Damiano, A. Russo, B. De Felice, Human miR-26a-5p regulates the glutamate transporter SLC1A1 (EAAT3) expression. Relevance in multiple sclerosis, *Biochim. Biophys. Acta Mol. Basis Dis.* 1864 (2018) 317–323.
- [39] P. Villoslada, C. Alonso, I. Agirrezabal, E. Kotelnikova, I. Zubizarreta, I. Pulido-Valdeolivas, A. Saiz, M. Comabella, X. Montalban, L. Villar, J.C. Alvarez-Cermeño, O. Fernández, R. Alvarez-Lafuente, R. Arroyo, A. Castro, Metabolomic signatures associated with disease severity in multiple sclerosis, *Neurol. Neuroimmunol. Neuroinflamm.* 4 (2017) e321.
- [40] S. Damiano, A. Sasso, B. De Felice, G. Terrazzano, V. Bresciamorra, A. Carotenuto, N.S. Orefice, G. Orefice, G. Vacca, A. Belfiore, M. Santillo, P. Mondola, The IFN- $\beta$  1b effect on Cu Zn superoxide dismutase (SOD1) in peripheral mononuclear blood cells of relapsing-remitting multiple sclerosis patients and in neuroblastoma SK-N-BE cells, *Brain Res. Bull.* 118 (2015) 1–6, <https://doi.org/10.1016/j.brainresbull.2015.08.009>.
- [41] C.R. Camara-Lemarroy, L.M. Metz, V. Wee Yong, Focus on the gut-brain axis: multiple sclerosis, the intestinal barrier and the microbiome, *World J. Gastroenterol.* 24 (2018) 4217–4223, <https://doi.org/10.3748/wjg.v24.i37.4217>.
- [42] S. Kumar, A. Molina-Cruz, L. Gupta, J. Rodrigues, C. Barillas-Mury, A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*, *Science* 327 (2010) 1644–1648, <https://doi.org/10.1126/science.1184008>.
- [43] S. Damiano, A. Morano, V. Ucci, R. Accetta, P. Mondola, R. Paternò, V.E. Avvedimento, M. Santillo, Dual oxidase 2 generated reactive oxygen species selectively mediate the induction of mucins by epidermal growth factor in erythrocytes, *Int. J. Biochem. Cell Biol.* 60 (2015) 889–893, <https://doi.org/10.1016/j.biocel.2014.12.014>.
- [44] T. Hosoi, R. Hirose, S. Saegusa, A. Ametani, K. Kiuchi, S. Kaminogawa, Cytokine responses of human intestinal epithelial-like Caco-2 cells to the nonpathogenic bacterium *Bacillus subtilis* (natto), *Int. J. Food Microbiol.* 82 (2003) 255–264, [https://doi.org/10.1016/s0168-1605\(02\)00311-2](https://doi.org/10.1016/s0168-1605(02)00311-2).
- [45] T. Tanabe, Rubin B.K. Chest, Airway goblet cells secrete pro-inflammatory cytokines, chemokines, and growth factors, *Chest.* 149 (2016) 714–720, <https://doi.org/10.1378/chest.15-0947>.
- [46] H. Danahay, A.D. Pessotti, J. Coote Brooke, E. Montgomery, Donghui Xia, A. Wilson, Haidi Yang, Zhao Wang, L. Bevan, C. Thomas, S. Petit, A. London, P. LeMotte, A. Dolemeier, G.L. Vélez-Reyes, P. Bernasconi, C.J. Fryer, M. Edwards, A.B. Jaffe, Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung cell, *Reports* 10 (2015) 239–252.
- [47] Y. Chen, G. Luo, J. Yuan, Y. Wang, X. Yang, X. Wang, G. Li, Z. Liu, N. Zhong, Vitamin C mitigates oxidative stress and tumor necrosis factor- $\alpha$  in severe community-acquired pneumonia and LPS-induced macrophages, *Mediat. Inflamm.* 2014 (2014), 426740.

- [48] A. Sorice, E. Guerriero, F. Capone, G. Colonna, G. Castello, S. Costantini, Ascorbic acid: its role in immune system and chronic inflammation diseases, *Mini Rev. Med. Chem.* 14 (2014) 444–452.
- [49] A.K. Al-Asmari, A.Q. Khan, A.M. Al-Qasim, Y. Al-Yousef, Ascorbic acid attenuates antineoplastic drug 5-fluorouracil induced gastrointestinal toxicity in rats by modulating the expression of inflammatory mediators, *Toxicol. Rep.* 2 (2015) 908–916, <https://doi.org/10.1016/j.toxrep.2015.06.006>(eCollection 2015).
- [50] V. Jaquet, L. Scapozza, R.A. Clark, K.-H. Krause, J.D. Lambeth, Small-molecule NOX inhibitors: ROS-generating, NADPH oxidases as therapeutic targets, *Antioxid. Redox Signal.* 11 (2009) 2535–2552, <https://doi.org/10.1089/ARS.2009.2585>.
- [51] N. Bunimov, O. Laneuville, Cyclooxygenase inhibitors: instrumental drugs to understand cardiovascular homeostasis and arterial thrombosis, *Cardiovasc Hematol Disord Drug Targets* 8 (2008) 268–277, <https://doi.org/10.2174/187152908786786250>.