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Title:

Bovine and soybean milk bioactive compounds: effects on inflammatory response of human intestinal Caco-2 cells.

Authors:

Rosa Calvello[1§*]; Antonella Aresta[2§]; Adriana Trapani[4]; Carlo Zambonin[2]; Rosaria Salvatore[1]; Maria Lisa Clodoveo[3]; Filomena Corbo[4*]; Carlo Franchini[4] ; Maria Antonietta Panaro[1]

Affiliations:

[1]*Dipartimento di Bioscienze, Biotecnologie e Biofarmaceutica - Università di Bari, via Orabona 4, 70126 Bari-Italy*

[2]*Dipartimento di Chimica - Università di Bari, via Orabona 4, 70126 Bari-Italy*

[3]*Dipartimento di Scienze Agro Ambientali e Territoriali - Università di Bari, via Orabona 4, 70126 Bari-Italy*

[4]*Dipartimento di Farmacia - Scienze del Farmaco- Università di Bari, via Orabona 4, 70126 Bari-Italy*

[§]These authors contributed equally to this work

E-mail address of each author:

***Corresponding authors:**

Filomena Corbo - Department of Pharmacy-Pharmaceutical Sciences, Università di Bari, via Orabona 4, 70126 Bari-Italy - filomenafaustinarina.corbo@uniba.it – Tel. +390805442746;

Rosa Calvello - Dipartimento di Bioscienze, Biotecnologie e Biofarmaceutica, Università di Bari, via Orabona 4, 70126 Bari-Italy - rosa.calvello@uniba.it – Tel. +390805442741;

22 rosa.calvello@uniba.it; antonellamaria.aresta@uniba.it; marialisa.clodoveo@uniba.it:

23 filomena.corbo@uniba.it; mariaantonietta.panaro@uniba.it; adriana.trapani@uniba.it;

24 carlo.zambonin@uniba.it; carlo.franchini@uniba.it; rosaria.salvatore@gmail.com.

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ABSTRACT

In this study, the effects of commercial bovine and soybean milks and their bioactive compounds, namely genistein, daidzein and equol, on the inflammatory responses induced by lipopolysaccharide (LPS) treatment of human intestinal Caco-2 cells were examined in terms of nitric oxide (NO) release and inducible nitric oxide synthase (iNOS) expression.

Both milks and their bioactive compounds significantly inhibited, in a dose-dependent manner, the expression of (iNOS) mRNA as well as protein expression, resulting in a decreased NO production. In addition, the activation of NF-κB in LPS-stimulated intestinal cells was examined. In all cases was observed that cell pre-treatment before LPS activation inhibited the phosphorylation, as well as the degradation, of the IkB complex. Accordingly, quantification of bioactive compounds by solid phase microextraction coupled to liquid chromatography have shown that they were absorbed, metabolized and released by Caco-2 cells in culture media.

In conclusion, we have demonstrated that milks and compounds tested are able to reduce LPS-induced inflammatory responses by intestinal cells, interfering with the activation of NF-kB dependent molecular mechanisms.

Keywords: Bovine and Soybean milks; isoflavones-aglycones; equol, Caco-2 cells; SPME-HPLC-UV/DAD.

1. INTRODUCTION

The intestinal mucosa is not only involved in absorbing nutrients, but represents an important route of entry for microbial pathogens, being enteric epithelial cells the initial sites of attack by entero-invasive microorganisms, such as Gram-negative bacteria (Yamamoto et al., 2003). A number of cells, both immune and non-immune, including intestinal cells, are involved in the production of mediators (cytokines, growth factors, adhesion molecules, etc.) which promote and amplify the inflammatory response (Nathan 2002).

Whereas an adequate inflammatory response is essential to protect the host from pathogens attach, an excessive production of inflammatory mediators may alter the homeostasis of bowel epithelium. Inflammatory bowel disease (IBD), including Crohn's disease (CD), and ulcerative colitis (UC), are chronic inflammatory disorders of the gut with complex etiologies (Kaser et al., 2010). One of the worst complications of IBD is the ultimate development of colon cancer. Therefore, the employments of agents that block the inflammatory responses have been particularly successful in IBD therapy. Currently there is no cure for IBD, thus the search for new molecules able to control IBD and their delivery to the site of inflammation are the goal of many investigators.

Flavonoids, natural phenolic compounds, are normal constituents of the human diet and are known for a variety of biological activities. Some of these act as enzyme inhibitors and antioxidants, and have been reported to have anti-inflammatory properties. However, the molecular mechanisms explaining how flavonoids suppress the inflammatory response are not known in detail yet. (Middleton et al., 2000; Havsteen, 2002).

There are studies showing that certain flavonoids down-regulate NO production in response to inflammatory stimuli (Panaro et al., 2012; Cianciulli et al., 2012) but no more precise mechanisms of action are known especially for that concerns intestinal cells. Genistein, an isoflavone, an important flavonoid class, very abundant in soy (*Glycine max*), has been found to play an important role in the prevention of various chronic diseases including cancer (Sarkar and Li, 2003). Various

epidemiological, *in vitro* and animal studies have evaluated the anti-cancer, cardio-protective, anti-osteoporotic, antioxidant, anti-inflammatory activities of genistein. It has been also associated with the inhibition of tyrosine-specific protein kinase in malignant cells (Caldarelli et al., 2005; Zhang et al., 2008; Park et al., 2010; Rusin et al., 2010). It was reported that genistein modulates the expression of NF-κB and MAPK (p-38 and ERK1/2), thereby attenuating D-Galactosamine induced fulminant hepatic failure in Wistar rats (Ganai et al., 2015)

Like genistein, daidzein is an isoflavone physiologically active that occurs mainly in free form and glycosylated in soy. Isoflavone glucosides of daidzein and genistein, namely daidzin and genistin, are more abundant and soluble but less actives than free forms (Tsangalis et al., 2002). In the digestive tract, each sugar-conjugated is hydrolysed by isoflavone β-glucosidase from gut bacteria to release the aglycone and sugar part. Then, all free forms are rapidly absorbed, metabolized or reconjugated to sugars by intestinal epithelial cells to assure their use or removal by the cells (Di Cagno et al., 2010). An interesting metabolite of daidzein is equol because it has a greater structural similarity to estrogens than its parent (Antignac et al., 2004; Di Cagno et al., 2010). It produces major effects on vascular reactivity, thrombosis, lipid profiles and cellular proliferation (Andres et al., 2011).

So, it could be hypothesized that the dietary intake of isoflavones may be helpful in the treatment of IBD. In this regard, bovine milk has been reported to have beneficial effects on gastrointestinal health both in infants and in the adult (Haug et al., 2007). Moreover, soy flavonoids may have some benefits for people with intestinal inflammation beyond simply nutritional support. Similarly, soy milk, the most popular alternative to cow's milk, could also possess anti-inflammatory activity, which is suitable in IBD therapy.

The aim of this study was to investigate the potential anti-inflammatory effects of bovine and soybean milks, as well as of the equol, daidzein and genestein using an *in vitro* model of human intestinal epithelial cells, Caco-2 cell line, submitted to Lipopolysaccharide (LPS) treatment as pro-

inflammatory stimulus. LPS, the principal component of the outer membrane of Gram-negative bacteria, plays a pivotal role in triggering an early inflammatory response through the interaction of several receptors for microbial products.

2. MATERIALS AND METHODS

2.1.Cell cultures and treatments

The Caco-2 cell line (ICLC HTL 97023-Interlab Cell Line Collection, Genoa, Italy) was grown in MEM medium supplemented with 10% fetal bovine serum (FBS, UE approved origin), 100 U/mL penicillin, 100 μg/mL of streptomycin, L-glutamine (2 mM), 1% nonessential amino acids (NEEA), referred to as complete medium (all reagents were purchased from Life Technologies-Invitrogen, Milan, Italy).

Cultures were maintained at 37° C in a humidified atmosphere containing 5% CO_2 and expanded in tissue culture flasks (75 cm^2, BD Bioscences, USA), changing the medium daily. The cells were seeded in 6-well cell culture plates at 5 x 10^5 cells/well and cultured to reach 80% confluency.

For the experiments, cells were treated with *Salmonella enterica* serotype typhimurium LPS (Sigma). Preliminary experiments were performed in order to establish the optimal dose (1 μg/mL) of LPS and time of exposure to LPS (48 h). Before LPS stimulation, some wells were pre-treated with different concentrations (5, 10, 50 μM) of daidzein (4′,7-dihydroxyisoflavone, Sigma), genestein (4′, 5, 7-trihydroxyisoflavone, Sigma) and equol (dihydroxyisoflavone, Sigma) or with different amounts (10, 30 and 50 μl) of the commercial (bovine or soy) milks. After 1h of incubation at 37°C, cell cultures were then stimulated with endotoxin as previously indicated. Untreated cells were used as control.

2.2. Cell Viability Assay

The viability of the cells was assessed by MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Cells (2.5×10^4) were seeded in a 96-

well plate (BD Biosciences, USA). After the treatment above described, culture media were carefully removed by aspiration. Then, 100 μL of 0.5 mg/mL MTT in cell culture medium was added to each well and incubated for 4 h. 100 μL of 10% SDS, 0.01 M HCl solution was added to each well to dissolve the formazan crystals formed. Amount of formazan was determined measuring the absorbance at 560 nm using a microplate reader.

2.3. Quantitative analysis of equol, daidzein and genistein from different samples by SPME-HPLC-UV7DAD method

In order to clarify the role of the isoflavones in the anti-inflammatory responses observed *in vitro,* their concentration have been determined both in cell culture media and cell lysates, as well as in bovine and soybean milk samples, by solid-phase micro-extraction coupled to liquid chromatography (SPME-LC).

Milk was diluted 1:100 with 0.2% formic acid with 30% (w/v) NaCl and stirred for 30 min before being subjected to SPME. An enzymatic deconjugation was performed to estimate the total amount of each isoflavone-aglycone. A solution (0.75 ml), containing 10 mg/ml β-glucosidase (≥2 units/mg solid, Sigma-Aldrich, Milano, Italy) in acetate buffer (0.1 M, pH 5.0), were added to 0.75 ml of milk, and incubated overnight at 37◦C. Finally, 30 μl, pure or diluted (1:10), were subjected to SPME. Culture media, taken from wells after 48 h, were diluted 1:10 with 0.2% formic acid with 30% NaCl before SPME analysis. Analytes extraction from the cell lysate supernatant was performed by diluting an aliquot of each sample 1:4 with saline after protein total assay. Then, variable aliquots were transferred into a vial containing the usual extraction mixture, in order to have 1.5 ml of solution at proteins total concentration of 0.025 mg/ml to be submitted to SPME analysis.

All extractions were carried out under magnetic stirring for 20 min using a 65 μm PDMS-DVB fiber (Supelco, Bellefonte, PA, USA). Desorption was performed in the static mode by soaking the

144 fiber in mobile phase directly into the desorption chamber of the interface for 15 min. Then, the
145 valve was changed to the inject position for 10 s.
146 Determination of analytes was conducted using a liquid chromatography system consisting in a
147 Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA), equipped a SPME manual
148 interface for compounds desorption in line (Supelco, Bellefonte, PA, USA), a KinetexTM C18 (2.6
149 μm, 100 x 4.6 mm i.d., Phenomenex, USA) column and a Spectra System UV6000LP photodiode
150 array (Thermo Finnigan, San Jose, CA).
151 Instrumental conditions were as follows: isocratic elution using 0.05 % (v/v) formic acid in
152 acetonitrile/methanol/water (8:32:60, v/v/v) as mobile phase; flow rate 0.7 ml/min; detection
153 wavelengths were 260 and 280 nm (5 nm band-width).
154 The quantification of the analytes in bovine and soybean milk samples was performed with the
155 standard addition method.
156 Fortified samples were equilibrated at 37 °C in a water bath for 30 min before being processed as
157 described. Three replicates for each concentration, including blank, were made.
158 Calibration curves for the target analytes in Caco-2 cells culture media were constructed in the
159 range 0.015 - 20 μM. Fortified samples were diluted 1:10 in 0.2% formic acid at 30% NaCl before
160 analysis.
161 *2.4. Nitric oxide (NO) production*
162 The stable nitrite (NO_2^-) concentration, being the end product of NO generation, was estimated by
163 measuring nitrite in the macrophage medium. Briefly, intestinal cells, cultured as indicated above,
164 were exposed to LPS for 48 hours. At the end of treatment, culture supernatants were collected and
165 incubated (1:1v/v) with the Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine
166 dihydrochloride, and 2.5% phosphoric acid) for 10 min at room temperature. Absorbance was
167 measured at 540 nm after incubation for 10 min. The NO_2^- concentration was determined by
168 extrapolation from a $NaNO_2$ standard curve and expressed as μmol/mL. To avoid interference by

nitrites possibly present in the medium, in each experiment the absorbance of the unconditioned medium was assumed as the "blank".

2.5. Electrophoresis and Western Blotting

After treatments, cells were lysed with lysis buffer [1% Triton X-100, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin hemisulfate salt, 0.2 U/ml aprotinin (all from Sigma-Aldrich)] for 30 min on ice. The lysate was vortexed for 15-20 sec and then centrifuged at 12,800x g for 20 min; the protein concentration in the supernatant was spectrophotometrically determined by Bradford's protein assay (Bradford, 1976). Protein samples were diluted with sample buffer (0.5 M Tris HCl pH 6.8, 10% glycerol, 10 % w/v SDS, 5% β2-mercaptoethanol, 0.05% w/v bromophenol blue) and then boiled for 3 min. Proteins (25 μg/lane) and prestained standards (BioRad Laboratories, Hercules, CA, USA) were loaded on 7% SDS precast polyacrylamide gels (BioRad Laboratories).

After electrophoresis, the resolved proteins were transferred from the gel to nitrocellulose membranes. A blotting buffer [20 mM Tris/150 mM glycine, pH 8, 20% (v/v) methanol] was used for gel and membrane saturation and blotting. A blocking solutions [bovine serum albumin (BSA), 0.2%–5% (w/v), Tween-20,(0.05–0.1%), non fat dry milk (0.5–5%), Casein(1%), all from BioRad Laboratories] was used in order to prevent nonspecific binding of unoccupied membrane sites. Then, membranes were incubated in the dark with (1:200 diluted) primary antibody anti human NOS II, anti human IKB α anti human pIKB α (all from Santa Cruz Biotechnology, Heidelberg, Germany), for 60 min at room temperature. The membranes were washed with T-PBS (for 20 min, 3 times) and then incubated with the secondary antibody anti-human IgG diluted 1:2000, horseradish peroxidase (HRP)-conjugate (Santa Cruz Biotechnology) for 60 min. Bands were visualized by the chemiluminescence method (BioRad, Laboratories).

2.6. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and quantitative real-time PCR analyses.

Briefly, total tissue RNA was extracted from treated and untreated cells by the Trizol isolation reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. Reverse transcription was performed in a final volume of 20 µl containing 3 µg of total RNA, 40 U of RNase Out (Invitrogen), 40 mU of oligo dT with 0.5 mM dNTP (PCR Nucleotide Mix, Roche Diagnostics, Milan, Italy), and 40 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Roche Diagnostics). The reaction tubes were incubated at 37°C for 59 min, and then at 95°C for 5 min and at 4°C for 55 min. cDNA obtained was then amplified by a thermal cycler (Eppendorf, Milan, Italy) under the following conditions: 95°C for 1 min, 55°C for 1 min and 72°C for 1 min (30 cycles of amplification). The reaction tube contained, in a final volume of 50 µl, 2 µl of cDNA, 200 µM dNTP (PCR Nucleotide Mix, Roche Diagnostics), 4 U TaqDNA Polymerase (Roche Diagnostics), 5µl of $MgCl_2$ buffer stock solution, and 50 pmol of the specific primers.

The mRNA levels of various genes were quantified using the SYBR Green QuantiTect RTPCR Kit (Roche, South San Francisco, CA, USA). β-actin was used as endogenous reference. Data were analyzed using the relative standard curve method according to the manufacturer's protocol. The mean value of each gene after β-actin normalization at the time point showing the highest expression was used as calibrator to determine the relative levels. The primers used for amplification for iNOS (NCBI reference sequence NM_000625.4) were: forward primer 5'-CGGCCATCACCGTGTTCCCC-3'; reverse primer, 5'-TGCAGTCGAGTGGTGGTCCA-3'; and for β-actin forward primer, 5'-GGCGGCACCACCATGTACCCT-3'; reverse primer, 5'-AGGGGCCGGACTCGTCATACT-3'.

2.7. Densitometric analysis

The bands obtained after immunoblotting were submitted to densitometric analysis using ID Image Analysis Software (Kodak Digital Science). Results were expressed as arbitrary units.

2.8. Data Presentation and Statistical Analysis

Student's t test and analysis of variance (one-way ANOVA) on the results of at least five independent biological replicates were performed. Values of $p < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Viability test

The MTT assay was used in order to test the viability of cells exposed to isoflavones and/or endotoxin treatment. Low concentrations of all tested isoflavones resulted no toxic for Caco-2 cells in comparison to untreated cells, whereas relatively higher concentration (50 μM) were resulted cytoxic (Fig. 1, Panel A). We also found that the viability of cells exposed for 48 h to 1 μg/mL LPS was significantly reduced in comparison to controls (Fig. 1, Panel B). Interestingly, the viability of LPS stimulated cells was significantly improved after 1h pre-treatment with isoflavones, in a dose-dependent manner (5-10 μM) (Fig. 1, Panel C). Cell viability of LPS stimulated cells resulted significantly improved by treatment with milks tested, in a dose dependent manner (data not shown).

3.2. Quantitative analysis of equol, daidzein and genistein from different samples by SPME-HPLC-UV/DAD method

To clarify the role-playing of equol, daidzein, genistein in the anti-inflammatory responses, firstly, their concentration in bovine and soybean milks was determined by a SPME-HPLC analysis; then, *in vitro* experiments were performed and compounds were also quantified in culture media and Caco-2 cells lysates, slightly modifying the previously validated method for the quantification of compounds in milk.

Table 1 shows concentrations of analytes in milks before and after β-glucosidase deconjugation.

Table 1

Isoflavones	Soybean milk	Bovine milk

	before	after	before	after
Daidzein	3.6± 0.3 μM	160.7± 13.5 μM	0.06± 0.01 μM	0.12± 0.05 μM
Genistein	0.9± 0.1 μM	121.8± 12.1 μM	0.03± 0.01 μM	0.04± 0.01 μM

241

242 As apparent, the predominant natural forms are sugar-conjugated and lower concentrations of
243 aglycones were found in cow's milk compared to soy. Equol was never detected in two milk types.
244 Calibration curves were obtained for quantitative analysis of compounds in culture medium.
245 Therefore, the method was validated in terms of linearity, limits of detection (LOD), quantification
246 (LOQ), precision, and repeatability.
247 The responses were found linear in the range of 0.05-15 μM for all analytes, with correlation
248 coefficients always >0.999 and intercepts not significantly different from zero at the 95%
249 confidence level.
250 The estimated limits of detection (LOD) and quantification (LOQ) obtained, calculated as three and
251 ten fold the standard deviation of the intercept of each calibration curve, were 0.02 μM for daidzein
252 and genistein and 0.03 μM for equol, and 0.07 μM for daidzein and genistein and 0.10 μM for
253 equol, respectively.
254 Precision of the method was investigated at the concentration levels of 0.5, 5 and 10 μM, by
255 performing daily three replicates (within day). The same solutions were analyzed three times each
256 day, for a period of five days (between days). The within-day and between days precision of the
257 method, estimated by an ANOVA test, ranged from 3.1 to 3.7% and from 5.1 to 6.7%, respectively.
258 Average amounts of compounds in 5 or 10 μM culture media, picked up after 48 h incubation by
259 wells with Caco-2 cells plus LPS, were 23.0 ± 2.0 16.0 ±1.8 and 2 ±0.2 % of estimated quantities in
260 corresponding control wells (without Caco-2 cells and with LPS) for equol, daidzein and genistein,
261 respectively.

To certify absorption analytes by cells, equol, daidzein and genistein were checked in related supernatants of cellular lysates. Given the complexity of the matrices, the method of standard additions was used for SPME analysis.

Caco-2 cells pretreatments have produced the results listed in table 2, where concentrations of compound are expressed in relation to total proteins estimated in each sample analyzed.

As apparent, all amounts estimated are lower than expected demonstrating their probable involvement in cellular responses at stimulus.

In case of cellular pretreatments with soybean milk, the concentrations of isoflavones-aglycones in culture media were found higher than original levels. For example, in culture media by Caco-2 cells pretreated with 50 μl of soybean milk before of the stimuli with LPS, daidzein and genistein were estimated at levels 2.54±0.09 and 2.67±0.10 μM, instead 0.23 ± 0.01 and 0.05 ± 0.01 μM of control, respectively. Whereas, in respective cellular lysates, only genestein was found at level of 2.29 nmoles/mg of total proteins. Similar results were obtained also with the other amounts tested (10, 30 μl) of soy sample.

Table 2

Pretreatment		[proteins] (mg/ml)	$[analyte]_{found}$ (nmoles/mg proteins)
equol	5 μM	8.6±0.5	0.44±0.25
	10 μM	6.8±0.3	1.16±0.54
daidzein	5 μM	9.4±0.6	0.46±0.19
	10 μM	18.4±1.5	0.44±0.36
genistein	5 μM	17.0±1.2	0.28±0.15
	10 μM	19.6±1.6	0.49±0.28

However, isoflavones-aglycones were not detected in samples relating to bovine milk pretreatments, probably due to their lower biological contents in cow's milk.

3.3. NO production and iNOS expression.

Undetectable levels of NO production were observed in cell cultures treated with isoflavones alone (data not shown). In Caco-2 cells, LPS treatment significantly increased the level of NO as compared to untreated cells (control). A significant reduction ($p<0.01$) of LPS-induced NO production was observed in cells submitted to 1h pre-treatment with isoflavones in comparison to cells treated with LPS alone; this effect was dose-dependent, where the maximal reduction ($p<0.01$) was observed at 10 μM, as shown in Fig.2 (Panel A). In particular, from our results emerged that both daidzein and genistein are more efficacious than equol in the reduction of LPS induced NO release.

We also investigated the effect of milks (bovine and soy) incubation on the NO release after LPS stimulation. Also in this case, 1h pre-treatment with milks, reduced LPS-induced NO production in a dose-dependent manner; the maximal reduction ($p<0.01$) was observed with 50 μl milk (Fig.2 Panels B and C). In cell cultures treated with milk alone we observed NO levels comparable to those detected in controls.

In addition, RT-PCR data indicated that treatment with LPS significantly increased the expression of iNOS at 6 hours; however, pre-treatment with isoflavones resulted in a significant dose dependent decrease of the iNOS gene expression (Fig. 3, Panel A). Similar results were obtained in the experiments performed in the presence of commercial milks tested. Also in this case we observed that milks pretreated cells before LPS stimulation exhibited a significant dose-dependent reduction of iNOS mRNA expression in comparison to cells stimulated with LPS alone (Fig. 3, Panels B and C).

Consistent with the results of RT-PCR, western blot analysis showed that treatment with LPS significantly increased iNOS protein synthesis at 48 hours; however, iNOS expression resulted

303 significantly down-regulated in the presence of compounds (Fig. 4, Panel A1). Similar results were
304 obtained in the experiments performed in the presence of commercial milks tested, where milk pre-
305 treatment of cell cultures determined a significant reduction of iNOS protein expression in
306 comparison to cells stimulated with LPS alone. Also in this case the effects was resulted dose
307 dependent (Fig. 4, Panels B1 and C1).
308 Taken together, these results indicate that isoflavones as well as milks, both bovine and soy, are
309 able to suppress the release of NO in LPS-stimulated intestinal cells modulating the expression of
310 iNOS both at transcriptional and at post-transcriptional levels.
311 *3.4. NF-κB activity in LPS-induced intestinal cells*
312 To gain insights regarding the effect of milks and their isoflavones on the inflammatory responses
313 of intestinal cells we investigated whether our compounds modulate NF-kB activity in LPS
314 stimulated Caco-2 cells, since activation of NF-κB is closely related to regulate the iNOS
315 expression.
316 The phosphorylation and degradation of IκB-α are essential in the translocation of NF-κB p65 in the
317 nucleus from cytosol, therefore we determined the effect of our compounds on LPS-induced
318 degradation and phosphorylation of IκB-α protein. For this aim, we evaluated the expression of p-
319 IkB in cell lysates obtained from LPS treated Caco-2 cells. In this context, we observed that cells
320 exposed to LPS exhibited a significant increase of p-IkB expression as compared to unstimulated
321 cells, after 48 h of cell stimulation. Little phosphorylation of IkB was observed in unstimulated
322 cells, as revealed by densitometric analysis (Fig. 4, Panels A2, B2, C2).
323 Pretreatment with isoflavones reduced, in a dose dependent manner, IκB-α phosphorylation,
324 reaching a maximal reduction at 10 μM (Fig. 4) in LPS stimulated Caco-2 cells (Fig. 4, panel A2).
325 Moreover, milk pre-treatment of LPS stimulated cells determined a dose-dependent inactivation of
326 the NF-kB (Fig. 4, Panels B2 and C2).

Overall, these data indicate that both isoflavones and milks inhibited NF-κB activity in LPS-stimulated intestinal cells by suppressing degradation of IκB-α, and, consequently, attenuating the expression of iNOS mRNA.

4. DISCUSSION

The Caco-2 cell line, which is derived from human colon adenocarcinoma, exhibits enterocyte-like characteristics and has been used widely as an *in vitro* model of absorption by intestinal epithelial cells. Using Caco-2 cell line, we previously examined the cellular uptake and metabolism of milk isoflavones. We found that compounds were taken up into Caco-2 cells and metabolized in accord to previously works (Steensma, Noteborn, &. Kuiper, 2004).

Several analytical methods have been suggested for the determination of isoflavones and their metabolic derivatives in biological fluids and food matrices. (de Rijke et al. 2006; Rostagno et al. 2009). Solid phase microextraction is a popular sample preparation technique employed to extract a wide range of analytes in many areas with good or high sensitivity. The extraction is almost selective because it depends by partition equilibrium of analyte between the thin polymeric phase coating a fiber in fused silica or alloy or in stable flex and the medium in which it is dispersed. Consequently, great importance is given to control of the optimum parameters affecting the extraction and desorption processes in order to obtained reproducible data.

Recently, it has proposed a new simple, selective, sensible, accurate and low cost method for determination of major isoflavones in soy drinks based on SPME coupled to liquid chromatography (Aresta et al., 2015). In this study, all SPME – HPLC-UV/DAD parameters suggested have been carefully considered and applied, obtaining results comparable to previous work. Equol was not detected in two type milks. However, by literature data, it is not a natural component of vegetable milks, unlike those of bovine, whose levels range from 0.236±0.004 to 4.140±0.152μM, quantities lower or near to LOD of method used (Antignac et al., 2004; Di Cagno et al., 2010).

The intestinal cells are also able to mount an adequate inflammatory response, by releasing of pro-inflammatory mediators, such as cytokines and free radicals, to protect against infections (Caricilli, Castoldi, & Câmara, 2014). However, when excessive, inflammatory responses may alter the homeostasis of the intestinal mucosa, destroying the intestinal epithelial monolayer. The reactive NO is a major mediator of the inflammatory responses. NO is enzymatically generated in a variety of cells from L-arginine pathway by three isoforms of NO synthetase. Three major isoforms of nitric oxide synthase include neuronal, endothelial and inducible nitric oxide synthase (nNOS, eNOS and iNOS, respectively). The iNOS form is calcium independent and inducible by inflammatory cytokines and bacterial products, such as LPS, in various cell types, including intestinal cells. Increased expression of iNOS leads to synthesis of micromolar quantities of NO, which causes damage to the cells and tissue through formation of NO-reactive products and used as measurement for toxicity marker (Haga et al., 2007). NO-induced oxidative stress is associated with many diseases, including IBD. Excessive production of NO, and the presence of nitric oxide synthase protein and iNOS mRNA have been demonstrated in intestinal mucosa in affected areas in patients with ulcerative colitis and Crohn's disease (Ikeda et al., 1997). This is consistent with data obtained from animal models of inflammatory bowel disease (Kankuri et al., 1999). Nitric oxide in conjunction with superoxide anion generates significant amounts of peroxynitrite anion, a potent oxidizing agent, which inflicts cellular injury and necrosis probably via mechanisms including DNA fragmentation and lipid oxidation (Valko et al., 2007). For these reasons, down-regulation of NO has been used to treat such diseases (Lanas et al., 2008). The anti-inflammatory agents like anti-inflammatory drugs (NSAIDs) have been widely used to treat inflammation-associated diseases. However, using NSAIDs to inhibit the expression of iNOS and NO can lead to severe gastrointestinal damage (Sasso et al., 2015). Thus, NSAIDs use is actually avoided for the treatment of IBD. Searching for effective treatments is urgent needed for the development of therapy and

prevention of IBD. Many people with IBD turn to alternative medicine including traditional plant based remedies.

In the current study, we found that equol, daidzein and genistein, isoflavones, which are present as natural components of milk, down-regulate the release of NO as well as the expression of iNOS mRNA and protein in LPS-stimulated intestinal cells in a dose-dependent manner. These effects on the LPS induced NO production was also observed in Caco-2 cells pre-treated with bovine and soy milks prior LPS stimulation. Similar results were obtained by Di Cagno et al., (2010) reporting that soy milk as well as equol, genistein and daidzein, markedly inhibited the inflammatory status of Caco-2/TC7 cells as induced by treatment with interferon-γ and LPS, although the exact molecular mechanism responsible for this action is not reported. These observations suggest that isoflavones present in the milks might be good candidate for the control of proinflammatory mediators, such as NO, in the IBD (Itzkowitz, 2006).

Interestingly, we also observed that isoflavones treatment, as well as milks treatment, of LPS stimulated cells are able to reduce, although no completely depressed, NO production, which remained yet significantly higher in comparison to control cells. This result is consistent to previous study reporting NO to play a protective role in the intestinal epithelium (Wallace & Miller, 2000; Zhang, Urbanski, & McCafferty, 2007). In this respect it was reported that in the iNOS-deficient mice iNOS induction the sites of intestinal injury result protective rather than detrimental, since an increase in the macroscopic damage was seen during acute phase of the experimentally-induced colitis (McCafferty et al., 1997). These data suggest that induction of iNOS plays a critical role in the healing and in the gut inflammatory processes. Thus, a controlled NO production by intestinal cells may contribute to the normal homeostasis of the intestinal epithelium.

It has been reported that NF-kB plays a critical role in the IBD and previous studies have shown that NF-kB inhibitors suppress pro-inflammatory responses thus ameliorating the clinical picture (Sun et al., 2015). In this context, it is well accepted that several natural antioxidants, such as

polyphenol compounds, mediate anti-inflammatory activities by directly suppressing the expression of NF-κB dependent pro-inflammatory mediators, including iNOS (Panaro et al., 2012; Bai et al., 2005).

In resting stage, NF-κB normally localizes in the cytosol as a complex with the inhibitory IκB protein. During inflammatory stimulus, IκB is phosphorylated by IκB kinase, subsequently degraded by proteasome, then gets released and translocates into the nucleus, where it triggers the transcription of multiple genes involved in inflammatory cascade (Kumar, Takada, Boriek, & Aggarwal, 2004). Therefore, blockade of NF-κB signal transduction pathways may be one of the major mechanisms underlying the prevention of the development of IBD (Andresen et al., 2005).

The isoflavones, daidzein and genistein, have been reported to inhibit iNOS protein and mRNA expression and NO production in LPS induced murine J774 macrophages in a dose-dependent manner down-regulating the NF-κB activation (Hämäläinen et al., 2007). Protective effects of genistein are also demonstrated in other experimental models. For example Valles et al. (2010) have found that genistein in astrocytes in primary culture attenuates inflammation, preventing the expression of inflammatory mediators, including iNOS. Also Comalada et al. (2006) have indicated that flavonoids inhibit TNF-α production as well as iNOS expression and NO production in LPS-activated macrophages, an effect associated with the inhibition of the NF-κB pathway. Furthermore, Lu et al. (2009), investigating the anti-inflammatory properties of genistein in primary astrocytes treated with hemolysate, demonstrated that genistein inhibited the expression of iNOS and COX-2 mRNA and the level of hemolysate-stimulated NF-κB.

Finally, other results demonstrate that equol is able to impair LPS-induced NO production and iNOS gene expression in RAW 264.7 murine macrophage and that these effects are mediated, at least in part, through NF-κB activity inhibition (Kang et al., 2007).

The present study demonstrated that isoflavones tested, as well as bovine and soy milks attenuate NO production in LPS activated intestinal cells, interfering to NF-kB activation. In particular we

observed that natural compounds used in this study, genistein, daidzein and equol, were able to reduce in a dose dependent manner NF-kB activation, thus leading to mRNA and protein reduced expression in LPS intestinal cells.

Many milk-derived components have immunomodulatory and anti-inflammatory properties, and some of these reduce intestinal inflammation when orally administered to animal models of colitis. The most commonly consumed milk in Western countries is bovine milk. Like human milk, bovine milk contains a large variety of saturated and unsaturated fatty acids with differing potential health effects, protein of high nutritional value and a number of potentially beneficial bioactivities, as well as carbohydrate (including lactose and oligosaccharides), vitamins, minerals and antioxidants (Haug et al., 2007).

Some leguminous plants such as soy have a natural high content of phytoestrogens (Franke, Custer, , Cerna, & Narala, 1995; Saloniemi et al., 1995), and they usually comprise a large proportion of the feed ration for dairy cows especially in organic milk production. Therefore, these plants are a potential source of phytoestrogens in milk and milk products. The content of different isoflavones (genistein, daidzein, equol, and others) in the bovine milk has been investigated (Antignac, Cariou, Bizec, & Andre 2004)

Milk composition varies between different species (Haug et al., 2007) and is affected by nutrition and health status (Mele et al., 2009). This could depend on different responses of each individual to the type of milk, which could depend on a variety of factors, such as genotype, milk protein allergies, or lactose intolerance (Haug et al., 2007). In this regard, the IBD patients are often likely to eliminate milk from their diet due to a widespread belief that is deleterious, using milk substitutes soy-based, that have been shown more benefits compared to cow milk. This would lead to the commercialization of soy as a healthy alternative to milk. For IBD patients who can tolerate dairy products, milk may provide valuable nutritional support. Some components of milk, such as equol, may also have beneficial actions in the control of the inflammatory status of the gut, although these

450 components may not be present in high enough concentrations in order to provide substantial
451 benefits. The impact of milk on human health and the functionality of each bioactive component
452 need to be further investigated, so that the use of bovine or soy milk as well as the milk
453 components, as isoflavones, in the diet can be optimised to provide maximum benefits for intestinal
454 inflammation minimizing adverse effects.

455 **Conclusions**

456 In conclusion, our present findings indicate that isoflavones present in the milk are able to control
457 LPS-induced NO down-regulating iNOS expression in activated intestinal cells. This action seems
458 to be directely attributable to the NF-kB impairment thus suggesting a possible signaling pathways
459 involved in the pro-inflammatory genes control of the intestinal cells. Thus, our data represent a
460 valid rationale for the use of isoflavone-enriched milks as a complementary approach, with lower
461 incidence of side effects, during the management of IBD. However, additional research is needed in
462 vivo to confirm these results in order to suggest the use of isoflavone-enriched milks as functional
463 beverages for medical purposes.

464

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593

Table and Figure Captions

Table 1

Concentration of isoflavones-aglycones in two commercial bovine and soybeans milks before and after β-glucosidase deconjugation.

Table 2

Concentration of proteins and analytes in Caco-2 cells lysates.

Figure 1

Cell viability assessed by MTT assay. Panel A: Caco-2 cells were incubated (48 h) in the presence of different concentrations of isoflanones (5-50 μM). Panel B: Caco-2 cells were incubated in absence (control) or in presence of LPS (0,5-1 μg/ml). Panel C: cells were stimulated with LPS alone or after pre-treatment of isoflavones. Ctr: control; LPS: LPS-treated cells. Data, expressing the percentage of cell viability, are reported as means ± SD of five independent experiments. *$p < 0.05$; **$p < 0.01$ compared with the control value (medium); #$p < 0.01$ versus LPS alone.

Figure 2

NO release in Caco-2 cells. Panel A: intestinal cells were exposed to LPS alone or after 1h pre-treatment with isoflavones. Panel B: effect of bovine milk (B) treatment (see in the text for concentration and time used) on NO release; Panel C: effect of soy milk (S) treatment (see in the text) on NO. Ctr: control; LPS: LPS-treated cells Results are expressed as means ± SD of five independent experiments. E: equol; D: daidzein; G: genistein. *$p < 0.01$ versus control value (medium); #$p < 0.01$ versus LPS alone; §$p < 0.05$ and §§$p < 0.01$ between LPS+isoflavones or LPS+milks.

Figure 3.

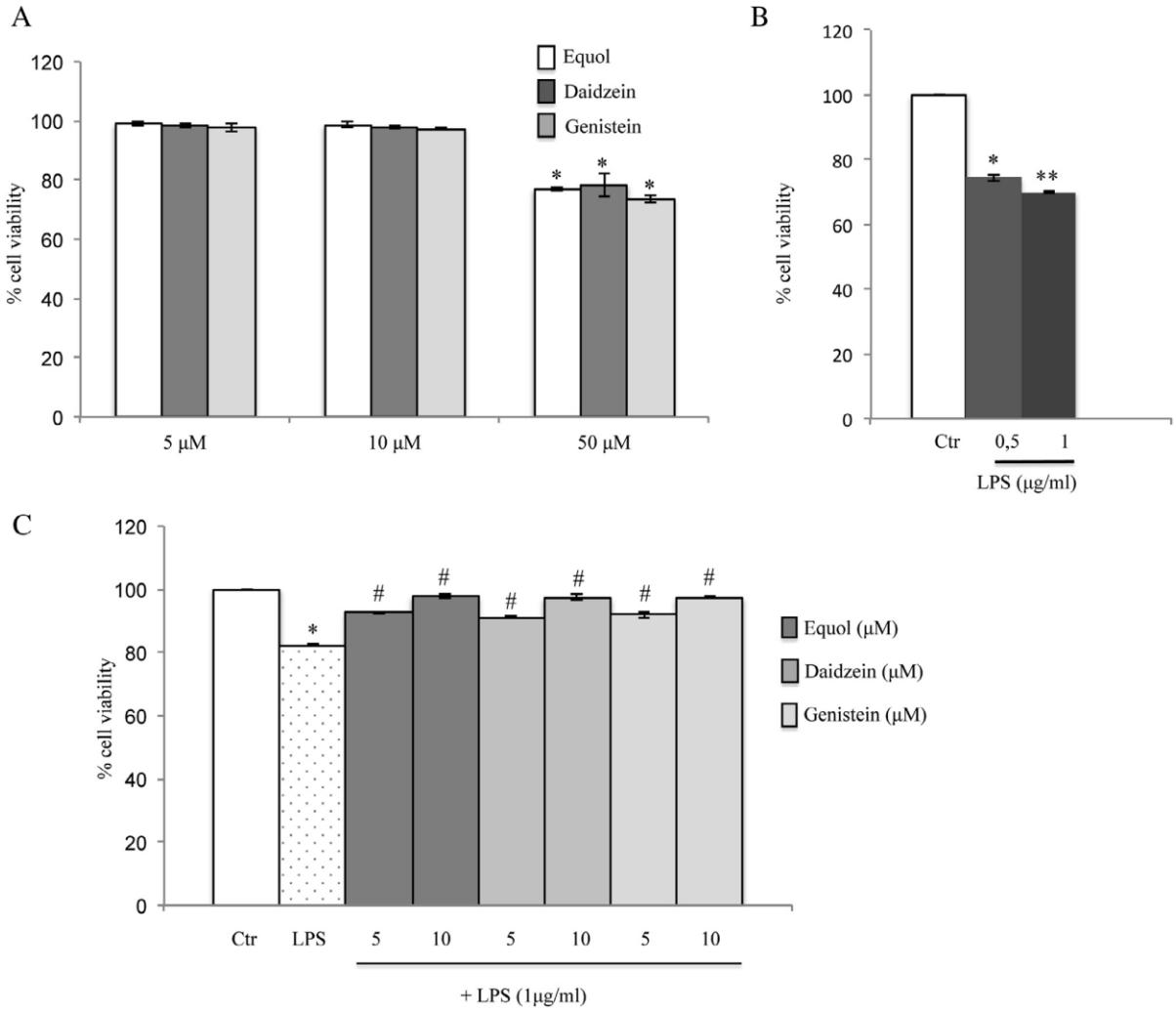
iNOS mRNA expression. Panel A: intestinal cells were treated with LPS alone or after 1h pre-treatment of isoflavones. Panel B: iNOS mRNA expression in Caco-2 cells treated with bovine milk (B) alone or in presence of LPS; Panel C: iNOS mRNA expression in Caco-2 cells treated with soy

619 milk (S) in absence or in presence of LPS. Values represent the mRNA fold changes relative to β-
620 actin used as resident control and expressed as means ±SD of five independent experiments. Ctr:
621 control; LPS: LPS-treated cells; E: equol; D: daidzein; G: genistein. *p < 0.01 compared with the
622 control value (medium); #p <0.01 versus LPS alone; §p <0.05 and §§p<0.01 between
623 LPS+isoflavones or LPS+milks.

624 **Figure 4**

625 Western blot analysis of iNOS in Caco-2 cells. Panel A1: intestinal cells were treated with LPS
626 alone or after 1h pre-treatment of isoflavones. Panel B1: Caco-2 cells were treated with bovine milk
627 (B) alone or in presence of LPS. Panel C1: Caco-2 cells treated with soy milk (S) in absence or in
628 presence of LPS. Protein expression levels were normalized to β-actin and results of densitometric
629 analysis are expressed as means ± SD of five independent experiments. E: equol; D: daidzein; G:
630 genistein; Ctr: control; LPS: LPS-treated cells *p < 0.01 compared with the control value (medium);
631 #p <0.01 versus LPS alone; §p <0.05 and §§p<0.01 between LPS+isoflavones or LPS+milks.
632 Signalling pathway analysis. Panel A2: Western blot analysis of p-IkBα in Caco-2 cells treated with
633 LPS alone or after 1h pre-treatment of isoflavones. E: equol; D: daidzein; G: genistein. Panel B2:
634 Immunoblot analysis of p-IkBα in Caco-2 cells treated with bovine milk (B) alone or in presence of
635 LPS. Panel C2: Immunoblotting analysis of cells treated with soy milk (S) in absence or in presence
636 of LPS. Protein expression levels were normalized to β-actin and results of densitometric analysis
637 are expressed as means ± SD of five independent experiments. *p < 0.01 compared with the control
638 value (medium); #p <0.01 versus LPS alone; §p <0.05 and §§p<0.01 between LPS+isoflavones or
639 LPS+milks.

640


A
120
100
80
60
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20
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% cell viability
Equol
Daidzein
Genistein
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5 µM
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50 µM
B
120
100
80
60
40
20
0
% cell viability
*
**
Ctr
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LPS (µg/ml)
C
120
100
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40
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0
% cell viability
*
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Ctr
LPS
5
10
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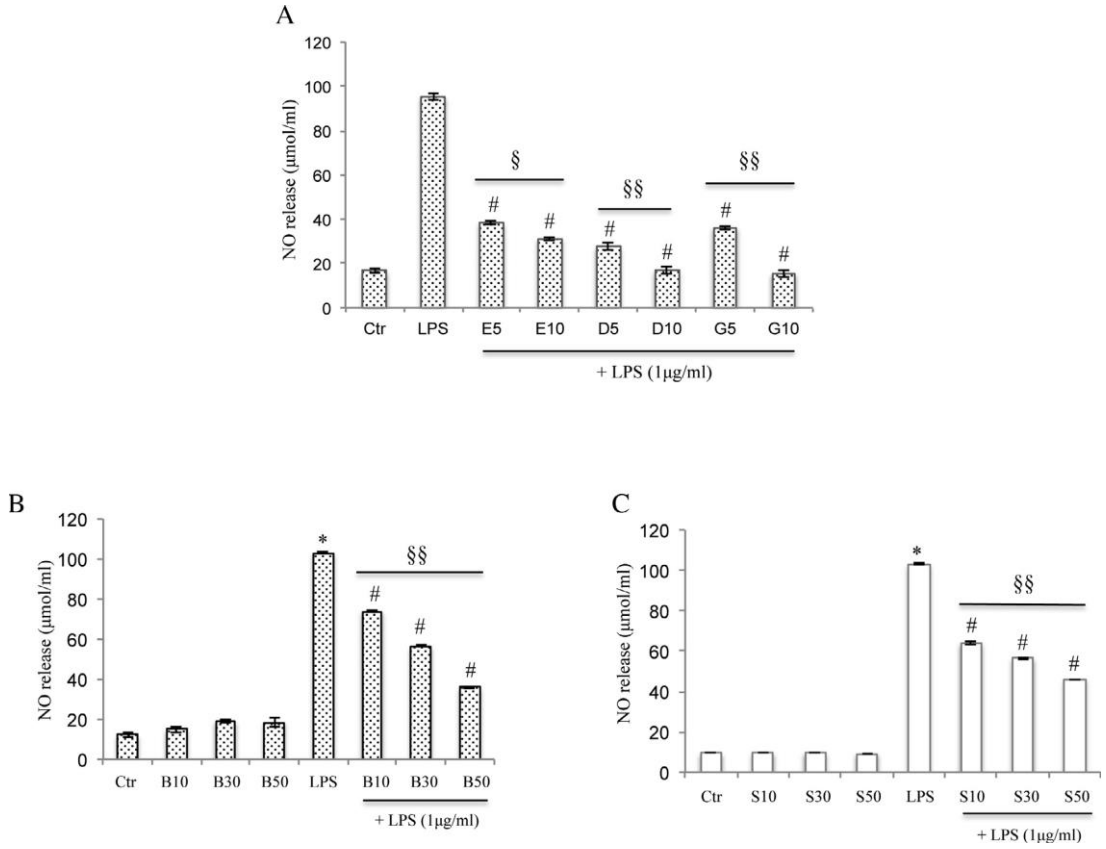
5
10
+ LPS (1µg/ml)
Equol (µM)
Daidzein (µM)
Genistein (µM)

Figure 1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80
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40
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0
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Ctr
LPS
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E10
D5
D10
G5
G10
+ LPS (1µg/ml)
B
NO release (µmol/ml)
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Ctr
B10
B30
B50
LPS
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B30
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+ LPS (1µg/ml)
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NO release (µmol/ml)
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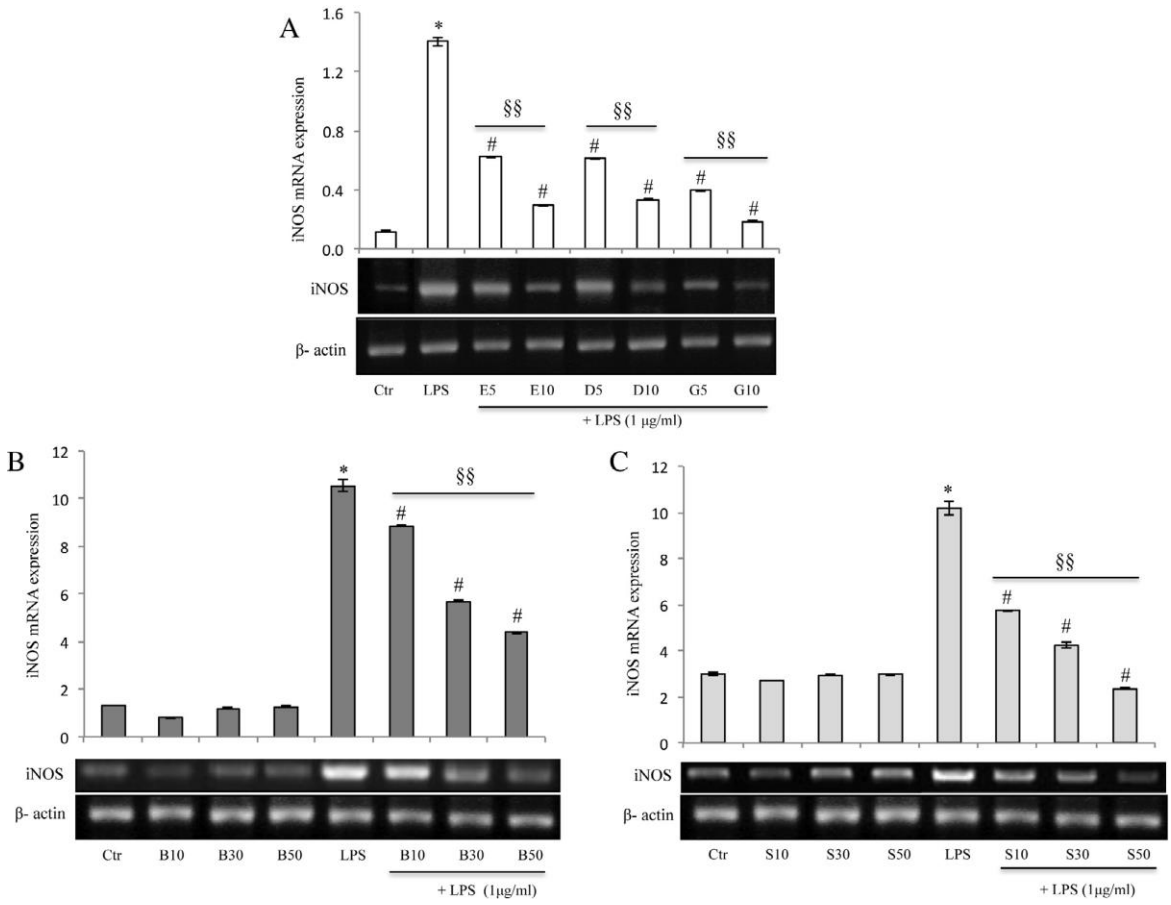
+ LPS (1µg/ml)

Figure 2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iNOS mRNA expression
1.6
1.2
0.8
0.4
0.0
*
§§
§§
§§
#
iNOS
β- actin
Ctr
LPS
E5
E10
D5
D10
G5
G10
+ LPS (1 μg/ml)
B
iNOS mRNA expression
12
10
8
6
4
2
0
iNOS
β- actin
Ctr
B10
B30
B50
LPS
+ LPS (1μg/ml)
C
iNOS mRNA expression
iNOS
β- actin
Ctr
S10
S30
S50
LPS
+ LPS (1μg/ml)

Figure 3

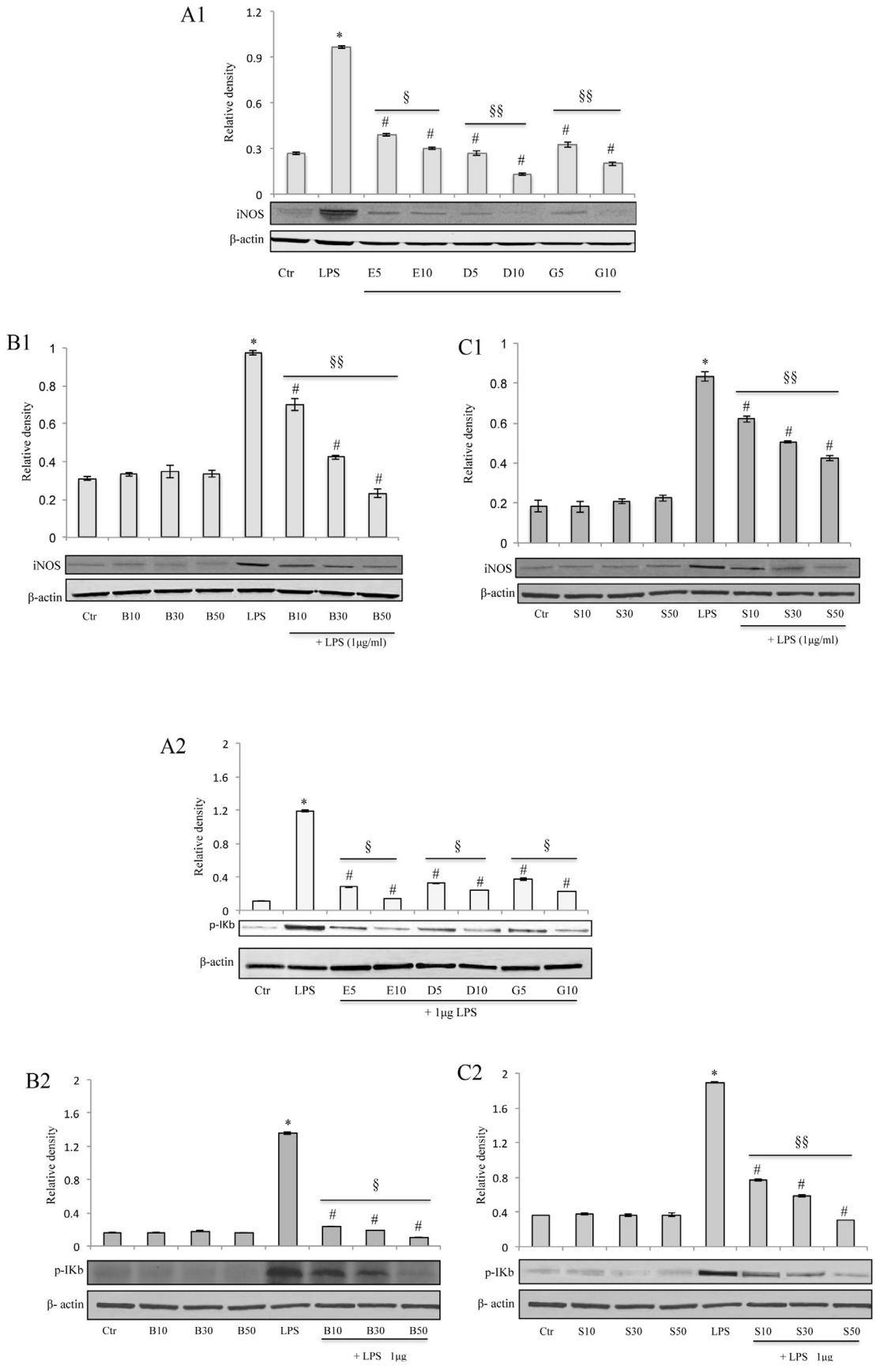
A1
Relative density
iNOS
β-actin
Ctr
LPS
E5
E10
D5
D10
G5
G10
B1
B10
B30
B50
+ LPS (1μg/ml)
C1
S10
S30
S50
A2
p-IKb
+ 1μg LPS
B2
C2
+ LPS 1μg

Figure 4