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Galacto-oligosaccharides exert a protective effect against heat stress in a Caco-2 cell model

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ABSTRACT

Thermal stress can evoke a stress response and enhance the synthesis of heat shock proteins, while gut barrier dysfunction is considered as an important adverse effect of thermal stress. Considering the previously described effects of galacto-oligosaccharides, nowadays mainly used in infant formulas, we hypothesized that galacto-oligosaccharides may protect the intestinal barrier against heat stress. Human epithelial colorectal adenocarcinoma cells were pre-treated with galacto-oligosaccharides prior to thermal stress exposure (40–42 °C) for 24 h. Pre-treatment of galacto-oligosaccharides prevented the heat stress-induced up-regulation of heat shock proteins and reduced the heat-induced stress response as observed by a decrease in haem oxygenase-1. Galacto-oligosaccharides partly prevented the heat-induced effects on monolayer integrity as measured by transepithelial electrical resistance, paracellular permeability and E-cadherin expression. In addition to their prebiotic effect, galacto-oligosaccharides may have beneficial potency to protect the intestinal epithelial barrier against heat stress and may be an attractive dietary application for people who are at high risk of developing heat stress.

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1. Introduction

The cellular stress response is a protective reaction of individual cells to potentially harmful internal and external stimuli. It is well established that exposure of cells to various stressors, including thermal stress, oxidative stress, or pathological conditions like ischaemia, tissue damage, infection and inflammation (Morimoto, 2011; Ragsdale & Proctor, 2000; Tsuji

et al., 2009) can evoke a stress response and enhance the synthesis of heat shock proteins (HSPs), via activation of heat shock factors (Akerfelt, Morimoto, & Sistonen, 2010). HSPs prevent stress-induced protein aggregation and misfolding, and promote their return to native conformations maintaining protein homeostasis (Kalmar & Greensmith, 2009). They are classified into different groups based on their molecular weight, structure and function, including families of small HSPs (molecular weight of 15–30 kDa), HSP60, HSP70, HSP90 and HSP110 (Joly, Wettstein,

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Abbreviations: AJ, Adherens Junction; E-cadherin, epithelial cadherin; GOS, galacto-oligosaccharides; HSPs, heat shock proteins; HO-1, haem oxygenase-1; LY, lucifer yellow; TEER, transepithelial electrical resistance; TJ, Tight Junction Proteins; ZO-1, zona occludens protein-1; °C, degrees Celsius

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Mignot, Ghiringhelli, & Garrido, 2010). Although HSPs are generally considered to improve cellular recovery, imbalances in HSP70 and HSP90 levels can induce cell growth arrest and developmental defects (Nollen & Morimoto, 2002). An alteration in the expression of HSPs, but also thermoregulatory failure and dysregulation of the acute-phase response may contribute to the progression of heat stress into heat stroke. Heat stroke is a potentially fatal disorder characterized by multi-organ injury and an elevated core body temperature that rises above 40 °C. Heat stroke may result from exposure to high environmental temperatures (classical heat stroke) or as a consequence of extensive exercise (exertional heat stroke) (Chan & Mamat, 2015). Heat-induced multi-organ injury may include varying degrees of central nervous system dysfunction, acute renal failure, liver failure, skeletal muscle injury and gut ischaemia (Leon & Helwig, 2010). An important early symptom of thermal stress is a dysfunction of the intestinal barrier leading to increased intestinal permeability and as a consequence increased entrance of toxic luminal substances (Dokladny, Moseley, & Ma, 2006; Xiao et al., 2013). With the prospect of increasing global warming and increase in frequency and intensity of heat stress (Bouchama & Knochel, 2002), it is important to investigate preventive measures that can alleviate adverse effects of exposure to high environmental temperatures. Food supplemented with non-digestible oligosaccharides, including galacto-oligosaccharides (GOS) are known to support the maintenance of the gut homeostasis, modulate the intestinal microbiome, protect the intestinal barrier integrity and stimulate gut associated immunity (Bruno-Barcena & Azcarate-Peril, 2015; Jeurink, van Esch, Rijnierse, Garssen, & Knippels, 2013; Zhong et al., 2009). Considering these effects of GOS on improving gut health, we hypothesized that dietary GOS might protect the epithelial barrier against the heat stress-induced effects on HSPs expression levels, on oxidative stress, and on the intestinal barrier integrity. In this study, an *in vitro* epithelial colorectal adenocarcinoma (Caco-2) cell culture model was used as a model to assess the effects of thermal stress on the expression of heat shock proteins as well as on the intestinal barrier function and to investigate the potential protective effects of GOS. Results show that in this *in vitro* model, dietary GOS prevented heat-induced up-regulation of HSPs and markers of oxidative stress. The heat stress-induced disruption of the intestinal barrier was mitigated by GOS especially by modulating epithelial-cadherin (E-cadherin) expression.

2. Materials and methods

2.1. Galacto-oligosaccharides (GOS)

The commercial product Vivinal® GOS syrup (FrieslandCampina Domo, Borculo, The Netherlands) containing galacto-oligosaccharides with a degree of polymerization (dp) of 2–8 was used. The final product contained approximately 59% (w/w) galacto-oligosaccharides, 21% (w/w) lactose, 19% (w/w) glucose and 1% (w/w) galactose on dry matter (dry matter of 75%) and dilutions (1% and 2.5% (w/v) GOS) were produced in complete cell culture medium. Before starting the experiments close to equimolar concentrations of 21% (w/v) lactose and 19% (w/v) glucose as present in the 2.5% GOS solution were tested in the

Caco-2 cell assays and no effect on the heat stress-induced transepithelial resistance (TEER) decrease and increase in paracellular Lucifer Yellow (LY) flux was observed (data not shown).

2.2. Cell culture

Caco-2 cells were obtained from the American Type Tissue Collection (Code HTB-37) (Manassas, VA, USA, passages 5–19) and were grown as a monolayer in Dulbecco's modified Eagle's minimum essential medium (DMEM), supplemented with 25 mM HEPES, 4.5 g/l glucose (Gibco, Invitrogen, Carlsbad, CA, USA), 10% (v/v) inactivated foetal calf serum (FCS) (Gibco), glutamine (2 mM, Biocambrex, Verviers, Belgium), 1% (v/v) non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Biocambrex) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Confluent cells (90%) were trypsinized using 0.05% trypsin and 0.54 mM ethylene diamine tetra acetic acid (EDTA). All heat shock experiments were performed with Caco-2 cells seeded on 0.3 cm² high pore density polyethylene terephthalate membrane transwell inserts with 0.4 µm pores (Falcon, BD Biosciences, Durham, NC, USA) placed in a 24-well plate. The Caco-2 cells were seeded at a density of 0.3×10^5 cells/transwell insert. All these transwell experiments were started after obtaining a confluent Caco-2 monolayer at day 17–19 of culturing with TEER values in the range of 400 Ω.cm².

2.3. Induction of heat stress

The cells were cultured at temperatures of 37 °C (normal temperature for cell culture), and alternatively at 40 °C, 42 °C for the induction of heat stress in a humidified atmosphere of 95% air and 5% CO₂ for 2, 4, 6, 8, 12 and 24 h. Temperatures of 40 °C and 42 °C were selected to reflect temperatures potentially causing slight or harmful heat stress to the cells, respectively. Prior to heat exposure, Caco-2 cells were pre-treated with either cell culture medium (control) or medium supplemented with GOS (1 or 2.5%) added to the apical and basolateral side for 24 h.

2.4. Cell viability assay

Cytotoxicity induced by heat stress exposure (24 h) and GOS (1% and 2.5%) was measured by the release of lactate dehydrogenase (LDH) in culture medium of the apical and basolateral compartment using the CytoTox 96 Non-Radioactive CytoToxicity Assay Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions.

2.5. Transepithelial electrical resistance (TEER) measurement

The integrity of the Caco-2 monolayer was determined prior to and after heat stress exposure (2, 4, 6, 8, 12 and 24 h) by measuring TEER levels using a Millicell-ERS voltohmmeter (Millipore, Temecula, CA, USA). Average TEER values for untreated cell monolayers were in the range of 412 ± 20 Ω.cm². The results are expressed as a percentage of initial value.

2.6. Paracellular permeability assay

Paracellular permeability across the Caco-2 cell monolayer was determined by measuring the flux of LY (molecular mass of

0.457 kDa). The transport studies from the apical side to the basolateral side were performed with 20 µg/ml of LY (Sigma Chemical Co, St Louis, MO, USA) which was added to the apical compartment (300 µl) of the transwells, 4 h prior to the end of heat stress exposure. Medium from the basolateral compartment was collected 4, 12 and 24 h after exposure to heat stress. The amount of LY in the basolateral compartment was determined by measuring the fluorescence intensity using a fluorometer (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany) at excitation and emission wavelengths of 410 and 520 nm.

2.7. RNA extraction and quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The levels of mRNA expression of different target genes [HSP70, HSP90, claudin-1, -3 and -4, occludin, zona occludens protein-1 (ZO-1), E-cadherin and haem oxygenase-1 (HO-1)] were measured by qRT-PCR in Caco-2 cells pre-treated with or without GOS for 24 h and exposed to heat stress for 6, 12 and 24 h. Cells were harvested with RNA lysis buffer containing β-mercaptoethanol. Total RNA was isolated using spin columns based on manufacturer’s instructions (Promega). RNA was reverse-transcribed to cDNA using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA).

For qRT-PCR, the PCR reaction mixture was prepared and amplifications were performed using iQSYBR Green Supermix (Bio-Rad Laboratories Inc.) according to manufacturer’s instructions using the MyiQ single-colour real time PCR detection system (Bio-Rad Laboratories Inc.) with the MyiQ System Software version 1.0.410 (Bio-Rad Laboratories Inc.). Commercially manufactured sets of gene specific primers (Eurogentec, Seraing, Belgium) were used after confirmation of specificity and efficiency analysis by qRT-PCR with dilution series of pooled cDNA at a temperature gradient (55 °C to 65 °C) for primer annealing and subsequent melting curve analysis (Table 1). The mRNA quantity was calculated relative to the expression of β-actin reference gene.

2.8. Western blot analysis

Caco-2 cells pre-treated with or without GOS for 24 h and exposed to heat stress for 12 and 24 h were lysed using 50 µl RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) containing protease

inhibitors (Roche Applied Science, Penzberg, Germany) and total protein concentration was assessed by a BCA protein assay kit (Thermo Scientific). Equal protein amounts of boiled samples were separated by electrophoresis (Criterion™ Gel, 4–20% Tris-HCl, Bio-Rad Laboratories Inc.) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands). Membranes were blocked with PBS containing 0.05% (v/v) Tween-20 (PBST) and 5% (w/v) milk proteins and incubated overnight at 4 °C with antibodies for HSP70, HSP90 (1:1000, Enzo Life Sciences, Farmingdale, NY, USA), claudin-1, -3, -4, occludin, ZO-1 (1:1000, Invitrogen, Carlsbad, CA, USA), E-cadherin (1:1000, eBioscience, San Diego, CA, USA) or caspase-9 (1:1000, Abcam, Cambridge, UK). After washing in PBST, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000, Dako, Glostrup, Denmark) for 2 h at room temperature. Finally, blots were washed in PBST and incubated in commercial ECL reagents (Amersham Biosciences, Roosendaal, The Netherlands) and exposed to X-ray film (Thermo Scientific, Antwerp, Belgium). The same membranes were probed with monoclonal rabbit anti-human β-actin antibody (1:2000, Cell Signaling, Danvers, MA, USA) to evaluate equality of loading. Films were scanned on a GS710 calibrated image densitometer (Bio-Rad Laboratories Inc.) and the optical density (OD) for the immune-reactive bands was quantified and expressed as relative protein expression (optical density normalized with β-actin).

2.9. Immunofluorescence staining

Immunofluorescence staining was performed to determine cellular localization of HSP70, HSP90 and E-cadherin. Caco-2 cells were grown on inserts and treated as described above. The inserts with Caco-2 cells were fixed with 10% formalin and after washing with PBS, the cells were permeabilized with PBS containing 0.1% (v/v) Triton-X-100 for 5 minutes, followed by blocking with 5% serum in 1% (w/v) bovine serum albumin (BSA)/PBS for 30 minutes at room temperature. Thereafter, Caco-2 cells were incubated (2 h at room temperature) with primary antibodies of HSP70 (1:50, Enzo Life Sciences), HSP90 (1:50, Abcam) and E-cadherin (1:50, BD Biosciences, San Diego, CA, USA) followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Nuclear counterstaining was performed with Hoechst 33342 (1:2000, Invitrogen) and subsequently the inserts were

Table 1 – Primer sequences used for quantitative real-time PCR.

Genes	Primer sequence (5'–3')		AT	References
	Forward	Reverse		
β-actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCTGTAAACAATGCA	63	NM_001101
Claudin-1	AGCTGGCTGAGACACTGAAGA	GAGAGGAAGGCACTGAACCA	63	NM_021101
Claudin-3	CTGCTCTGCTGCTCGTGTC	CGTAGTCCTTGCGGTCGTAG	63	NM_001306
Claudin-4	GTCTGCCTGCATCTGCTCTGT	CCTCTAAACCGGTCATCCA	62.5	NM_001305
E-cadherin	TGGACCGAGAGAGTTCCCT	CCCTGTACGTGGTGGGATT	60	BC-144283.1
HO-1	GCCACCAAGTTCAAGCAGCT	CAGTGCCACGGTAAGGAAG	61.2	NM_002133.2
HSP70	AGAGCCGAGCCGACAGAG	CACCTTGCCGTGTTGGAA	57	NG_011855.1
HSP90	CCTTCTATTGTCCACG	ATCCTCCGAGTCTACCAC	58.7	NM_005348.3
Occludin	TTGGATAAAGAATTGGATGACT	ACTGCTTGAATGATTCTTCT	57	NM_002538
ZO-1	GAATGATGGTTGGTATGGTGCG	TCAGAAGTGTGTCTACTGTCCG	55.8	NT_010194.17

washed and mounted with ProLong Gold anti-fade reagent (Invitrogen). HSPs and E-cadherin were visualized and images were taken using the Nikon Eclipse TE2000-U microscope equipped with a Nikon Digital Sight DS-U1 camera.

2.10. Statistical analysis

Analyses were performed by using GraphPad Prism (version 6.0) (GraphPad, La Jolla, CA, USA). Experimental results are expressed as mean \pm SEM and differences between groups are statistically determined by using one-way or two way analysis of variance (ANOVA), with Bonferroni post-hoc test. For single factor experiments one way analysis is performed, while for experiments composed of two factors a two-way ANOVA is used. Results are considered statistically significant when $P < 0.05$. Changes in mRNA relative expression between groups are statistically determined when the expression ratio of two-fold or higher is observed (Karlen, McNair, Perseguers, Mazza, & Mermoud, 2007).

3. Results

3.1. Heat stress does not affect cell viability

To determine the effects of GOS as well as heat stress exposure without treatment on the survival of the Caco-2 cell monolayers, a LDH leakage assay was performed. The results indicated that neither GOS in the used test concentration nor heat stress at 40 °C and 42 °C for 24 h did impair Caco-2 cell viability (Supplementary Fig. S1).

3.2. Heat stress up-regulates the mRNA expression of HSPs and disrupts intestinal barrier integrity

Before investigating the effects of GOS, the effect of heat stress on HSP gene expression and intestinal barrier integrity was investigated at different time points to design an *in vitro* heat stress model. Exposure of Caco-2 cells to 40 °C and 42 °C induced an up-regulation of mRNA expression levels of HSP70 and HSP90 after 6, 12 and 24 h (Fig. 1A, B). A temperature-dependent effect could be clearly observed for both HSPs, and early stage responses (6 and 12 h) showed higher HSP mRNA expression levels compared to the 24 h heat stress response. The HSP70 mRNA levels were more pronounced after heat stress exposure compared to HSP90, since a 77-fold increase was achieved in the levels of HSP70 mRNA expression, whereas the mRNA expression levels for HSP90 was increased 11-fold after 6 h heat stress exposure (Fig. 1A, B). Furthermore, changes in intestinal monolayer integrity were determined by TEER measurement and paracellular transport of LY (0.457 Da) across the Caco-2 cell monolayer. Results indicated that after heat stress exposure (40 °C and 42 °C), TEER levels decreased temperature- and time-dependently during 24 h compared to the control group of 37 °C (Fig. 1C). In line with these results, heat stress exposure also induced a temperature-dependent increase in the translocation of LY from the apical to the basolateral side after 12 and 24 h heat stress (Fig. 1D).

3.3. GOS prevent the heat-induced up-regulation of HSPs on mRNA and protein level

Confluent Caco-2 cells were pre-incubated with GOS for 24 h prior to 6 h (qRT-PCR analysis) or 24 h (Western blot analysis and immunofluorescence staining) heat stress exposure. qRT-PCR analysis revealed that treatment with GOS significantly decreased HSP70 and HSP90 mRNA expression levels induced by 42 °C heat treatment for 6 h (Fig. 2A, B). Western blot analysis showed an increased protein expression of HSP70 and HSP90 after 24 h heat stress exposure. The increased HSP70 protein levels were more pronounced after heat stress exposure as compared to HSP90 (Fig. 2C, D). Pre-treatment with GOS could concentration-dependently suppress the induction of HSP70 and HSP90 by thermal stress (Fig. 2C, D). The effect of GOS on the heat stress-induced HSP70 and HSP90 protein levels was confirmed by an immunofluorescence staining, since the HSP70 and HSP90 expression induced by heat stress was clearly decreased in the Caco-2 cells pre-incubated with GOS (Fig. 2E, F).

3.4. Heat stress induces cellular oxidative stress and GOS suppress the heat-induced increase in mRNA expression of haem oxygenase-1 (HO-1)

HO-1 is considered as a cellular marker for oxidative stress, and to evaluate whether heat stress with or without GOS pre-treatment could affect the oxidative stress response, the mRNA expression of HO-1 was measured by qRT-PCR. Results indicated that 42 °C heat stress for 6 h resulted in an immediate up-regulation of HO-1 as compared to the Caco-2 cells exposed to 37 °C. Pre-treatment with GOS could dose-dependently suppress this induction of HO-1 mRNA expression levels (Fig. 3A).

3.5. A GOS-induced decrease in HSP levels does not lead to apoptosis

Since the heat shock protein expression can interfere with the process of apoptotic cell death, the effect of thermal stress on caspase-9 production was measured to exclude a direct effect of GOS on apoptosis. Heat stress exposure (40 °C and 42 °C) to Caco-2 cells for 24 h did not affect the caspase-9 protein expression measured by Western blot analysis (Fig. 3B). However, a slight decrease in caspase-9 protein expression was observed in cells pre-treated with GOS, although these differences were statistically non-significant.

3.6. GOS partly prevent the heat-induced disruption of the intestinal barrier

Since thermal stress disrupts intestinal barrier integrity as observed in Fig. 1A, B, the effect of 24 h GOS pre-incubation was investigated on the confluent Caco-2 cells exposed to heat stress for 24 h. Results indicated that pre-treatment with GOS modulated the heat stress-induced TEER decrease in a dose-dependent manner (Fig. 4A). Besides the effect of GOS on 24 h heat stress exposure, the TEER levels were also measured at earlier time points (2, 4, 6, 8, and 12 h), where the effect of GOS was still present, albeit less pronounced (data not shown). Furthermore, pre-incubation with GOS could partly suppress the

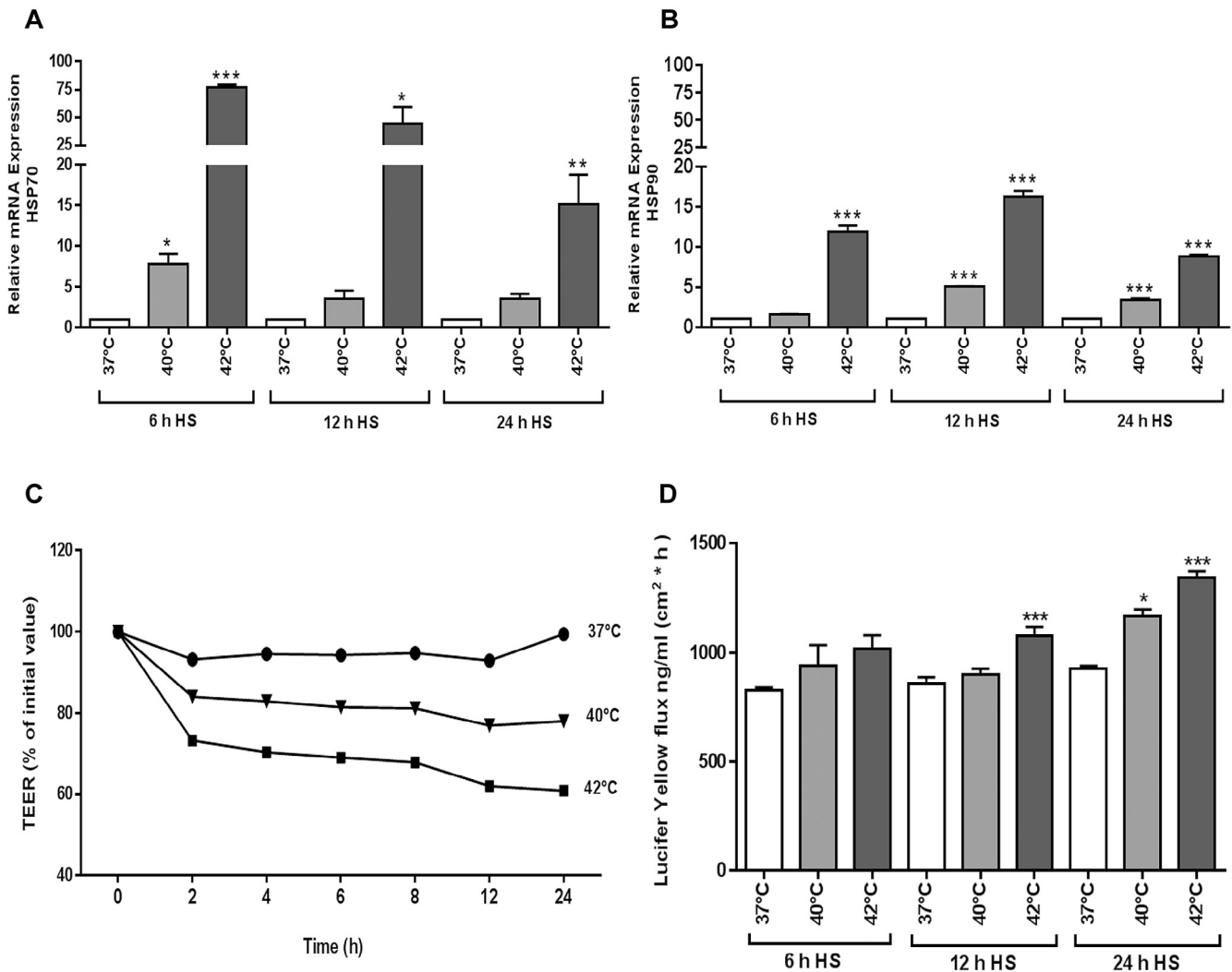


Fig. 1 – Heat stress up-regulates the HSPs mRNA expression and disrupts intestinal barrier integrity. Caco-2 cells were grown on inserts and exposed to heat stress (40 °C and 42 °C) for 24 h. The mRNA expression levels of HSP70 (A) and HSP90 (B) were measured by qRT-PCR after 6, 12 and 24 h heat stress. TEER (C) and transport of LY (D) from the apical to the basolateral chamber were measured. Data are presented as percentage of initial value (TEER), the amount of tracer transported [LY (ng/(cm² × h)) or relative mRNA expression as means ± SEM of three independent experiments, each accomplished in triplicate (*P < 0.05, **P < 0.01, ***P < 0.001; significantly different from the 37 °C-exposed cells at the same exposure time).

heat-induced increase in translocation of LY from apical to the basolateral side in 40 and 42 °C (Fig. 4B).

3.7. Heat stress modulates E-cadherin mRNA and protein level, while the Tight Junction (TJ) proteins remain unaffected

Since intestinal barrier integrity is intimately related with TJ and Adherens Junction (AJ) proteins, the mRNA expression levels of different TJ proteins (claudin-1, -3, -4, occludin and ZO-1) and E-cadherin, as typical AJ protein, were evaluated in Caco-2 cells exposed to heat stress. No significant changes in mRNA levels of the TJ proteins (claudin-1, -3, -4, occludin and ZO-1) were observed after thermal stress for 24 h (Fig. 5A–E). However, after exposure to 42 °C for 24 h, a temperature-dependent up-regulation in E-cadherin mRNA expression levels was observed (Fig. 5A–E). At the earlier time points of 6 and

12 h heat exposure, no remarkable changes were detected in mRNA expression levels of TJs and E-cadherin (Supplementary Fig. S2). The effect of heat stress exposure on TJ and AJ proteins in Caco-2 cells was further examined on protein level via Western blot analysis. Although thermal stress did not significantly affect the TJ protein expression, a slight decrease in occludin protein expression was observed after 24 h heat stress (42 °C) (Supplementary Fig. S3). Interestingly, the E-cadherin protein levels were clearly decreased by exposure to 42 °C (Fig. 6B).

3.8. GOS prevent the heat-induced effects on E-cadherin

Heat stress did not significantly affect the TJ mRNA and protein expression, and hence pre-treatment with GOS resulted only in minor alterations, such as a tendency towards an

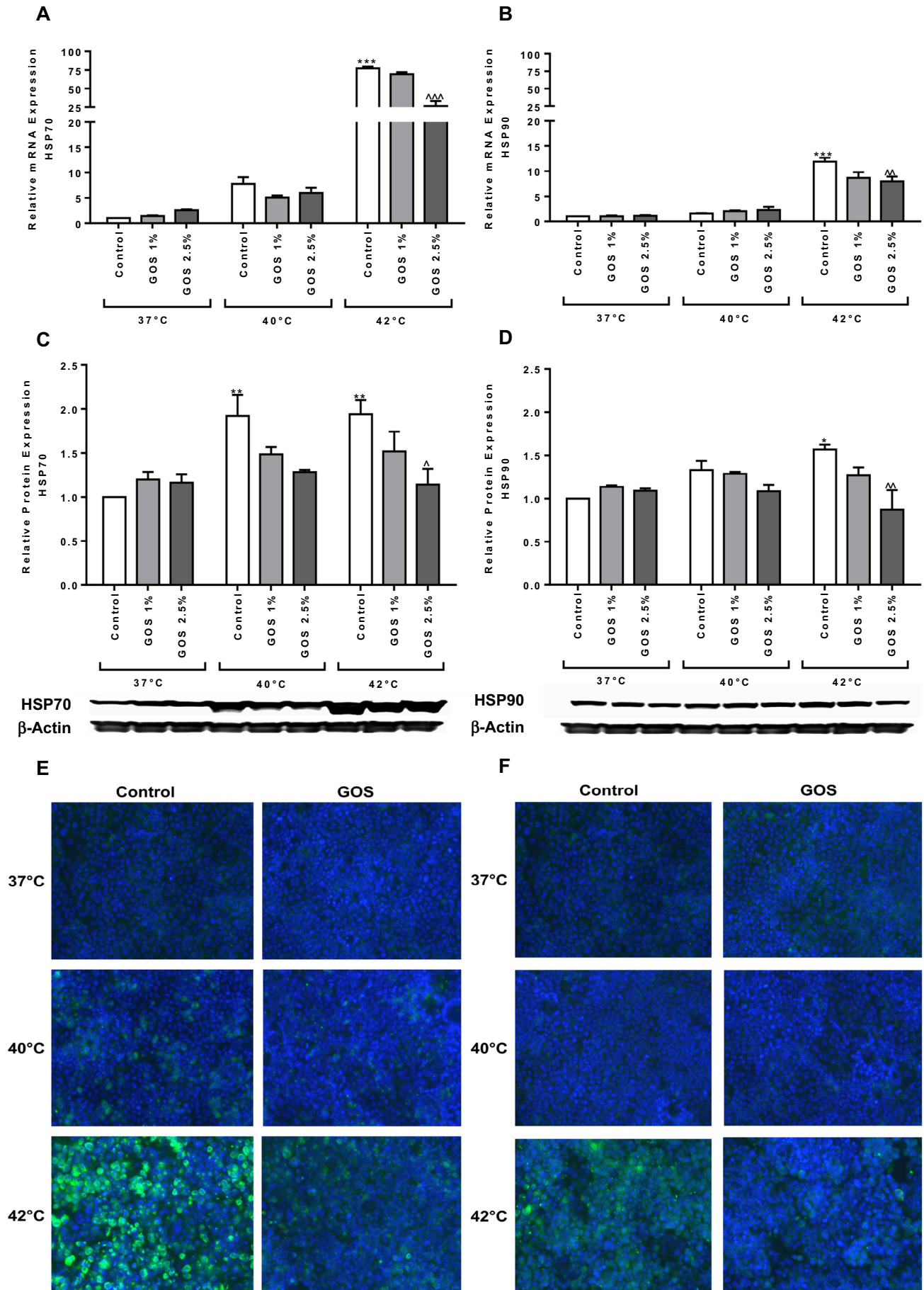


Fig. 2 – GOS prevent heat-induced up-regulation of HSP70 and HSP90 on mRNA and protein level. Caco-2 cells grown on inserts were pre-treated with GOS (1% and 2.5%) and exposed to heat stress (40 °C and 42 °C) for 6 h (qRT-PCR) or 24 h (Western blot analysis and immunofluorescence staining) to evaluate HSP70 and HSP90 mRNA (A, B), protein levels (C, D) or distribution pattern (E, F). Results are expressed as relative mRNA expression (qRT-PCR) or relative protein expression (Western blot, optical density normalized with β -actin) as mean \pm SEM of three independent experiments (* P < 0.05, ** P < 0.01, *** P < 0.001; significantly different from the control cells exposed to 37 °C. $\wedge P$ < 0.05, $\wedge\wedge P$ < 0.01, $\wedge\wedge\wedge P$ < 0.001; significantly different from the control cells exposed to 42 °C). Results of the immunofluorescence staining with antibodies for HSP70 and HSP90 are presented at 200 \times magnification.

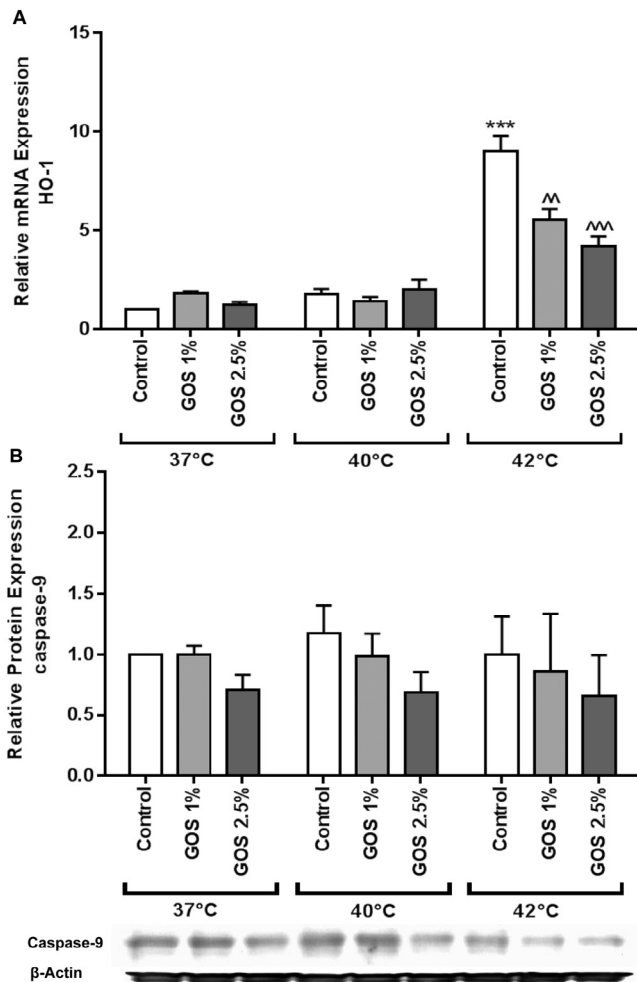


Fig. 3 – GOS suppress the heat-induced increase in HO-1 mRNA expression and do not affect caspase-9 protein expression. Caco-2 cells grown on inserts were pre-incubated with GOS for 24 h and exposed to heat stress (40 °C and 42 °C) for 6 h (qRT-PCR) to evaluate the mRNA expression of HO-1 (A) or for 24 h (Western blot) to measure the protein expression of caspase-9 (B). Results are expressed as relative mRNA expression (qRT-PCR) or relative protein expression (Western blot, optical density normalized with β -actin) as mean \pm SEM of three independent experiments (*** P < 0.001; significantly different from the control cells exposed to 37 °C. $\wedge\wedge P$ < 0.01, $\wedge\wedge\wedge P$ < 0.001; significantly different from control cells exposed to 42 °C).

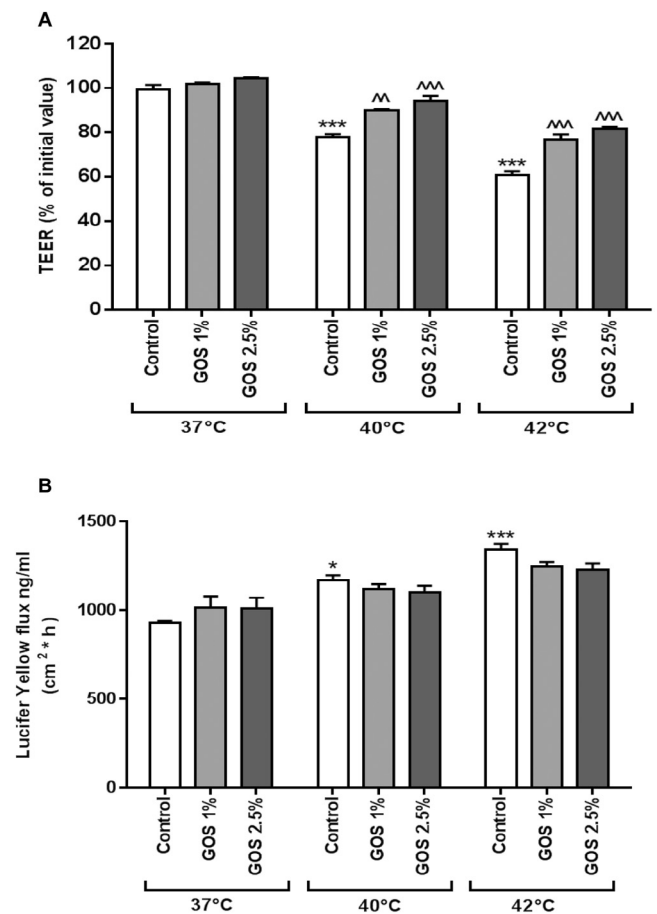


Fig. 4 – GOS partly prevent the heat-induced disruption of the intestinal epithelial barrier. Caco-2 monolayers grown on inserts were pre-treated with GOS and exposed to heat stress (40 °C and 42 °C) for 24 h. TEER levels (A) as well as translocation of LY (0.457 Da) from the apical to the basolateral chamber (B) were measured. Data are presented as percentage of initial value (TEER) or in the amount of tracer transported [LY (ng/cm² × h)] as means \pm SEM of three independent experiments, each performed in triplicate (* P < 0.05, *** P < 0.001; significantly different from the control cells exposed to 37 °C. $\wedge\wedge P$ < 0.01, $\wedge\wedge\wedge P$ < 0.001; significantly different from corresponding control cells exposed to 40 °C or 42 °C).

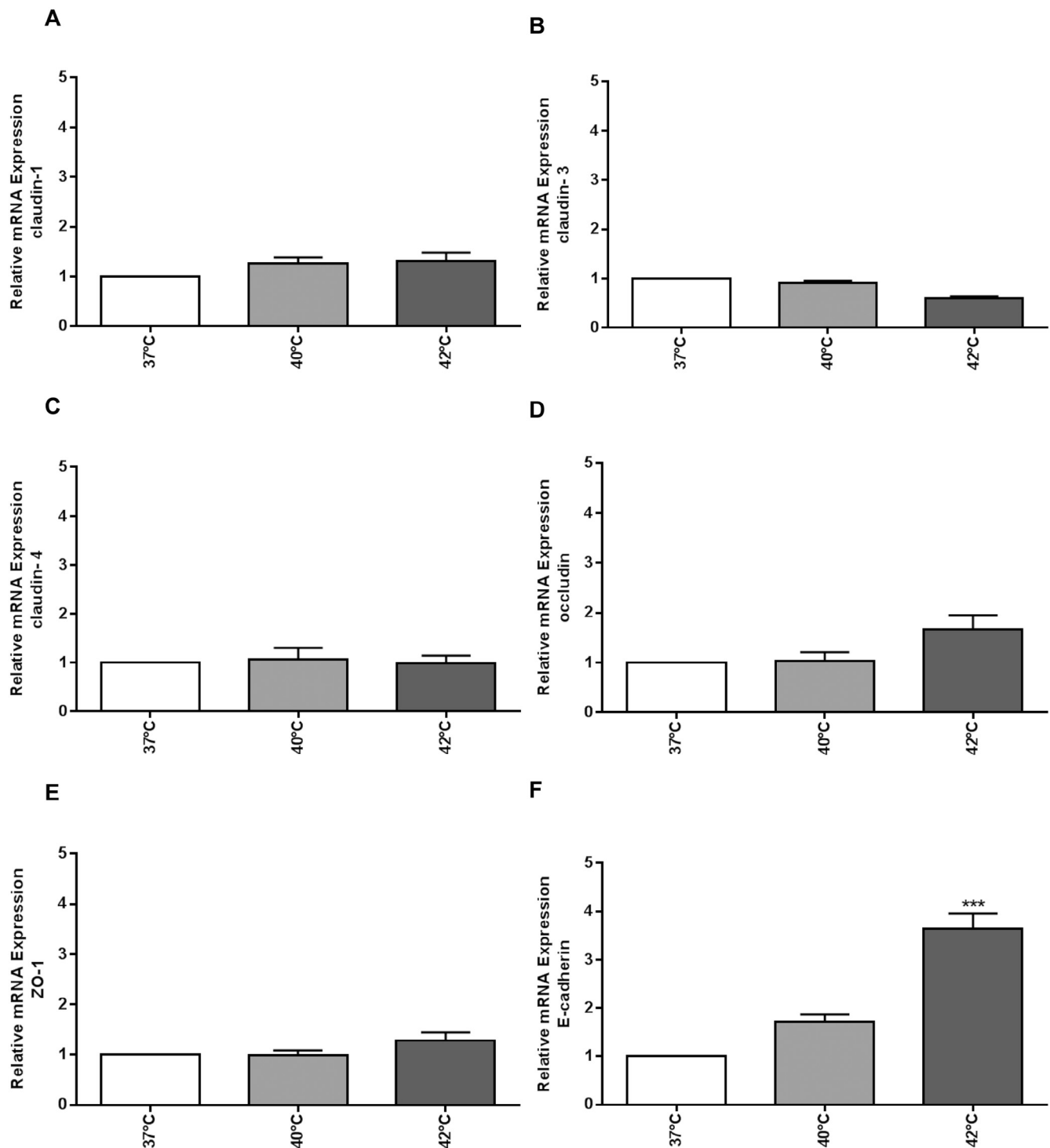


Fig. 5 – Heat stress modulates E-cadherin mRNA expression, while the TJ proteins remain unaffected. Caco-2 cells were grown on inserts and exposed to different temperatures (37 °C, 40 °C and 42 °C) for 24 h. mRNA levels of TJ proteins claudin-1 (A), claudin-3 (B), claudin-4 (C), occludin (D), ZO-1 (E) and E-cadherin AJ (F) were measured by qRT-PCR. Results are expressing relative mRNA expression as mean \pm SEM of three independent experiments performed in triplicate (***) $P < 0.001$; significantly different from the control cells exposed to 37 °C).

enhancement of the protein expression of claudin-3 and occludin (Supplementary Fig. S3).

In contrast, qRT-PCR analysis indicated that pre-treatment with GOS significantly decreased the E-cadherin mRNA

expression levels induced by 42 °C heat treatment for 24 h (Fig. 6A), while the heat-induced decrease in E-cadherin protein expression could be clearly prevented by GOS (Fig. 6B). The effect of GOS on the heat stress-induced decrease in E-cadherin

protein levels was confirmed by an immunofluorescence staining, showing that E-cadherin was translocated from the cellular membrane to the submembraneous space. This irregular cellular distribution was partly prevented by GOS (Fig. 6C), as in the cells pre-treated with GOS and exposed to 42 °C, more Caco-2 cell clusters were observed with E-cadherin localized at the cell membrane (red arrows) compared to the heat-exposed cells without pre-treatment.

4. Discussion

Non-digestible oligosaccharides, like GOS, are known as functional food ingredients that can modify the gut function by enhancing the growth of beneficial bacteria, stimulating immune responses and maintaining the intestinal barrier integrity (Al-Sheraji et al., 2013; van Hoffen et al., 2009; Zhong et al., 2009). Therefore, we hypothesized that dietary GOS could protect the intestinal epithelial barrier against heat-induced effects on intestinal barrier integrity, oxidative stress and associated HSP production.

HSPs have been recognized as markers of thermal stress (Dokladny et al., 2006; Tomanek & Sanford, 2003). In our *in vitro* model, thermal stress of 42 °C induced an increase in HSP70 and HSP90 mRNA and protein expression in Caco-2 cells, which was prevented by pre-incubation of the cells with GOS. These effects were confirmed by immunofluorescence staining for HSP70 and HSP90. The HSP70 mRNA and protein expression in Caco-2 cells was more pronounced after thermal stress as compared to HSP90. HSP70 is known as the most temperature sensitive and highly conserved member of the HSP family (Kregel, 2002). Previous *in vitro* studies in K562 cells also indicated that exposure to high temperatures would induce significantly more HSP70 and to a lesser extent HSP90 (Holmberg, Leppä, Eriksson, & Sistonen, 1997; Leppä, Kajanne, Arminen, & Sistonen, 2001). HSP70 controls in a non-specific manner protein folding, while HSP90 interacts with specific client proteins such as hormone receptors and protein kinases [reviewed in (Pratt & Toft, 2003; Zhao & Houry, 2005)]. These differences could be possible explanations for differences in HSP70 and HSP90 expression after thermal stress. Our findings related to the HSP expression are in line with Dokladny et al. (2006) who also showed a significant increase in protein expression of HSP70 and HSP90 in Caco-2 cells exposed to 41 °C for 24 h. Various previous studies focused on strategies to decrease HSPs to stimulate apoptosis, or increase HSP to suppress cell death, depending on the pathological conditions, as for example in cancer therapy, induction of apoptosis might be beneficial (Shelton, Dillard, & Robertson, 2010).

In the intestines and many other organs, HSP70 and HSP90 are known as negative regulators of apoptosis during stressful conditions and interact with the caspase-mediated death signalling pathways by inhibiting effector-caspases (Gao & Newton, 2002; Joly et al., 2010). To exclude that decreasing HSP70 and HSP90 expression levels induced by GOS in our study were not provoking apoptosis of the Caco-2 cells, the expression of caspase-9 was measured. Our results demonstrated that the decreased expression of heat shock proteins caused by GOS pre-treatment did not induce apoptosis through an

up-regulation of caspase-9 protein levels. It has previously been shown that within the caspase-mediated apoptosis chain, caspase-9 activation is strictly required for apoptosis induced by thermal stress in Jurkat cells (Shelton et al., 2010).

HSPs are also considered as sensitive biomarkers that directly represent the degree of oxidative stress (Stacchiotti et al., 2004). Previous studies already demonstrated that the protective effect of radical scavengers against oxidative stress resulted in suppressed expression of HSPs in different cell lines (Núñez, Osorio, Tapia, Vergara, & Mura, 2001; Tsuji et al., 2009). Another, independent and sensitive marker of oxidative stress is HO-1. Comparable to the HSPs, HO-1 is known to be transcriptionally induced in response to heat exposure in intestinal cells (Naito, Takagi, Uchiyama, & Yoshikawa, 2011). Hence we measured the relative mRNA expression of HO-1 and indeed could show that exposure to 42 °C significantly induced HO-1 expression in Caco-2 cells and GOS prevented the heat-induced increase in HO-1 mRNA levels. A protective role of GOS against oxidative stress was also reported in T84 intestinal cells (Van den Ende, Peshev, & De Gara, 2011). Moreover, *in vivo* supplementation of diets with fructo-oligosaccharides (FOS) or GOS alleviated the oxidative stress injury in hepatocytes and renal cells (Chen, Wang, Kuo, & Tsai, 2011; Furuse et al., 2014; Nakamura et al., 2014). Although in these *in vivo* studies, the protecting role of oligosaccharides against oxidative stress has been dedicated to the improvement of the intestinal microflora, we here demonstrated that also a direct cell-protective effect should be considered.

It is already known that exposure to thermal stress can induce gut barrier dysfunction and increase intestinal epithelial permeability to luminal antigens and lead to bacterial translocation through the gut epithelium (Dokladny, Ye, Kennedy, Moseley, & Ma, 2008; Hall et al., 2001; Lambert et al., 2002). In line with these findings, our results showed a temperature- and time-dependent decrease in TEER levels and temperature-dependent increase in paracellular permeability to LY, indicating physical impairment of the cell monolayer. The integrity of the intestinal barrier largely depends on the expression and function of TJ and AJ proteins facilitating the efficient cell–cell adhesion (Hartsock & Nelson, 2009). Previous studies indicated that the heat-induced increase in paracellular permeability is mainly associated with TJ proteins assembly (Dokladny et al., 2006, 2008). Xiao et al. (2013) described that the occludin and ZO-1 mRNA and protein levels were decreased in Caco-2 cells exposed to severe thermal stress for 1 h (43 °C) and n-3 polyunsaturated fatty acids protected this heat-induced permeability dysfunction.

In contrary to these previous reports, we did not find any significant effects on TJ protein mRNA or protein expression after heat stress exposure. These differences suggest that different heat exposure times, temperatures and recovery periods may result in different expression levels of TJ proteins. Nevertheless, GOS pre-treatment could partly prevent the thermal stress-induced decrease in TEER levels as well as LY translocation from the apical to the basolateral compartment of the Caco-2 monolayer. PCR analysis of E-cadherin showed an up-regulation in mRNA levels after heat stress, which may be recognized as a compensatory response to heat shock-induced changes in E-cadherin protein levels. Further experiments demonstrated that heat stress could markedly reduce the protein

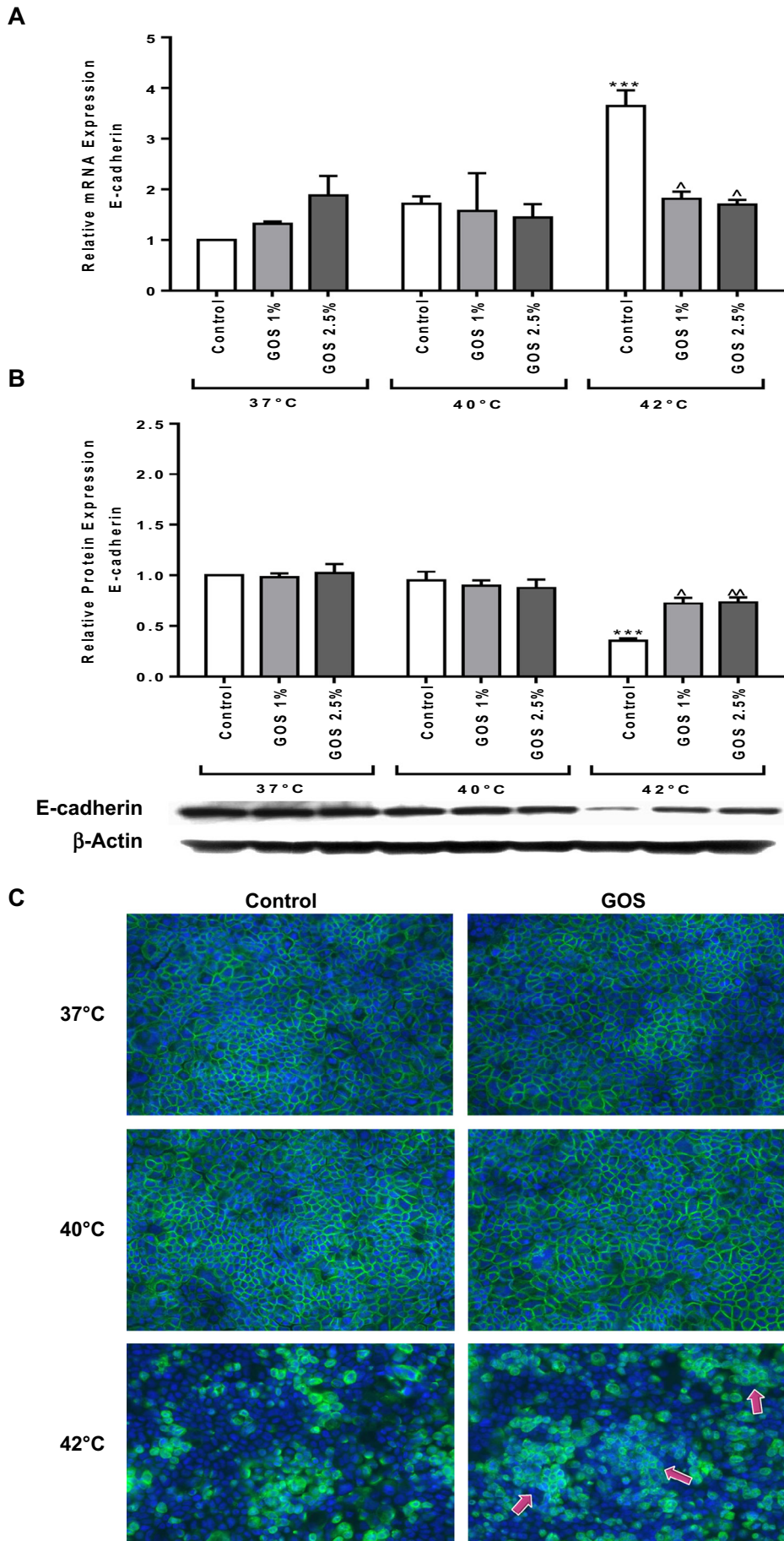


Fig. 6 – GOS prevent the heat-induced effects on E-cadherin. Caco-2 cells grown on inserts were pre-incubated with GOS (1% and 2.5%) and exposed to heat stress (40 °C and 42 °C) for 24 h. The expression of E-cadherin in mRNA level (A), protein level (B) and cellular distribution (C) was evaluated. Results are expressed as relative mRNA expression (qRT-PCR) or relative protein expression (Western blot, optical density normalized with β -actin) as mean \pm SEM of three independent experiments ($P < 0.001$; significantly different from the control cells exposed to 37 °C. $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$; significantly different from the control cells exposed to 42 °C). Immunofluorescence results are performed using an antibody for anti E-cadherin and are presented at 200 \times magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

expression and altered the cellular localization of E-cadherin. These results confirmed previous findings indicating that heat shock could alter cell–cell adhesion by down-regulating E-cadherin protein levels (Lang et al., 2012). Recently, Chen, Chen, Chen, Lee, and Huang (2013) demonstrated that the underlying mechanism of E-cadherin expression during thermal stress corresponds with the HSP90-induced TCF-12 gene expression as negative regulator of E-cadherin protein. Moreover, our results clearly indicated that the heat stress-induced mRNA expression, protein derangement and delocalization of E-cadherin could be prevented by GOS pre-treatment.

Further research is needed to unravel the mechanisms behind this microbiota-independent protective effect of GOS. *In vivo* heat stress models with chickens also reported that mannan-oligosaccharides and probiotic mixtures can partially lessen the heat-induced changes in intestinal morphology and intestinal barrier function (Sohail et al., 2012; Song et al., 2014). Moreover, Xu et al. (2002) showed that a certain size of oligosaccharides, tetrasaccharides of hyaluronan, upregulated HSP72 expression under heat stress conditions and suppressed cell death. This finding suggests that the size of oligosaccharides could be important for the HSP-related effect. The currently applied GOS is a complex mixture of oligosaccharides produced with different degrees of polymerization (DP) and glycosidic linkages (Hernández-Hernández, Calvillo, Lebrón-Aguilar, Moreno, & Sanz, 2012). Li et al. (2015) found that various structures of galacto-oligosaccharides differently affect the *in vitro* fermentation by human intestinal microbiota. Nevertheless, it remains to be completely elucidated whether there are significant differences in the biological activity of GOS related to the structures, molecular weight and type of glycosidic linkages. As yet it is only known that non-digestible oligosaccharides, like GOS, might interact with peptidoglycan recognition protein 3, peroxisome proliferator-activated receptor γ or carbohydrate receptors, such as C-type lectin (Jeurink et al., 2013; Vos, M'Rabet, Stahl, Boehm, & Garssen, 2007; Zenhom et al., 2011). Furthermore, there might be a similarity between the protection of GOS against heat stress and the thermal protection of fungal species by accumulation of high levels of trehalose-based oligosaccharides (Wyatt et al., 2015). Trehalose may act as an anti-oxidant and is capable of reducing protein carbonylation during oxidative stress. It also stabilizes the structure of lipid bilayers and of proteins, avoids protein aggregation, and prevents (oxidative) changes in large molecules (reviewed by Eleutherio, Panek, De Mesquita, Trevisol, & Magalhães, 2014). Possibly, GOS, like other sugars, have a macromolecule-stabilizing character that protect cells against oxidative and heat stress (Cray, Russell, Timson, Singhal, & Hallsworth, 2013).

5. Conclusion

Our results indicate that galacto-oligosaccharides protect the intestinal epithelial barrier against heat stress as observed by a decrease in heat-induced HSP70 and HSP90 on mRNA and protein levels, and by a suppression of the heat-induced oxidative stress response. Furthermore, in the absence of measurable changes in expression of TJ proteins, thermal stress-induced disruption of the intestinal epithelial barrier can be particularly associated with the derangement of E-cadherin, which is mitigated by pretreatment of cells with GOS. It can be concluded that dietary GOS, nowadays mainly used in infant formulas, may be an attractive dietary application for people who are at high risk of developing heat stress. However, further investigations are necessary to identify the molecular targets of oligosaccharides (from different origins) in the intestines and to establish a structure–activity relationship.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.04.045.

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