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Polyphenol - enriched grape pomace extract and lactobacilli combination restores epithelial integrity in LPS-induced inflammation in intestinal Caco-2 cells

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Abstract

Inflammatory bowel disease is a chronic pathology resulting from uncontrolled inflammation that ultimately leads to mucosal disruption and ulceration. Nutritional therapy using biologically active compounds was taken into account as alternative to medical treatment of intestinal inflammation. The aim of our study is to investigate the role of polyphenol-rich grape pomace extract and *Lactobacilli* sp on the genes associated with epithelial integrity using LPS-treated intestinal Caco-2 cells. After induction of intestinal inflammation, cells were cultured in presence of grape pomace extract, *Lactobacilli* species mixture or combination of them for 24 hours. The gene expression of markers for epithelial integrity (Occl, ZO-1, MMP-2, MMP-9 and TIMP-1) was evaluated by qPCR. Treatment either with grape pomace extract or lactobacilli probiotics was able to restore the expression of important markers of epithelial integrity in inflamed intestinal cells (decrease of Occl, ZO-1, MMP-2 and MMP-9 gene expression and increase of TIMP-1 mRNA). Also, the combination of prebiotics and probiotics could induce a favorable modulation of studied genes in LPS-treated cells. In conclusion, the polyphenols from grape pomace extract and probiotics or their combination could be a useful nutritional strategy in intestinal inflammation management.

Introduction

The rapid increase of the incidence of intestinal inflammatory pathologies has a great impact on the quality of life. Inflammatory bowel disease (IBD) is a chronic pathology resulting from uncontrolled inflammation that ultimately leads to mucosal disruption and ulceration (Shimshoni E 2015). The sustained immune response in IBD is mainly facilitated by defects in the function of the intestinal epithelial barrier and in the regulation of mucosal immunity. Between the important regulators of epithelial integrity in the intestinal tissue are junction proteins Occludin (Occl), Zonula occludens-1 (ZO-1) matrix metalloproteinases (MMPs) and their natural inhibitors (TIMPs). Current therapies to treat IBD have limited efficacy and potentially serious side-effects (e.g. long-term use of glucocorticoid treatment cause epithelial thinning).

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Nutritional approaches could be an alternative strategy for IBD management. Polyphenols are abundant in plant foods, with antioxidant and anti-inflammatory properties. Grape pomace (GP) is an industrial waste which is enriched in beneficial polyphenols. The recent studies demonstrated the beneficial role of polyphenols in modulating the intestinal inflammation (Boussenna A 2016). On the other hand, probotics (*Lactobacilli* sp) are another promising alternative which could reduce the magnitude of epithelial disruption in intestinal inflammation (de Vrese M 2008).

Starting from these data, the aim of our study is to investigate the role of polyphenol-rich grape pomace (GP) extract and *Lactobacilli* sp in restoring the genes associated with epithelial integrity using LPS- treated Caco-2 cells as *in vitro* model of intestinal inflammation.

Materials and Methods

Preparation of grape pomace extract: Red grape pomace was obtained as a dried material from a mix of *Vitis Vinifera* sp. variety delivered by a local winery producer from Valea Calugareasca, Romania. The polyphenols from GP were extracted using acetone 80% solution (1g GP/ 7 ml acetone solution). The GP-acetone mixture was shaken for 24 hours. After centrifugation for 10 minutes at 4000 rpm, the resulting extract was concentrated on a RVC 2-18 CDplus mini concentrator and the total polyphenol concentration of the resiude was determined using the Folin-Ciocalteu method. The extract residue was then aliquoted and stored at -20ºC until further analyses.

Bacterial strains and culture conditions The *Lactobacillus* strains (L. rhamnosus (ID IBNA02), L. paracasei (ID 13239), L. acidophilus (ID 11692)) were kindly offered by Dr. Olguta Dracea (Cornelli), from Cantacuzino NIRDMI, Bucharest, Romania and used in this study. The bacteria were cultured in MRS broth at 37°C for 16 h. After cultivation, the cells were harvested by centrifugation (4000 rpm, at 4°C). The cell density was determined by measuring the absorbance at 600 nm. The *Lactobacillus* cells were finally suspended Caco-2 culture medium (MEM without antibiotic, see below), adjusted at a concentration of $1x10^8$ CFU/mL for each strain and added to the Caco-2 cells ($3x10^8$ CFU/mL *Lactobacillus*/well).

Cell culture: Caco-2 intestinal cells were purchased from the American Type Culture Collection (ATCC). The cells were cultured in Minimum Essential Medium (MEM), supplemented with 10% Fetal Bovine Serum and 1% antibiotic and were maintained at 37 C in a 5% CO_2 atmosphere. The inflammatory condition was simulated by treating cells with LPS 5 µg/ml, for 4 hours. After induction of intestinal inflammation, cells were cultured in presence of GP extract (50µg/ml), *Lactobacilli* (Lb) mixture ($3 x10^8$ CFU/mL total Lb) and combination between them for 24 hours. For *Lactobacilli* addition, the medium containing 1% antibiotic was aspired and was replaced with MEM without antibiotic.

Extraction of total RNA and cDNA synthesis. The Caco-2 cells were lysed with Lysis buffer. Total RNA was extracted using Qiagen RNeasy mini kit (QIAGEN GmbH, Germany), according to the manufacturer's recommendations. The total RNA isolated from each sample was further used to generate cDNA using M-MuLV Reverse Transcriptase kit (Thermo Fischer Scientific, USA) according to the manufacturer's protocol.

qPCR analysis. To evaluate the gene expression for markers involved in epithelial integrity (junction proteins Occl, ZO-1, matrix metalloproteinases MMP-2, -9, the inhibitor of MMPs (TIMP-1), the qPCR reaction was performed in Rotor-Gene-Q (QIAGEN GmbH, Germany) machine using 1µl of cDNA, 12.5 µl SYBR Green qPCR Master Mix and 0.3µM each of gene-specific primer. The nucleotide sequences of the primers used are presented in Table 1. The PCR cycling conditions consisted of: initial denaturation step at 95°C 15s, followed by 40 cycles of 95°C 15s, 60°C 15s with a single fluorescence measurement; a final elongation step was carried out at 72°C 10 min. Two reference genes, β-actin and GAPDH (selected from a panel of four references genes, using Excel-based NormFinder software) were used for data normalisation. The results were expressed as relative fold change (Fc) compared with untreated cells.

Table 1. The characteristics of primers used for qPCR amplification

Gene	Primer characteristics		Amplicon length
	Orientation	Sequence	
Occl	Fw	aagagttgacagtcccatggcatac	133
	Rv	atccacaggcgaagttaatggaag	
ZO-1	Fw	ttaagccagcctctcaacagaaa	83
	Rv	Ggttgatgatgctgggtttgt	
MMP-2	Fw	cgctcagatccgtggtgag	72
	Rv	ttgtcacgtggcgtcacag	
MMP-9	Fw	ttgacagcgacaagaagtgg	134
	Rv	gtacatagggtacatgagcg	
TIMP-1	Fw	cacccacagacggccttct	67

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	Rv	cttctggtgtccgcacgaa	
Beta2 microglobulin	Fw	gtgctcgcgctactctctc	150
	Rv	gtcaacttcaatgtcggat	
defensin, beta 1 (DEFB1)	Fw	ctctgtcagctcagcctc	279
	Rv	cttgcagcacttggccttccc	
Beta - actin	Fw	cctggcacccagcacaat	70
	Rv	gccgatccacacggagtact	
GAPDH	Fw	tgtggtcatgagtccttcca	136
	Rv	catgggtgtgaaccatgaga	

Statistic analysis: One way ANOVA analysis was used to determine the statistical differences between groups for all parameters analysed. Values of P< 0.05 were considered significant.

Results and Discussion

The effect of GP extract, Lb mixture and combination between them on junction proteins Occl and ZO-1 gene expression in LPS-treated Caco-2 cells

LPS treatment alone induced a reduction of mRNA levels coding for Occl and ZO-1 (in comparison with control Caco-2 cells (Figure 1). This reduction of gene expression of junction proteins was also induced by LPS in different cellular models of intestinal inflammation and enteritis (Guo H 2016). GP extract added to LPS-treated Caco-2 cells was associated with an increase of Occl and ZO-1 gene expression (1.3±0.1Fc, P=0.044; 1.0±0.2Fc, Figure 1) in comparison with LPS-treated cells, while Lb mixture resulted in a significant up-regulation of Occl (3.0±0.6 Fc, P=0.050) and ZO-1 (4.6±0.3Fc, P=0.001, Figure 1) mRNAs in Caco-2 cells, when compared with LPS treatment. Our results are in accordance with studies showing the role of polyphenols such as tea flavonoids (Guo H 2016) and of the *Lactobacilli* sp. (Anderson 2010) in the restoration of junction protein levels in LPS-affected cells. In our experiments, the Occl and ZO-1 gene expressions were increased by pre- and probiotic administration in LPS-inflamed cells (2.8±0.7Fc; 3.6±0.6, P=0.029, Figure 1). On our knowledge, this is the first study demonstrating the role of the combined pre- and probiotic administration in counteracting the LPS-affected junction protein gene expressions.

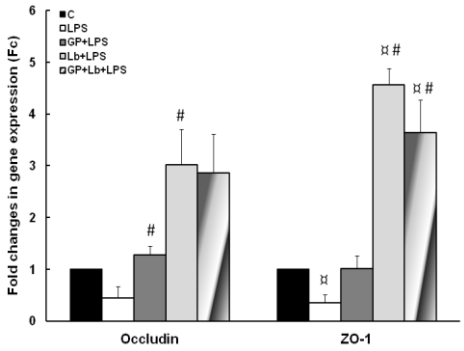

Figure 1. The effect of GP extract, Lb mixture and combination between them on junction proteins Occl and ZO-1 in LPS-treated Caco-2 cells. After treatment with LPS 5 µg/ml, for 4 hours, Caco-2 cells were cultured in presence of GP extract (50µg/ml), Lactobacilli (Lb) mixture (3 x10^8 CFU/mL total Lb) and combination between them for 24 hours. The bars represent average±SEM for three independent experiments. The symbols represent the statistical differences between the experimental groups and control samples (¤) and prebiotic/probiotic/combination of them and LPS group (#), respectively.

The effect of GP extract, Lb mixture and combination between them on MMP-2 and MMP-9 matrix metalloproteinases and TIMP-1 mRNAs in LPS-treated Caco-2 cells

As shown in Figure 1, LPS treatment alone induced a significant up-regulation of MMPs gene expression (MMP-2: 4.7±0.3Fc, P=0.009; MMP-9: 5.7±0.1Fc, P=0.001) in Caco-2 cells, while TIMP-1 mRNA level was reduced (0.4±0.0Fc, P=0.013) in LPS-pre-treated cells. The gelatinases (MMP-2 and MMP-9) have been found to be the predominant upregulated MMP in intestinal tissue of patients with IBD (Shimshoni E 2015). In concordance with our results, Strup-Perot et al found that LPS treatment of intestinal cells was associated with overexpression of MMPs, which exceeded that of their inhibitor, TIMP-1 (Strup-Perrot C 2004). The addition of 50µg/ml of GP extract to the inflamed Caco-2 cells was associated with a decreased level of MMPs mRNAs (MMP-2: 1.2±0.2Fc, P=0.009; MMP-9: 0.5±0.1Fc, P<0.001) and with an increase TIMP-1 gene expression (1.3±0.03, P=0.030) in comparison with LPS-treated cells. A recent study demonstrated that green tea polyphenols reduced the gene expression for MMP-2 and -9 in the gastric MKN-28 cell line (Arcone R. 2016). Lb mixture (1x10^8 each Lb) has a similar effect on Caco-2 LPS-treated cells (decrease of the levels of mRNAs coding for MMP-2: 1.3±0.1, P=0.003 and for MMP-9: 0.5±0.1Fc, P<0.001; increase of TIMP-1 gene expression: 1.9±0.1Fc, P<0.001, Figure 2). Previously, the role of different probiotics such as *E.coli* Nissle 1917 strain in the modulation of MMPs expression was established (Fábrega M-J 2017). To our knowledge, no data on the

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effects of the Lactobacilli sp. on MMPs expression in LPS-induced inflammation has been published yet. The combination between GP enriched in polyphenols and probiotics (*Lactobacilli* sp) could also restore the gene expression for markers affected by LPS treatment in Caco-2 cells. Briefly, the expression of MMPs genes was decreased (MMP-2: 0.3±0.1Fc, P=0.003; MMP-9: 1.0±0.1Fc, P<0.001), while mRNA for TIMP-1 remained unaltered (2.5±0.6Fc, P>0.05) in LPS-treated cells which received pre- and probiotic combination. The combination of some probiotics (*E coli* strain Nissle 1617) with other treatments increase the effects of probiotics, reducing MMP-2/MMP-9 expression in an dextran sodium sulphate (DSS)-model of colitis (Garrido-Mesa N 2011). In our experiments, the reduction of MMP-2/MMP-9 gene expression in the inflamed cells could be associated with a reduction of local inflammation by cellular treatment with the tested prebiotic/probiotic combination.

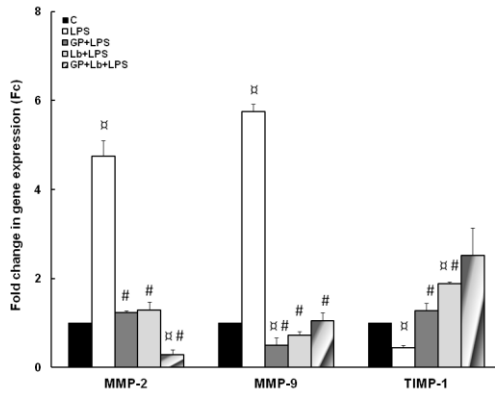

Figure 2. The effect of GP extract, Lb mixture and combination between them on MMP-2 and MMP-9 matrix metalloproteinases and TIMP-1 mRNAs in LPS-treated Caco-2 cells. After treatment with LPS 5 µg/ml, for 4 hours, Caco-2 cells were cultured in presence of GP extract (50µg/ml), Lactobacilli (Lb) mixture (3 x10^8 CFU/mL total Lb) and combination between them for 24 hours. The bars represent average±SEM for three representative experiments. The symbols represent the statistical differences between the experimental groups and control samples (¤) and prebiotic/probiotic/combination of them and LPS group (#), respectively.

Conclusion

Our study showed that GP enriched in polyphenols could restore the gene expression for important markers associated with epithelial integrity in inflamed intestinal cells. The same effect was observed in case of probiotics treatment. Moreover, the combination of prebiotics and probiotics could induce a favourable modulation of genes involved in epithelial damage in LPS-treated cells. In conclusion, the polyphenols from GP extract as well as Lactobacilli sp mixture, alone or their combination could be a useful nutritional strategy in the management of IBD-associated intestinal disruption.

Acknowledgements

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