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Full Length Article

Microbe-derived Antioxidants Promote the Expression of Nutrient Transporters Through Regulating Apoptosis and Autophagy in Mice Challenged with Diquat

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Abstract

We evaluated the effects and underlying mechanisms of microbe-derived antioxidants (MA) on the expression of nutrient transporters in mice challenged with diquat. Thirty C57BL/6 female mice were randomly divided into 3 groups: Control and Diquat group, both orally administered with 0.9% saline each day; MA+Diquat group, administered with 0.065 mg/mL day^{-1} MA. At day 22, the mice were injected with 0.9% saline (Control) or 1.8 mg/mL diquat (Diquat and MA+Diquat groups) and sacrificed for jejunum samples after 3 h. Results showed that oral administration of MA alleviated diquat-induced intestinal oxidative stress, as evidenced by the increased (P < 0.05) activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), enhanced (P < 0.05) content of total glutathione (T-GSH) and total antioxidant capacity (T-AOC), and decreased (P < 0.05) contents of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). Compared with the Diquat group, pretreatment with MA elevated (P < 0.05) the mRNA expression of glucose transporters, fatty acid transporters and oligopeptide and amino acid transporters. Notably, diquat supplementation increased (P < 0.05) the concentrations of caspase-3, caspase-8 and caspase-9, whereas such increases were inhibited (P < 0.05) by MA supplementation in diquat challenged mice. In addition, complementation of MA elevated (P < 0.05) the mRNA and protein expression of microtubule-associated protein light chain 3 (LC3) and beclin-1 in diquat challenged mice. Collectively, our findings indicate that the promoted expression of nutrient transporters in MA-pretreated mice appears to be associated with the enhanced autophagy and the inhibited apoptosis.
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Key words: Microbe-derived antioxidants; Diquat; Nutrient transporters; Jejunum; Mice

Introduction

Nutrient transporters play a vital role in the intestinal transportation and absorption of glucose, fatty acids, oligopeptides, and amino acids, both in human and animals (Losacco *et al.*, 2018; Oswald, 2019; Saleh *et al.*, 2018). Impairment of these transporters results in inadequate provision of nutrients (Wan *et al.*, 2018) and subsequently causing various intestinal diseases, including inflammatory bowel diseases and irritable bowel syndrome (Camilleri *et al.*, 2012), and even growth restriction (Andrade *et al.*, 2018b). Many factors, such as oxidative stress, especially high levels of reactive oxygen species (ROS), can impair nutrient transporters. Hence, maintaining antioxidant capacity is crucial for preserving nutrient transporters. Currently, supplementation with antioxidants seems to be feasible options for promoting nutrient transport and preventing oxidative stress-related intestinal diseases.

Microbe-derived antioxidants (MA) were produced with fruits (*e.g.*, roxburgh rose and sea-buckthorn) by microbial (*e.g.*, *Bacillus subtilis*, *Lactobacillus*, *Clostridium butyricum*, and *Saccharomyces cerevisiae*) solid-liquid fermentation, extraction, concentration, inactivation, and freeze-drying (Cai *et al.*, 2011). And the antioxidant activity of MA could be associated with multiple ROS scavengers such as vitamin C, vitamin E, isoflavones and glutathione (Cai *et al.*, 2011). We previously found that MA could scavenge hydroxyl radical and superoxide anion ($O_2^{\cdot-}$) (Cai *et al.*, 2011). Nevertheless, the effects and underlying mechanisms of MA on nutrient transporters remain obscure. Autophagy and apoptosis are the two major stress-response cellular pathways, which regulate cell survival and cell death (Martinez-Outschoorn *et al.*, 2014). Diquat is a bipyridyl herbicide that could induce oxidative stress (Cao *et al.*, 2018; Mo *et al.*, 2018; Xu *et al.*, 2018). Accordingly, this study was undertaken to ascertain whether MA could promote the expression of nutrient transporters through its antioxidant effects on apoptosis and autophagy in mice challenged with diquat.

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Materials and Methods

The present study was approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (Shanghai, China). MA (KB-120) were provided by Shanghai Jiang Han Biotechnology (Shanghai, China) and the main components were listed as follows: 13,600 mg/kg Mg, 503 mg/kg Fe, 367 mg/kg Mn, 1.07 mg/kg Cu, 0.18 mg/kg Se, 1,940,000 U/kg super oxide dismutase (SOD), 3,220 mg/kg vitamin C, 9,080 μg/kg vitamin E, 4.43% total isoflavones, 1.37% isoflavones, 8,860 mg/kg glutathione, 824 mg/kg total saponins, 4.21% total amino acids, 0.146% taurine.

Animals and Experimental Design

Thirty C57BL/6 female mice (16-18 g, 7 weeks old) were purchased from Shanghai Jiesjie Experimental Animals Co., Ltd. (Shanghai, China). The mice were randomly divided into 3 groups of 10 each and kept in 6 cages. Each cage contained the same group of 5 mice. The size of the cages is 290 mm x 178 mm x 160 mm, with stainless steel mesh cover and corncob padding. Meanwhile, the bottle type drinker and feed were placed on the stainless steel mesh cover. All mice were kept at a constant temperature of 23±2°C with 12 h light/dark cycles and had *ad libitum* access to feed and water. Three groups of mice were entitled according to the different oral administration of supplementation, control and diquat group, both orally administered with 0.9% saline each day, and MA+Diquat group, administered with the same dose of 0.065 mg/mL day^{-1} MA. At day 22, the Control received an intraperitoneal injection of 0.9% saline while the Diquat and MA+Diquat groups were intraperitoneally injected with the same dose of 1.8 mg/mL diquat (Innochem, Beijing, China).

Sample Collection

After 3 h injected, the mice were killed by cervical dislocation and the jejunum samples were collected for biochemical assay, histological analysis, real-time quantitative polymerase chain reaction (PCR) assay and western blot analysis.

Biochemical Assay

Tissue samples preparation: The jejunum samples of each mice were mixed with 0.9% saline at a ratio of 1:9 and then the mixtures were centrifuged at 3,000 g for 15 min at 4°C to acquire the supernatants. The concentration of protein in the supernatants was determined by Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). The indices were standardized to the protein concentration in each sample.

Antioxidant assay: The contents of hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), malondialdehyde (MDA), and total glutathione (T-GSH), the activities of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in the supernatants of jejunum were measured with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. Among them, the H_2O_2, T-GSH and CAT assay was determined by the spectrophotometric method with an absorbance at 405 nm. The $O_2^{\cdot-}$ and SOD assay was determined through the hydroxylamine method at the absorbance of 550 nm. The MDA assay was detected using the thiobarbituric acid (TBA) method and the absorbance was recorded at 532 nm. The T-AOC and GSH-Px assay were determined by the colorimetric method at the absorbance of 520 nm and 412 nm respectively.

Enzyme-linked immunosorbent assay (ELISA): ELISA was performed as described in the recent study (Qiu *et al.,* 2018; Rehman *et al.,* 2019).The concentrations of caspase-3, caspase-8 and caspase-9 in the supernatants of jejunum were measured with specific ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and determined at the absorbance of 450 nm, according to the manufacturer's instructions. The values were calculated referred to the standard curve.

Histological Analysis

Paraformaldehyde-fixed jejunum samples were embedded in paraffin and cut 5-μm thick with a microtome. After staining with hematoxylin and eosin (H&E), three discontinuous sections were selected for each sample and the villus height and crypt depth were measured within the 200X field view using a light microscope.

Real-time Quantitative PCR Assay

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, C.A., U.S.A.) according to the manufacturer's protocol and reverse transcribed into cDNA using the PrimeScript^{RT} reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) in accordance with the manufacturer's protocol. All the specific primers of genes detected by real-time quantitative PCR assay are listed in Table 1. Real-time quantitative PCR was performed using an Applied Biosystems StepOnePlus Real-Time PCR thermocycler instrument (Applied Biosystems, CA, USA) with a SYBR Premix Ex TaqTM II PCR kit (TaKaRa, Dalian, China). The following PCR conditions were applied: 95°C for 30 s and 40 cycles using a step program (95°C for 5 s and 60°C for 34 s). Then melting curve analysis was performed at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The relative mRNA expression in the jejunum was evaluated by $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Each sample was lysed in lysis buffer (Sigma-Aldrich, M.O., U.S.A.) according to the manufacturer's instructions and the concentration of protein was determined by Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Total protein (30 μg) from each sample was separated

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Table 1: Primer sequences for real-time quantitative polymerase chain reaction (PCR)

Genes	Forward (5′ to 3′)	Reverse (5′ to 3′)
SGLT-1	CAGTAACATTGGAAGTGGTCA	GGGACAGAACGGAAAGGT
GLUT-2	TTGCTGGACGAAGTGTATC	GACTAATAAGAATGCCTGTGAC
GLUT-5	GAGCAACGATGGAGGAAAAA	CCAGAGCAAGGACCAATGTC
FATP-4	GGCTTCCCTGGTGTACTATGGAT	ACGATGTTTCCTGCTGAGTGGTA
FABP-1	CCATGACTGGGGAAAAAGTC	GCCTTTGAAAGTTGTCACCAT
FABP-2	ACGGAACGGAGCTCACTG	TTACCAGAAACCTCTCGGACA
PEPT-1	CCACGGCCATTTACCATACG	TGCGATCAGAGCTCCAAGAA
NBAT	CCATGTCAACGGTGTAACCA	GCCAGCTGGAGTTTCCATAC
LAT-2	TTACTCTTATGTGAAGGACATCTTCG	CCAGCACAGCAATCCACA
ASC-1	TGGCTGGAACTTCCTCAACT	GATGGCACGAGGTAGGTTCT
LC3	CCGAGAAGACCTTCAAGCAG	ACACTTCGGAGATGGGAGTG
Beclin-1	TGATCCAGGAGCTGGAAGAT	CAAGCGACCCAGTCTGAAAT
β–actin	TGGAATCCTGTGGCATCCATGAAA	TAAAACGCAGCTCAGTAACAGTCCG

Sodium-glucose cotransporter 1, SGLT-1; Glucose transporter 2, GLUT-2; Glucose transporter 5, GLUT-5; Fatty acid transporter 4, FATP-4; Fatty acid binding protein 1, FABP-1; Fatty acid binding protein 2, FABP-2; Oligopeptide transporter 1, PEPT-1; Neutral amino acid transporter, NBAT; Cationic amino acid transporter, LAT-2; Alanine-serine-cysteine transporter 1, ASC-1; Microtubule-associated protein light chain 3, LC3

by electrophoresis (Tanon Science and Technology, Shanghai, China) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, M.A., U.S.A.). The membrane was blocked for 2 h and incubated all the night at 4°C with the primary antibodies: microtubule-associated protein light chain 3 (LC3), 1:1,000 dilution of rabbit anti-LC3 (cat. no. NB100-2220; Novus Biologicals, Centennial, CO, U.S.A.); beclin-1, 1: 10,000 dilution of rabbit anti-beclin-1 (cat. no. NB110-87318; Novus Biologicals, Centennial, CO, U.S.A.). After washing 3 times for 10 min, the blots were incubated with 1: 2,000 dilution of goat anti-rabbit IgG secondary antibodies (ab97051; Abcam, Cambridge, U.K.) for 1.5 h and developed using ECL (GE Healthcare, W.I., U.S.A.). The protein bands were quantified by using an enhanced chemiluminescence detection system (Tanon, Shanghai, China) and ImageJ software (National Institutes of Health, M.D., U.S.A.). The relative abundance of each protein was expressed as the ratio of targeted protein to β-actin.

Statistical Analysis

Statistical analysis was performed by one-way applying variance (ANOVA) followed by LSD in S.P.S.S. 22.0. All data were expressed as means with their standard errors (Mean ± SE) and the statistical significance was set at $P < 0.05$.

Results

MA Alleviated Diquat-induced Intestinal Oxidative Stress

The contents of H_2O_2 and MDA in the jejunum were increased ($P < 0.05$) after the challenge with diquat. However, supplementation of MA reduced ($P < 0.05$) the contents of H_2O_2 and MDA and elevated ($P < 0.05$) the T-GSH content and T-AOC in diquat challenged mice. There was no difference ($P > 0.05$) in the jejunal $O_2^{\cdot -}$ content among the three groups. Diquat stimulation decreased ($P < 0.05$) the activities of SOD, CAT, and GSH-Px in the jejunum, while MA treatment reversed ($P < 0.05$) their activities in diquat challenged mice (Table 2).

Effects of MA on the Intestinal Morphology of Jejunum

There was no difference ($P > 0.05$) in the jejunal villus height, crypt depth and the ratio of villus height and crypt depth among the three groups (Table 3 and Fig. 1).

Effects of MA on the mRNA Expression of Nutrient Transporters in the Jejunum

Diquat supplementation decreased ($P < 0.05$) the mRNA expression of NBAT in the jejunum. Compared with the Diquat group, pretreatment with MA elevated ($P < 0.05$) the mRNA expression of glucose transporters (SGLT-1, GLUT-2 and GLUT-5), fatty acid transporters (FATP-4, FABP-1 and FABP-2), oligopeptide transporters (PEPT-1), amino acid transporters (LAT-2 and ASC-1) (Fig. 2).

MA Down-regulate the Concentrations of Caspase in the Jejunum

Diquat supplementation increased ($P < 0.05$) the concentrations of caspase-3, caspase-8 and caspase-9 in the jejunum, whereas such increases were inhibited ($P < 0.05$) by MA supplementation in diquat challenged mice (Fig. 3).

MA Elevate the mRNA and Protein Expression of LC3 and Beclin-1 in the Jejunum

Compared with the Diquat group, complementation of MA elevated ($P < 0.05$) the mRNA and protein expression of LC3 and beclin-1 in the jejunum (Fig. 4).

Discussion

Previous studies have proved that intestinal oxidative stress was involved in the impairment of nutrient transporters

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Table 2: Effects of microbe-derived antioxidants on the antioxidant status in the jejunum of mice challenged with diquat

Item	Control	Diquat	MA + Diquat
[1]H_2O_2 (mmol/g protein)	8.59 ± 0.75^{b}	12.11 ± 1.06^{a}	8.72 ± 0.51^{b}
[2]$O_2^{\cdot-}$ (U/g protein)	23.53 ± 1.18^{a}	27.77 ± 3.26^{a}	25.84 ± 0.84^{a}
[3]MDA (nmol/mg protein)	0.63 ± 0.07^{b}	1.07 ± 0.18^{a}	0.71 ± 0.08^{b}
[4]T-AOC (U/mg protein)	0.99 ± 0.04^{ab}	0.80 ± 0.05^{b}	1.13 ± 0.11^{a}
[5]SOD (U/mg protein)	92.05 ± 4.63^{a}	73.05 ± 2.07^{b}	101.11 ± 5.33^{a}
[6]CAT (U/mg protein)	34.76 ± 2.12^{a}	27.10 ± 3.18^{b}	37.21 ± 1.18^{a}
[7]GSH-Px (U/mg protein)	462.77 ± 23.68^{a}	319.21 ± 12.22^{b}	487.19 ± 20.89^{a}
[8]T-GSH (μmol/l)	334.58 ± 25.78^{a}	203.75 ± 16.93^{b}	266.25 ± 32.66^{a}

Mean ± standard errors. Values sharing same letters differ non-significantly ($P > 0.05$)
[1]H_2O_2, hydrogen peroxide; [2]$O_2^{\cdot-}$, superoxide anion; [3]MDA, malondialdehyde
[4]T-AOC, total antioxidant capacity; [5]SOD, superoxide dismutase; [6]CAT, catalase
[7]GSH-Px, glutathione peroxidase; [8]T-GSH, total glutathione

Table 3: Effects of microbe-derived antioxidants on the intestinal morphology of jejunum of mice challenged with diquat

Item	Control	Diquat	MA + Diquat
Villus height (μm)	224.17 ± 7.15^{a}	199.59 ± 8.96^{a}	209.17 ± 14.44^{a}
Crypt depth (μm)	72.92 ± 4.43^{a}	85 ± 5.00^{a}	82.08 ± 5.38^{a}
Villus height/Crypt depth	3.19 ± 0.28^{a}	2.39 ± 0.13^{a}	2.79 ± 0.53^{a}

Mean ± standard errors. Values sharing same letters differ non-significantly ($P > 0.05$)

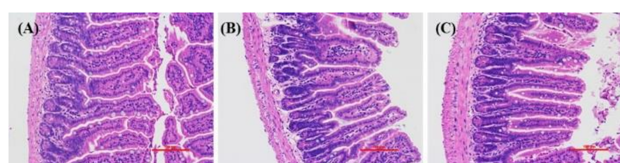

Fig. 1: Photomicrographs showing intestinal morphology of jejunum of control mice (**A**), mice treated with diquat (**B**) and mice treated with microbe-derived antioxidants and diquat (**C**) (H&E staining; 200X)

(Andrade *et al.*, 2018a). However, reports on the mechanisms of ROS on nutrient transporters are rare. Diquat has been widely used to induce oxidative stress (Cao *et al.*, 2018; Mo *et al.*, 2018; Xu *et al.*, 2018) while the positive effect of MA partly related to its antioxidant activity. Therefore, we established an oxidative stress mice model by diquat injection to evaluate the effects and underlying mechanisms of MA on nutrient transporters.

The generation of ROS, such as O_2^- and H_2O_2, are considered as the key oxidation mechanism induced by diquat (Zhang *et al.*, 2016) and the peroxidation damage to lipid can be reflected by MDA (Castillo *et al.*, 2006). Furthermore, T-AOC indicates the total antioxidant capacity of various antioxidant enzymes (*e.g.*, SOD, CAT, and GSH-Px) and non-enzyme substances (*e.g.*, T-GSH) (Castillo *et al.*, 2006). Our results showed that exposure to diquat increased the contents of H_2O_2 and MDA, and decreased T-AOC, the activities of SOD, CAT, and GSH-Px and the content of T-GSH in the jejunum, which indicated intestinal oxidative stress in mice. Here, MA treatment not only reduced the ROS contents and lipid peroxidation but also elevated the antioxidant capacity in the jejunum.

Glucose, fatty acid, amino acid and oligopeptide transporters, which are along the brush border and basement

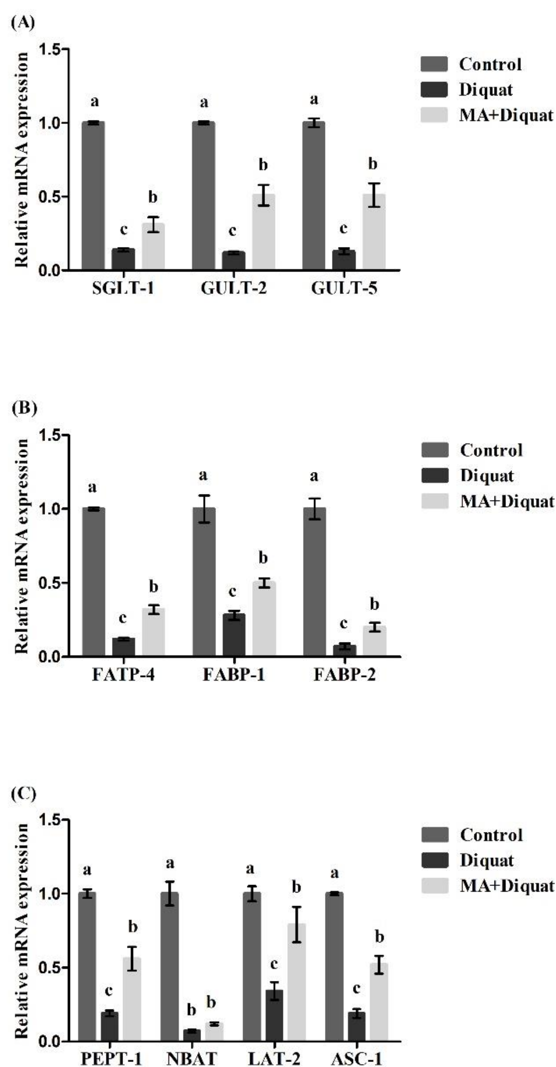

Fig. 2: Relative mRNA expression of glucose transporters (**A**), fatty acid transporters (**B**) and oligopeptide and amino acid transporters (**C**) in the jejunum of mice challenged with diquat and mice treated with microbe-derived antioxidants and diquat in comparison to control mice. Error bars represent standard errors. Different letters represent significant differences ($P < 0.05$)

membrane of the small intestinal epithelium, play a vital role in the transportation and absorption of nutrients (He *et al.*, 2017). Villus height and crypt depth indicate the rates of growth and development of enterocytes, which can affect nutrient transport (He *et al.*, 2017). The present results showed a small, yet not statistically significant increase in the ratio of villus height and crypt depth of jejunum of mice fed MA, supporting the notion that pretreatment with MA could improve intestinal morphology of mice. Many studies have shown that overproduction of ROS could induce oxidative stress, impair membrane transporters and alter cell signaling (Andrade *et al.*, 2018a). In present study, we found that diquat-induced oxidative stress down-regulated the mRNA expression of glucose transporters

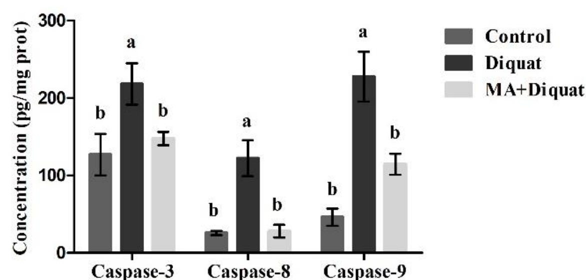

Fig. 3: Concentrations of caspase-3, caspase-8 and caspase-9 in the jejunum of mice challenged with diquat and mice treated with microbe-derived antioxidants (MA) and diquat in comparison to control mice. Error bars represent standard errors. Different letters represent significant differences ($P < 0.05$)

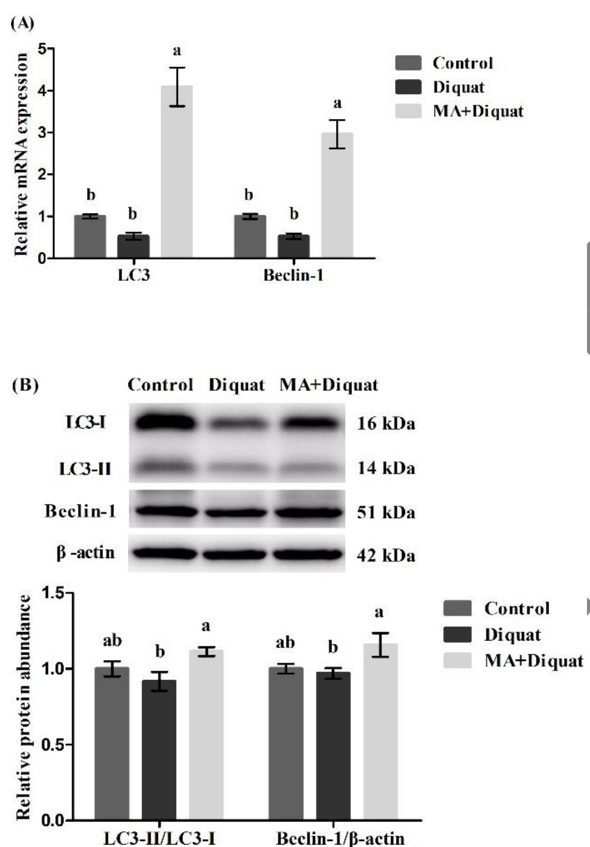

Fig. 4: Relative mRNA (**A**) and protein (**B**) expression of microtuble-associated protein light chain 3 and beclin-1 in the jejunum of mice challenged with diquat and mice treated with microbe-derived antioxidants and diquat in comparison to control mice. Error bars represent standard errors. Different letters represent significant differences ($P < 0.05$)

(SGLT-1, GLUT-2 and GLUT-5), fatty acid transporters (FATP-1, FABP-1 and FABP-2), amino acid transporters (LAT-2 and ASC-1) and oligopeptide transporter (PEPT-1) in the jejunum, while, MA administration enhanced the mRNA expression of these nutrient transporters in diquat challenged mice. In agreement, increased oxidative stress has been previously found to have a negative impact in nutrient transporters (Rashid and Sil, 2015). Taken together, these results indicated that MA, exerting antioxidant effects, could promote the expression of nutrient transporters in mice challenged with diquat.

Autophagy and apoptosis are the two major stress-response cellular pathways, which regulate cell survival and cell death (Martinez-Outschoorn *et al.,* 2014). It has been widely studied that ROS-induced apoptosis would impair intestinal function (Zhu *et al.*, 2013). Apoptosis can be induced through the activation of caspase (*e.g*., caspase-3, caspase-8, and caspase-9) (Badr *et al.*, 2014). In the present study, the diquat treatment increased the concentrations of caspase-3, caspase-8, and caspase-9, which confirmed an increased ROS-mediated apoptosis in enterocytes. In addition, we found that the increased phenomenon was reversed by MA supplementation. And as reported, basal levels of autophagy can protect the cells from oxidative stress (Han *et al.*, 2014). LC3-II is the membrane marker for autophagic vacuoles and beclin-1 contributes to the formation of autophagosomes (Luo *et al.*, 2019). In this study, we demonstrated that pretreatment with MA enhanced LC3-II and activated beclin-1 to promote autophagy. For the first time, we found that MA could eliminate excessive ROS, further trigger a beneficial autophagic process and inhibit apoptotic cell death, which was beneficial to promoting the expression of nutrient transporters. In support of our results, previous reports demonstrated that N-acetyl cysteine, exerting antioxidant effects, could maintain intestinal structure and function through inhibiting caspase-dependent apoptotic pathway (Zhu *et al.*, 2013). Similarly, the structure and function of intestine was reported to be maintained by the enhanced autophagy and transport of glutamine (Hu *et al.*, 2018).

Conclusion

Microbe-derived antioxidants exerted antioxidant effects in promoting the expression of nutrient transporters of mice challenged with diquat. Their protective effects were closely related to the enhanced autophagy and the inhibited apoptosis.

Acknowledgments

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