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Effect of *Enterococcus faecium* 1 (EF1) on Antioxidant Functioning Activity of Caco-2 Cells under Oxidative Stress

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Abstract: The free radical scavenging systems remove most peroxide which shows antioxidantion capacity of body and lactic acid producing bacteria have capacity to support the body in the mechanism. The present study was initiated to investigate the antioxidantion functioning property of *Enterococcus faecium* 1 (EF1) to Caco-2 cells under oxidative stress condition. The cells were cultured and randomly divided into 4 groups, the control group (T_0), the oxidative stress group (T_1), Tert-Butyl Hydroquinone (TBHQ) with addition of H_2O_2 (T_2) and EF1 with combination of H_2O_2 (T_3). The results showed that Total Antioxidation Capacity (T-AOC), Catalase (CAT), Superoxide Dismutase (SOD) activities, Glutathione (GSH) contents in the cultured supernatant and SOD activity of the cells lysate at 12 h increased ($p<0.05$) in T_3 as compared to T_1. The supernatant of cells cultured at 12 h significantly improved the SOD, GSH-Px activities and GSH contents in T_3. While, Anti Superoxide Anion Free Radical (ASAFR), CAT, SOD and Glutathione Peroxidase (GSH-Px) activities ($p<0.05$) increased in supernatant at 48 h, conversely Malondialdehyde (MDA) contents was significantly reduced ($p<0.05$). The SOD activity and GSH contents of cells lysates at 48 h showed similarly reduced ($p<0.05$). The comparative findings of T_3 to T_2 supernatant and lysate of cells at 48 h showed significant increase in T-AOC, CAT, SOD, GSH-Px activities and GSH contents of supernatant and in lysate POD activity and GSH contents significantly increased. While, decline ($p<0.05$) was observed in the MDA contents in supernatant and lysates of T_3. The findings revealed that *Enterococcus faecium* 1 could increase the antioxidation functioning activity of Caco-2 cells under oxidative stress condition.

Key words: *Enterococcus faecium*, antioxidation, TBHQ, stress, Caco-2 cells, China

INTRODUCTION

Since the free radical theory of aging was conceived by Harman (1988), it has been known that oxidative free radicals can lead to aging, cancer, atherosclerosis increase intestinal permeability and easily induce gastrointestinal diseases, also closely relate to the growth performance, carcass characteristics and disease resistance in farm animals. Conversely, in order to cope with the excess of free radicals produced by oxidative stress and neutralize the radical production by the body is a sophisticated mechanism that is most important to understand. The sustaining mechanisms of protection is secured via detoxifying Reactive Oxygen Species (ROS), blocking of their fabrications and apposite conversion of the metals. Whilst, some of enzymes also contribute in antioxidant barricade activities, generated by the body for homeostasis (Masella *et al*., 2005). Among the varity of synthetic phenolic antioxidant food additives, Tert-Butyl Hydroquinone (TBHQ) is extensively used as an antioxidant (Ryu, 2010). Some previous published literatures reported that *Enterococcus faecium* 1 (EF1) as one of natural probiotics could improve animal health and growth performance, modify the immune response and the intestinal microbial flora and reduce post-weaning diarrhea (Huang *et al*., 2012; Pollmann *et al*., 2005; Schareka *et al*., 2005; Taras *et al*., 2006; Strompfova and Laukova, 2009). However, few studies described that probiotic *Enterococcus faecium* has ability of oxidation resistance (Moon *et al*., 2007; Wen *et al*., 2011). Therefore, the present study was conducted to evaluate the effects of *Enterococcus faecium* 1 on antioxidative function of Caco-2 cells under oxidative stress compared with TBHQ.

MATERIALS AND METHODS

Bacteria: The EF1 used during experiment was isolated and identified by Institute of Feed Science, Zhejiang University. The bacterial strain was cultured in de

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Man-Rogosa-Sharpe (MRS) broth (Oxoid; England) in anaerobic condition at 37°C till log phase. Centrifugation at 2000×g for 15 min applied to separate the bacterial strain. Furthermore, bacteria were washed twice with Phosphate-Buffered Saline (PBS) pH 7.4 and re-suspended in PBS to prepare required concentration (1×10^8 CFU mL^{-1}).

Caco-2 cells culturing: The human colon carcinoma cell line Caco-2 cells were obtained from Institute of Biological Sciences, Cell Resource Center (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Co. USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sijiqing Biological Engineering Materials, China) and antibiotics were added (penicillin 100 U mL^{-1} and streptomycin 100 μg mL^{-1}; Sigma Co., USA). The cells were seeded at a concentration of 5×10^5 CFU mL^{-1} in collagen-treated 6-well plates (Cambridge, MA) at 37°C with 5% CO_2 and 95% air constant humidity in an incubator (Thermo Electron Corporation, USA). While, fresh prepared media was changed at alternative days.

Experimental design: Caco-2 cells were cultured and randomly divided into 4 groups: The control group (T_0); Oxidative stress group, (T_1) negative control, treated with 100 μM hydrogen peroxide (T_2) TBHQ group (2.75 μg mL^{-1}), positive control in addition of 100 μM H_2O_2 and EF1 (1×10^8 CFU mL^{-1}) with 100 μM H_2O_2 as a (T_3) treatment group. Whilst, each group was consist of six replications, respectively. The culturing duration was designed on the basis of pre-experiment results. The cells were cultured for 12 and 48 h and further divided into two parts, cell supernatant and cell lysate in all the groups, respectively. After completion of culturing duration seeding was removed with gentle pippeting and each well was washed with PBS thrice. The cells were collected and centrifuged at 2000×g for 15 min. The supernatant and suspension (lysate) collected separately in Ependrof tubes for further analysis.

Antioxidtive indicators measurement: The Total Antioxidation Capacity (T-AOC), Anti Superoxide Anion Free Radical (ASAFR), Catalase (CAT), Malondialdehyde (MDA), Superoxide Dismutase (SOD), Peroxidase (POD), Glutathione Peroxidase (GSH-Px) activities and Glutathione (GSH) contents were determined in culture supernatants of Caco-2 cells at 12 and 48 h. While, Superoxide Dismutase (SOD), Peroxidase (POD) activity and Glutathione (GSH) contents in lysate of Caco-2 cells were analyzed at 12 and 48 h. All indicators were detected using kits provided by Jiancheng Bioengineering Institute (Nanjing, China).

Statistical analysis: The data was analyzed using the one-way analysis of variance procedure of SPSS 16.0 Inc., Chicago, USA. Differences between treatments were evaluated with unpaired t-test. Data were expressed as mean±SD, a significant value ($p<0.05$) considered statistically in all the results.

RESULTS AND DISCUSSION

Antioxidant activity in cells supernatant at 12 h: The results showed that (Fig. 1) no significantly difference was observed in the T-AOC, ASAFR, CAT activities and MDA contents in between T_1 and T_0; adding of TBHQ significantly increased the T-AOC and CAT activities (370.89 and 578.81%), respectively and reduced ASAFR activity (37.88%). The MDA contents improvement observed but no significant difference in comparison with T_1. Addition of EF1 (T_3) significantly increased the T-AOC and CAT activities (176.71, 67.58%) and reduced the MDA contents (49.09%) in comparison with T_1. However, T_3 showed significant reduction in T-AOC, ASAFR, CAT activities and MDA contents (41.24, 21.11, 75.31 and 52.94%) as compared to T_2.

The results manifested that T_1 increased the GSH-Px activity and decreased POD activity (93.46 and 49.75%) significantly as compared to T_0 but no difference was found in SOD activity and GSH contents (Fig. 2). The significant reduction was found in GSH-Px activity (38.51%) of T_2 to T_1. The T_3 increased ($p<0.05$) the SOD activity and GSH contents (27.31 and 30.47%, respectively) and decreased the POD activity significantly (33.49%) in comparison of T_1. Whilst, T_3 increased

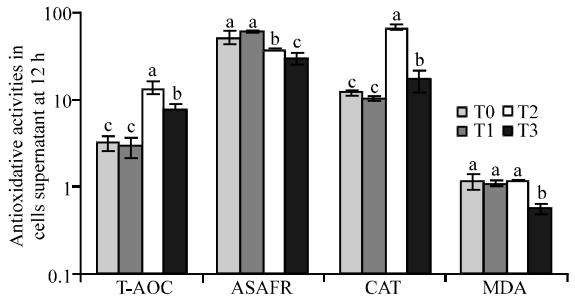

Fig. 1: Total Antioxidation Capacity (T-AOC), (U mL^{-1}), Anti Superoxide Anion Free Radical (ASAFR) (U L^{-1}), Catalases (CAT) (U mL^{-1}), Malondialdehyde (MDA) (nmol mL^{-1}) were determined at 12 h. The data was expressed as l g values are means±SD of results obtained with six independent cultures. The superscript letters in the columns indicate values which differ significantly among the groups ($p<0.05$)

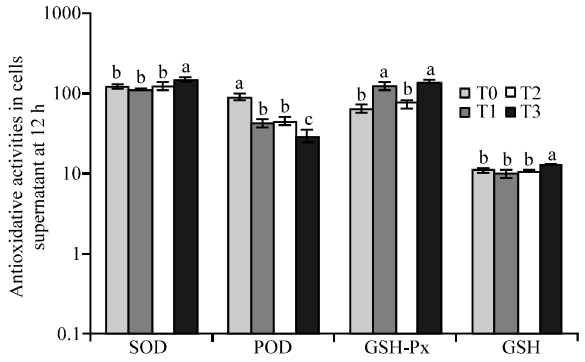

Fig. 2: Expressed Superoxide Dismutase (SOD) (U mL^{-1}), Peroxidase (POD) (U mL^{-1}), Glutathione Peroxidase (GSH-Px) (U mL^{-1}), activities and Glutathione (GSH) contents (mg L^{-1}) were determined at 12 h. The data was expressed as values are means±SD of results obtained with six independent cultures. The superscript letters in the columns indicate values which differ significantly among the groups ($p<0.05$)

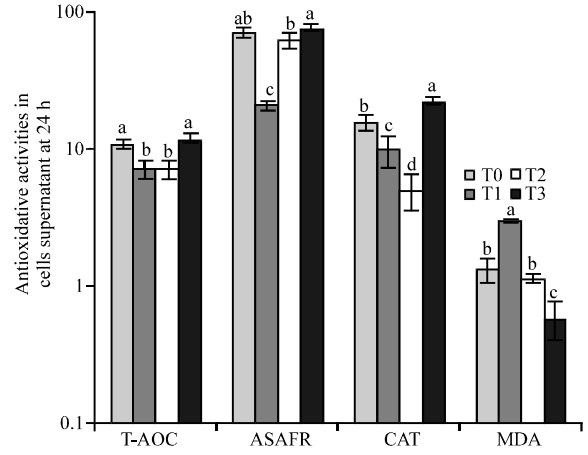

Fig. 3: Total Antioxidation Capacity (T-AOC), (U mL^{-1}), Anti Superoxide Anion Free Radical (ASAFR) (U L^{-1}), Catalase (CAT) (U mL^{-1}), Malondialdehyde (MDA) (nmol mL^{-1}) were determined at 48 h. The data was expressed as means±SD of results obtained with six independent cultures. The superscript letters in the columns indicate values which differ significantly among the groups ($p<0.05$)

($p<0.05$) the SOD, GSH-Px activities and GSH contents (17.97, 84.11 and 25.10%), respectively and reduced in (T_2) POD activity (34.99%) significantly as compared to T_2.

Antioxidant activity in cells supernatant at 48 h: As shown in Fig. 3, MDA was increased ($p<0.05$) in T_1

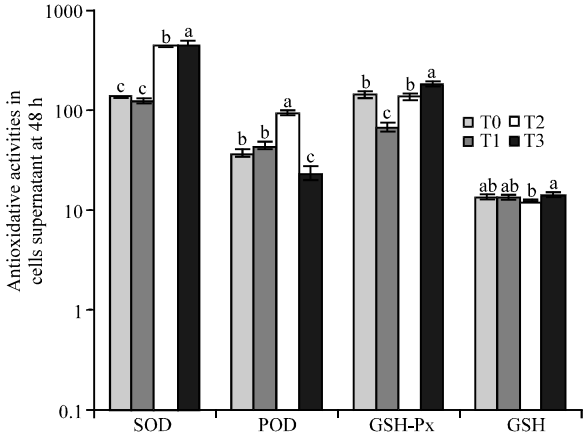

Fig. 4: Superoxide Dismutase (SOD) (U mL^{-1}), Peroxidase POD (U mL^{-1}), Glutathione Peroxidase (GSH-Px) (U mL^{-1}), activities and Glutathione (GSH) contents (mg L^{-1}) were determined at 48 h. The data was expressed as means±SD of results obtained with six independent cultures. The superscript letters in the columns indicate values which differ significantly among the groups ($p<0.05$)

(127.07%) and decreased ($p<0.05$) the T-AOC, ASAFR and CAT activities (34.82, 70.02, 36.92%) significantly as compared to T_0. Significantly increased was found in ASAFR activity and reduction in CAT activity and MDA contents (47.91 and 61.92%) in T_2. And decrease was appeared in T-AOC activity but no significant difference to T_1.

Moreover, T_3 significantly decreased (80.80%), MDA contents and increased ($p<0.05$) T-AOC, ASAFR and CAT activities (67.78, 269.20, 131.97%), respectively as compared to T_1. The significant increase revealed in T-AOC, ASAFR and CAT activities (70.16, 23.03, 336.08%), respectively and (49.57%) decrease ($p<0.05$) observed in MDA contents in comparison of T_2.

Figure 4 showed that addition of H_2O_2 declined ($p<0.05$) the GSH-Px activity (52.44%) than T_0 and no significant difference was observed between GSH contents of T_2 and T_1. The significant increase appeared in SOD, POD and GSH-Px activities (258.96, 109.51 and 103.30%, respectively) as compared to T_1. The fourth group showed highly significant increase in SOD, GSH-Px activities (287.21 and 169.55%) and decrease in POD activity (46.41%) than T_1 group. The T_3 was higher ($p<0.05$) to T_2 in SOD and GSH-Px activities (7.87 and 15.63%) and GSH contents (32.59%). The significantly decrease observed in POD activity by 74.42%.

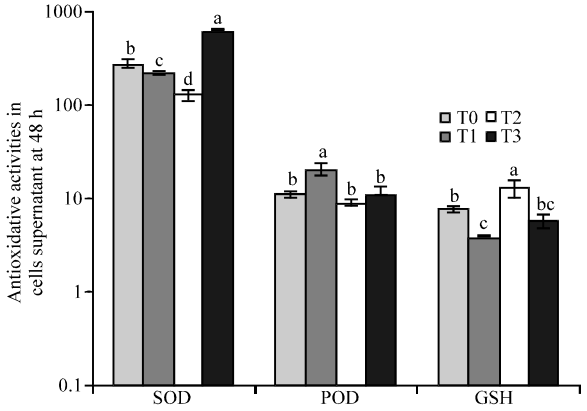

Fig. 5: Superoxide Dismutase (SOD) (U mL^{-1}), Peroxidase POD (U mL^{-1}) and Glutathione (GSH) contents (mg L^{-1}) were determined after treatment at 12 h. The data was expressed as means±SD of results obtained with six independent cultures. The superscript letters in the columns indicate values which differ significantly among the groups ($p<0.05$)

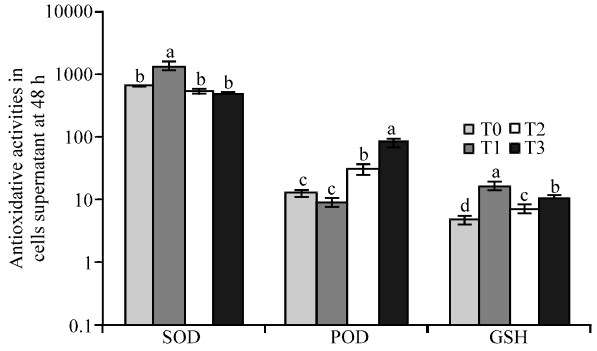

Fig. 6: Superoxide Dismutase (SOD) (U mL^{-1}), Peroxidase POD (U mL^{-1}) and Glutathione (GSH) contents (mg L^{-1}) were determined after treatment at 48 h. The data was expressed as means±SD of results obtained with six independent cultures. The superscript letters in the columns indicate values which differ significantly among the groups ($p<0.05$)

Antioxidant activity in cells lysate at 12 h: The results of (Fig. 5) lysate at 12 h showed that T_1 increased (79.96%) POD activity and reduction observed (20.95, 48.46%) in SOD activity and GSH contents ($p<0.05$) to T_0. The TBHQ outcomes in comparison with T_1 showed (22.64%) increase in GSH contents and decrease in SOD and POD activities (41.65 and 56.30%) ($p<0.05$), respectively. The T_3 increased the SOD activity (76.45%) and decreased the POD activity (44.39%), significantly as compared to T_1. Whereas, GSH contents decreased (56.36%) and SOD activity increased (373.81%) significantly in comparison with T_2.

Antioxidant activity in cells lysate at 48 h: As observed from Fig. 6, the SOD activity and GSH contents of T_1 increased ($p<0.05$) (112.70 and 245.13%, respectively). Whilst, the POD activity decreased ($p>0.05$) in comparison with T_0. The T_2 showed (62.46%) and (57.23%) decrease in SOD activity and GSH contents significantly, whereas POD activity significantly increased (241.91%) rather than T_1. The POD activity increased (78.16%) while, SOD activity and GSH contents decreased significantly (63.43 and 36.05%), respectively in T_3 to T_1. The POD activity and GSH contents significantly increased (186.09%) and (49.51%) as compared to T_2.

The free radical scavenging systems in body includes SOD, GSH-Px, CAT and other non-enzymatic antioxidants like vit. C and E. These could capture and remove most peroxide of the body (Ko *et al*., 2004), therefore the T-AOC including all enzymes and non-enzymatic antioxidants are an indicator reflecting the antioxidative capacity. However, MDA is the main product of lipid peroxides could damage proteins, DNA and other biological macromolecules, resulting in the aging and a variety of diseases (Draper and Hadley, 1990; Bilici *et al*., 2001). Specific antioxidant enzymes have been designed by nature to destroy superoxide and hydroperoxides. They play an important role in the regulation of metabolic pathways and specific functions of aerobic cells. The enzymatic degradation of superoxide is ensured by Superoxide Dismutases (SOD) while hydroperoxides is ensured by catalase, Glutathione Peroxidases (GPx) or ascorbate peroxidases (Chaudiere and Ferrari-Lliou, 1999). The important components of the cellular defense system reduce Glutathione (GSH) and antioxidative enzymes like Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px) and Catalase (CAT) (Frei *et al*., 1988; Halliwell *et al*., 1992; Vervaart and Knight, 1996; Miller and Britigan, 1999). Reports indicated that Some lactobacilli, particularly on the Cytoplasmic Fraction (CF) of them (Saide and Gilliland, 2005) had antioxidative activity, protected themselves by decreasing the risk of accumulation of ROS (Kaizu *et al*., 1993; Peuhkuri *et al*., 1996) and degrade the superoxide anion and hydrogen peroxide (Ahotupa *et al*., 1996; Korpela *et al*., 1997).

H_2O_2 is an intermediate product of oxidative metabolism which has a strong oxidation activity leading to a series of physiological function and generates hydroxy radicals in the presence of transition metal ions and formats of lipid peroxides. Thus, H_2O_2 has been

considered as an appropriate model of oxidative stress stimulator *in vitro* (Lee *et al*., 2002; Farombi *et al*., 2004). The current findings showed that the activities of the T-AOC, ASAFR, CAT, SOD and MDA contents were not significantly different from T_0 and T_1; whether at 48 h, the T-AOC and some major antioxidative enzymes decreased. However, significant increase of MDA contents observed in the state of oxidative stress, meanwhile, cells increased antioxidative function and reduced oxidative stress on cell injury through regulating the activity of intracellular antioxidative enzymes and the contents of antioxidant substances just like the increasing activity of the SOD and the contents of GSH of the lysate at 48 h.

The oxidative stress occurs when abnormally high levels of Reactive Oxygen Species (ROS) are generated, resulting in DNA, protein and lipid damage (Seifried *et al*., 2007; Kullisaar *et al*., 2002). Researchers found that EF1 can improve the antioxidant function of cells better than the oxidative stress group in mild or severe. It could enhance the total antioxidant activity by some major antioxidant functions of the supernatant, increasing the SOD, CAT activities and GSH contents significantly in cells lysate at 12 h. Whilst it also increased the T-AOC, ASAFR, CAT, SOD, GSH-Px activities and reduced the MDA contents significantly, though the SOD activity and GSH contents of cells lysate were reduced at 48 h. The findings manifested that with the time disparity, EF1 plays a prominent role by their components in antioxidantion to maintain the homeostasis of cell. The similarity of outcomes, reported by Moon *et al*. (2007) and Wen *et al*. (2011) who evaluated that probiotic *Enterococcus faecium* also has oxidation resistance, scavenging hydroxyl radical and increased antioxidant capacity (Capcarova *et al*., 2010).

TBHQ is a chemically synthetic food grade antioxidant which reduces the oxidative damage by directly rapid response against H_2O_2 by their consumption. But duration showed antioxidant activity which declined in the antioxidant systems of the cells. This study showed that EF1 as an antioxidant are more moderate and long-lasting than TBHQ, it is probable that EF1 is a living organism which might secrete by itself or promote other organisms to synthetize some antioxidase (Wen *et al*., 2011). While, TBHQ is a synthetic antioxidant with anti-oxidation consumption causing the corresponding decline in antioxidant capacity after a time, depends on concentration. To understand the mechanisms, more future researches need to be studied.

CONCLUSION

The findings provided direct evidence that *Enterococcus faecium* 1 can reduce the oxidative damage and have antioxidant effects on Caco-2 cells under H_2O_2-induced oxidative stress. It is feasible that *Enterococcus faecium* will be developed to be used in stabilizing food or feed products against oxidative deterioration, for further investigations, in order to comprehend the mechanisms of antioxidative effects, studies are persisting based on different fractions of *Enterococcus faecium* 1 to explore the molecular prospects of antioxidative activities.

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