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Investigating the effects of oryzatensin on gut microbiota growth using a novel quantitative standard curve-fitting method: an ex-vivo study

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ABSTRACT

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Objective: Oryzatensin (ORZ) is a refractory, bioactive peptide that is isolated from rice. In the current study, we investigated the effect of ORZ on the growth of gut microbiota.

Methods: Ten apparently healthy subjects were enrolled in the current ex vivo study. Casual stool specimens were obtained from the subjects, then Enterobacteriaceae, Bacilaceae, and probiotic bacterial families were isolated using MAC, MYP, and MRS agars, respectively. The effect of exposure to different concentrations of ORZ (10-4, 10-5, 10-6, and 10-7 mol/L) on the growth of these bacteria was evaluated using the standard curve-fitting method, instead of standard qualitative methods such as MBC and MIC, because of the absence of detectable optical differences among the wells. The bacterial quantity was determined by evaluation of the suspensions turbidity at 492 to 630 nm.

Results: ORZ had an antibacterial effect and negatively controlled the growth of all isolated species compared with the control group. The inhibitory effect of ORZ was most significant at the dose of 10-7 mol/L (not significant at 10-5 and 10-6 mol/L and significant at 10-4 mol/L) for bifidobacteria and 10-4 mol/L for Enterobacteriaceae, Bacilaceae, and lactic acid bacteria.

Conclusion: The findings of the current study reveals that ORZ and possibly rice can reduce intestinal total bacterial count. Moreover, considering the carcinogenic effects of final metabolites of the enteral bacteria, such as free radicals, ORZ and rice may have an antineoplastic effect.

key words:
Oryzatensin,
gut microbiota,
rice,
standard curve-fitting,
bioactive peptides,
neoplasm

Introduction

Refractory peptides with biologic activities (bioactive peptides [BAPs]) escape from digestion and remain intact in gastrointestinal tract (GIT) [1, 2]. BAPs can play a potential role in health and are classified as antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulatory, mineral-binding, and antioxidant agents based on their functional properties [3]. The structural characteristics of BAPs, including the number of amino acids, may affect their intestinal absorption [4, 5]. Undigested BAPs have been reported by a number of studies to affect gut microbiota after entering the colon [5-8].

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Oryzatensin (ORZ) is a BAP isolated from rice (amino acid sequence: Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg [MW = 1093.3]). As a BAP, it can potentially escape the intestinal digestion and absorption, reach the colon, and consequently affect the existing gut microbiota. This may be one of the possible mechanisms explaining the association between rice consumption and several gastrointestinal diseases (GIDs) [9]. Moreover, because of the role ORZ plays in appetite regulation, there have been growing effort to introduce new ORZ-based anorectic drugs [10, 11]. It could be suggested that the pharmaceutical administration of such ORZ-based drugs would result in a higher concentration of ORZ in the colon, which may affect the quantity and quality of microbiota.

A great number of publications have reported the association between specific foods and GID. For instance, Asakura et al. reviewed several case-control and epidemiological studies conducted in Japan and observed an inverse association between rice consumption and the prevalence of inflammatory bowel disease (IBD). Several mechanisms were considered to contribute to this relationship, including the presence of a wide spectrum of bioactive compounds in rice that could interact with both gastrointestinal mucosa and microbiota [12].

The human microbiota consists of about 100 trillion symbiotic microbes harbored by each person. The microbiota potentially has a pivotal role in health and disease. The effect of bacterial overgrowth on the pathogenesis of GIDs such as small intestinal bacterial overgrowth (SIBO), IBD, and cancer has been investigated in several studies, and both microbial quantity and diversity have been shown to contribute to GID. Luminal microflora release many harmful molecules (e.g., H_2O_2, diacetyl, organic acids, sterols and gases such as CO_2, H_2, CH_4, and H_2S) into the intestinal environment as metabolic by-products. In addition to remarkable beneficial effects, probiotic bacteria also produce harmful molecules; therefore, excessive numbers of probiotic bacteria could have unwanted effects on the body [13-16].

The current study was designed to investigate the effect of ORZ on the gut microbiota by assessing its effects on the growth of lactic acid bacteria (LAB), *bifidobacteria* (BB), *Bacillus cereus* (BC) and *Enterobacteriaceae* (EB), isolated from apparently healthy male volunteers.

Methods

Study protocol

The current study was conducted in September 2016. Figure 1 illustrates the study protocol. EB, BC, and probiotic bacteria including LAB and BB were isolated from stool specimens obtained from 10 healthy male volunteers None of them had any obvious symptoms of gastrointestinal or other diseases. One gram of fresh stool specimen from each participant was diluted with 9 mL of sterilized 0.1% peptone water, and serial dilutions (1:10) up to 1 × 10-15 CFU/mL were prepared. The diluted specimens were then transferred to MacConkey agar (MAC) (Merk, Germany), mannitol-egg yolk polymyxin (MYP) agar (Merk, Germany), and de Man, Rogosa, and Sharpe (MRS) agar (Merk, Germany) to isolate EB, BC, and probiotic bacteria, respectively. Considering the sensitivity of BB to oxygen, anaerobic jars were used for their growth during all study procedures. The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee, and written informed consent was obtained from each subject.

For bacterial isolation, 100 μL of each suspension was spread over the plates and incubated for 24 hours. Specific bacterial suspensions (Suspension I in Figure 1) were prepared by inoculating the

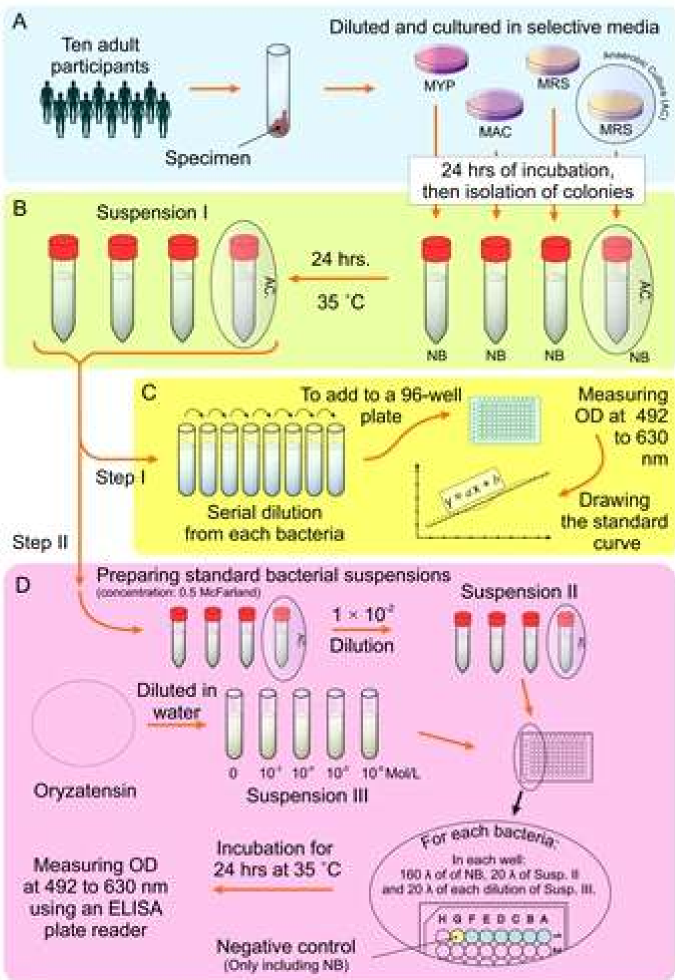

Figure 1. Illustration of the study design.

isolated bacterial colonies into Nutrient Broth (NB) (Merk, Germany) followed by incubation for 24 hours at 35˚C. Total bacterial counts of the suspensions were determined, and serial dilutions (1:10) were prepared from each suspension. Then, 200 µl of each concentration was added to a 96-well plate (Sigma-Aldrich, USA), the absorbance was measured at 492 to 630 nm using an ELISA plate reader (Bio-Rad, USA), and a standard curve was generated for each bacterium.

In the next step, the effect of ORZ on the growth of EB, BC, LAB, and BB was investigated. For this purpose, two suspensions were prepared. First, a standard bacterial suspension was prepared spectrophotometrically (at 600 nm) in NB to reach a concentration of half McFarland. Then, each of the suspensions was diluted to 1.5×10^{6} CFU/mL (Suspension II). Second, different concentrations of ORZ (99% purity, Biomatic, USA) (0, 10^{-3}, 10^{-4}, 10-^{5}, and 10-6 mol/L) were prepared with sterilized water and passed through a 0.22-µm membrane for sterilization (Suspension III). Finally, a mixture of three components was prepared in each well: 20 µl of Suspension II (bacterial suspension), 20 µl of Suspension III (different ORZ concentrations), and 160 µl of NB, to reach a total volume of 200 µl. The final concentration of ORZ in each well was 10 times diluted (i.e. 0 (positive control), 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} mol/L). The plates were then incubated for 24 hours at 35˚C, and the absorbance was measured at 492 to 630 nm using an ELISA plate reader. The standard curve-fitting method was used to estimate the number of bacteria after the peptides exposure. All experiments were performed in triplicate, along with appropriate negative and positive controls for each peptide dilution.

The standard curves were created using Microsoft Office Excel 2010, and the equations corresponding to each curve were used to estimate bacterial count in each well. The data analysis was performed with SPSS software v. 19.0 (SPSS Inc., Chicago, Illinois, USA). Repeated-measures analysis of variance was done for intra-group statistical analysis. P values less than 0.05 were considered statistically significant. Charts were drawn using GraphPad Prism v. 6.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Standard curves and corresponding equations are illustrated in Figure 2. ORZ treatment at various concentrations reduced the growth of BC (Figure 3), BB (Figure 4), EB (Figure 5), and LAB (Figure 6) in a dose-dependent manner; however, the inhibitory effect was statistically significant only at the concentration of 10^{-4} mol/L. Between-groups statistical analyses are shown in Figure 7.

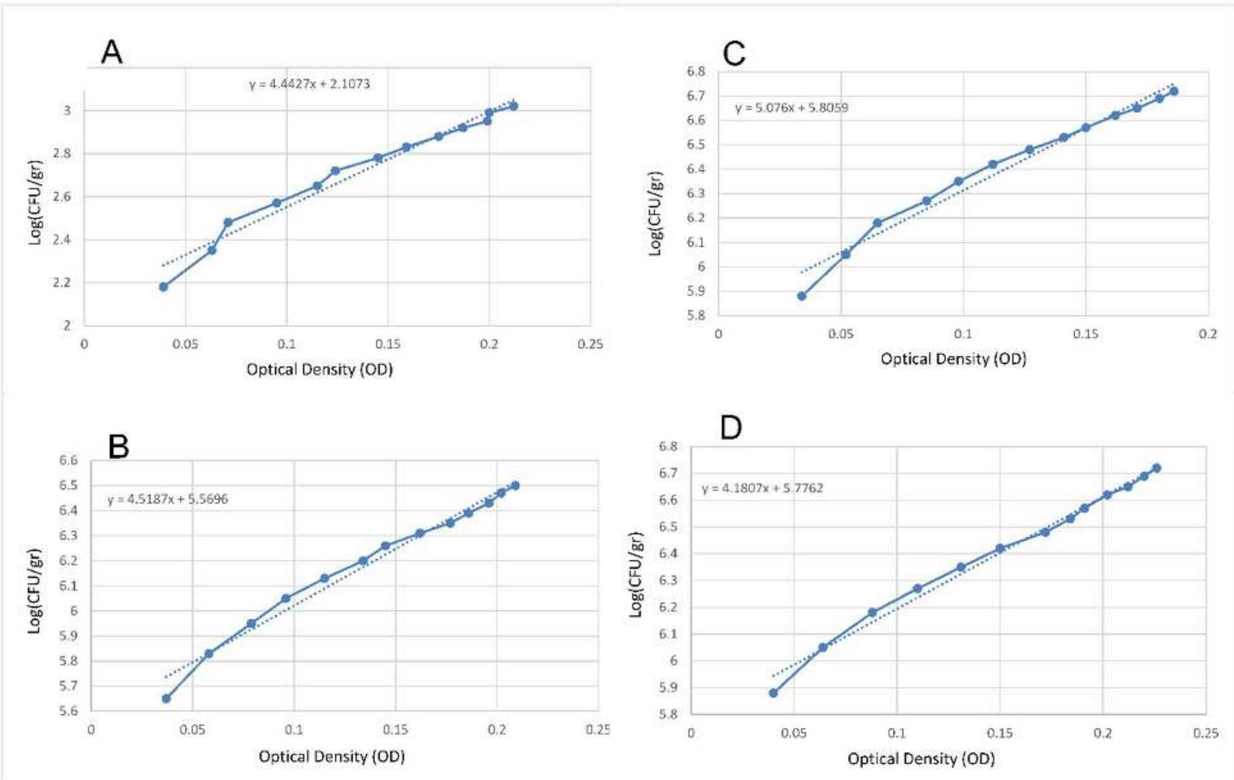

Figure 2. The standard curves for estimation of total bacterial count. (A) Bacillus cereus, (B) Lactic acid bacteria, (C) Enterobacteriaceae, (D) Bifidobacteria.

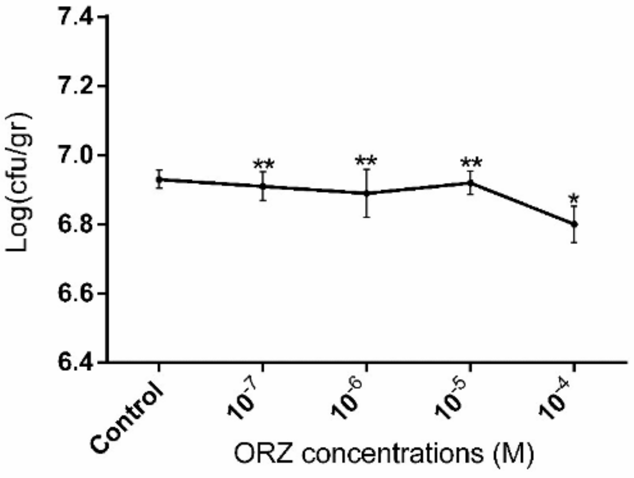

Figure 3. The effect of oryzatensin (ORZ) on Bacillus cereus growth. Statistical analysis was done by the repeated-measures test. Each point represents th mean ± SD. ** P > 0.05 vs control and * P < 0.05 vs control.

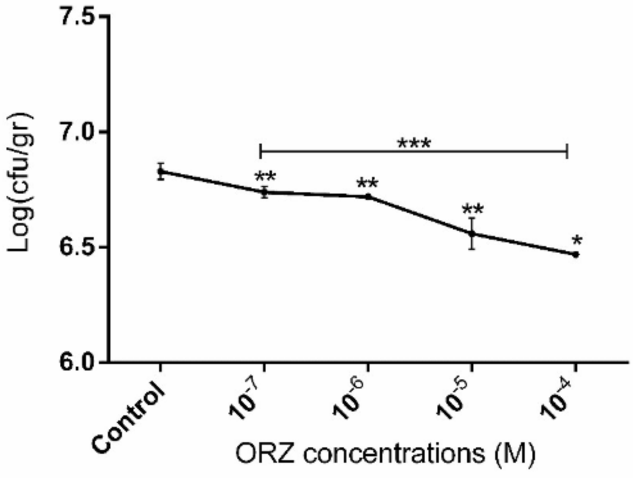

Figure 4. The effect of oryzatensin (ORZ) on bifidobacteria growth. Statistical analysis was done by the repeated-measures test. Each point represents the mean ± SD. ** P > 0.05 vs control. * P < 0.05 vs control. ***P < 0.05.

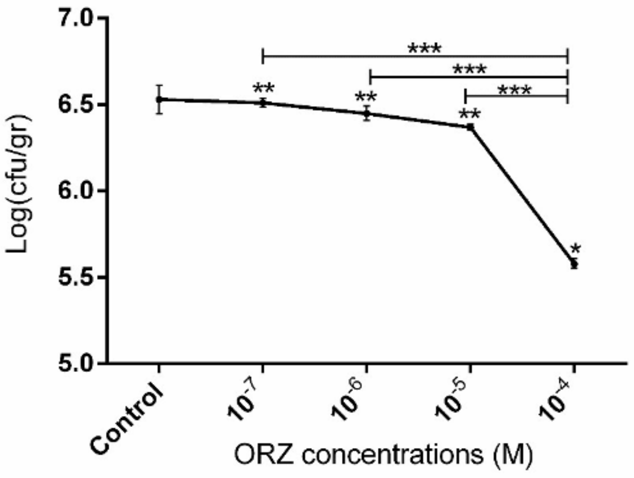

Figure 5. The effect of oryzatensin (ORZ) on Enterobacteriaceae growth. Statistical analysis was done by repeated measure test. Each point represents the mean ± SD. ** P > 0.05 vs control. * P < 0.05 vs control. ***P < 0.05

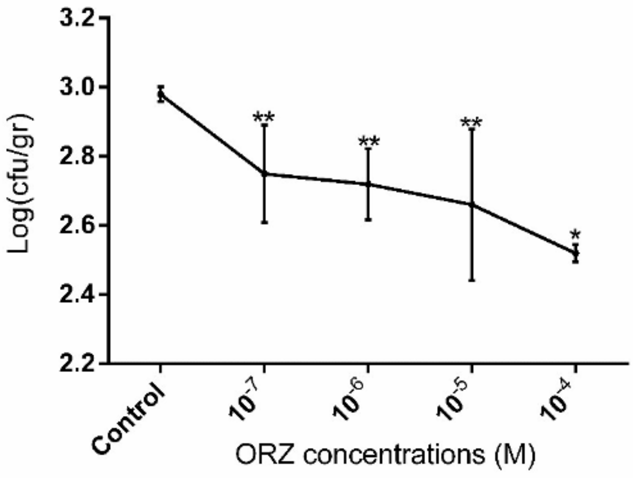

Figure 6. The effect of oryzatensin (ORZ) on lactic acid bacteria growth. Each point represents the mean ± SD. ** P > 0.05 vs control. * P < 0.05 vs control.

Discussion

In the current study, ORZ reduced the quantity of all types of enteral bacteria, both probiotics and nonprobiotics. Owing to the absence of a detectable optical difference among the wells, we were not able to calculate minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) for ORZ. Therefore, the bacterial quantity was measured by comparing the turbidity of the suspension in each well with that of the control group. It appears that this technique could be an alternative quantitative, feasible, and inexpensive method for estimation of bacterial count.

There is a wide range of BAPs with antimicrobial and immunomodulatory effects. Zeng et al. isolated antiviral BAPs from the Pacific oyster [17]. Protective effects of BAPs extracted from bovine milk have been reported in mice with *Staphylococcus aureus* and *Klebsiella pneumonia* infections [18]. In addition, antimicrobial, anticarcinogenic, and immunomodulatory effects of proteins have been widely confirmed [19-22]. Based on the results of the present experiment, ORZ seems to be one of these antimicrobial BAPs [22, 23].

Current evidence indicates that BAPs exert their antibacterial effect by two main mechanisms: directly affecting the bacterial cell and influencing the immune system of the gut [24, 25]. From the first point of view, the antibacterial effect of ORZ could be attributed to its potential role in decreasing the bacterial opportunity to consume simple sugars such as glucose, lactose, etc. [9, 10]. In addition, ORZ digestion by the bacteria spends too muchenergy and therefore leads to the inhibition of bacterial growth population. Considering the negative effects of the increased intestinal bacterial population [37], it is suggested that rice ORZ could possibly have an anti-inflammatory and anticarcinogenic effect.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent:

Informed consent was obtained from all individual participants included in the study.

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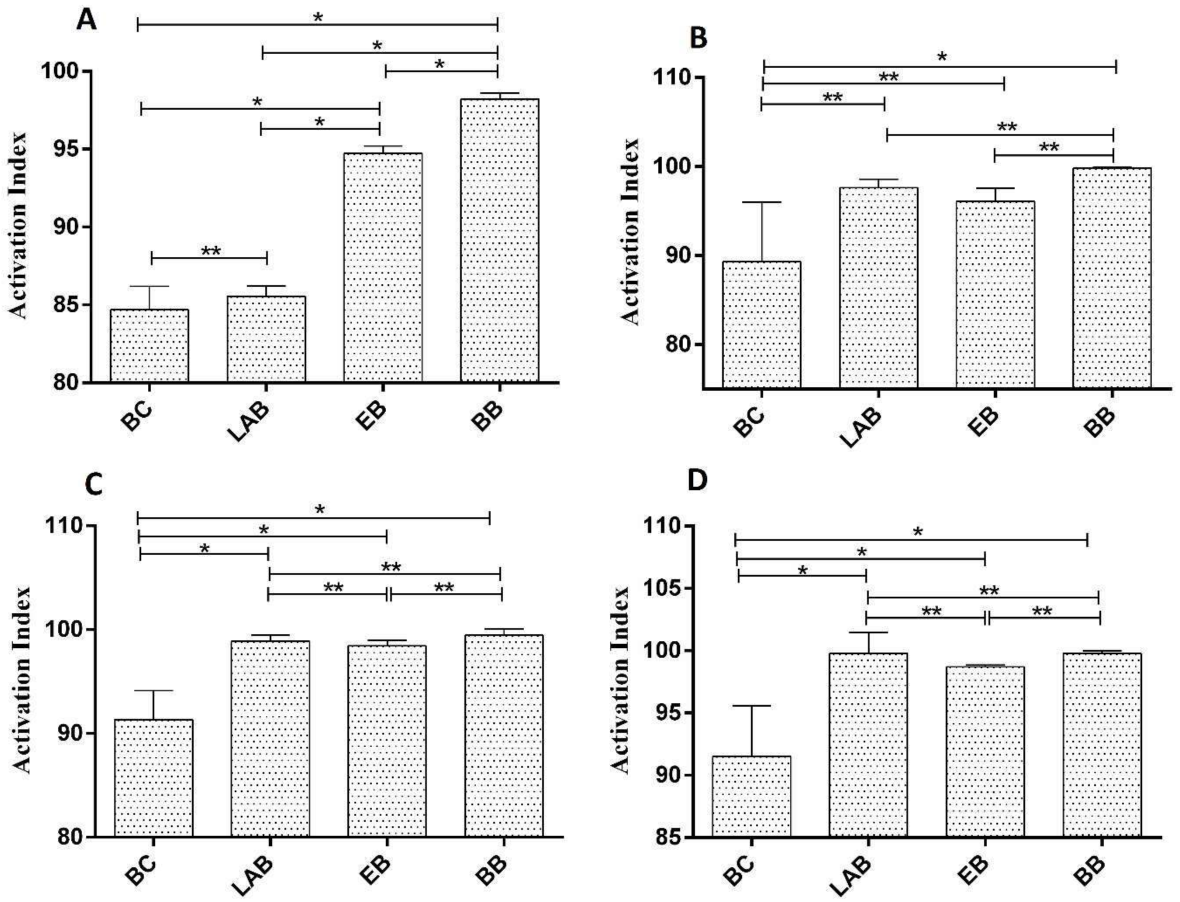

Figure 7. Effect of various concentrations of oryzatensin (ORZ) on the growth of bacteria. (A) ORZ at 10-4 mol/L, (B) ORZ at 10-5 mol/L, (C) ORZ at 10-6 mol/L, and (D) ORZ at 10-7 mol/L. The activation index = (Growth of ORZ-treated bacteria / growth of control bacteria) × 100. Statistical analysis was done by the repeated-measures test. Each point represents the mean ± SD. ** P > 0.05. * P < 0.05. BC: Bacillus cereus, LAB: Lactic acid bacteria, EB: Enterobacteriaceae, and BB: Bifidobacteria.

and eventually death [9, 10, 23]. Unfortunately, there is limited evidence on the latter mechanism and further investigation seems necessary. BAPs neutralize the toxic effects of lipopolysaccharides and are also able to improve phagocytosis and immune cell infiltration [12, 13]. Owing to their low molecular weight, antimicrobial peptides are easily diffused through cell membranes, stimulating the immune response against pathogens [14, 15].

The importance of this antibacterial effect is that the final metabolites (organic acids, acetaldehyde, free radicals, etc.) of the enteral bacteria—whether pathogenic or nonpathogenic—have shown both favorable and adverse effects on the body [26-28]. Nonpathogenic bacteria, as well as other GI bacteria, also contribute to the production of free radicals [29, 30]. Thus, the reduction of the entire bacterial population by ORZ could possibly reduce oxidative stress and the risk of GIDs such as SIBO, IBD, and cancer. Nevertheless, there is limited prospective or retrospective evidence regarding the association between rice consumption and neoplasms [31]. For example, in a cohort study conducted in Japan, Hirayama et al. showed that higher intake of rice in adults may decrease the risk of sigmoid cancer [31].

Despite the contradictory findings on the effect of increasing the nonpathogenic bacteria (e.g., by probiotic supplementation) on oxidative stress, nonpathogenic bacteria theoretically may release free radicals as metabolic by-products as they are metabolically active microorganisms [32]. Abnormal quality and quantity of gut microbiota could directly influence gastrointestinal health and lead to diseases such as SIBO and IBS. Therefore, antibiotic therapy has been widely used to overcome the situation, leading to the reduction of total bacterial count [33, 34]. It seems that any other compound with a similar function may have a beneficial effect on gastrointestinal inflammatory diseases including IBD [35, 36].

In our study, the inhibitory effect of ORZ on bacterial families isolated from human feces was observed. This suggests that rice may have a beneficial effect by reducing the intestinal bacterial

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