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Effect of Oxygenated Water and Probiotic Administration on Fecal Microbiota of Rats

INGRID SURYANTI SURONO [1,2*], ALI KHOMSAN[2], ENOK SOBARIAH[2] AND DARTI NURANI[3]

[1]*Southeast Asian Ministers of Education Organization-Tropical Medicine and Public Health Network, Regional Center for Community Nutrition University of Indonesia, Jalan Salemba Raya 6, Jakarta 10430, Indonesia ;*
[2]*Department of Community Nutrition, Faculty of Human Ecology, Institut Pertanian Bogor, Kampus Darmaga, Bogor 16680, Indonesia;* [3]*Institut Teknologi Indonesia, Jalan Raya Puspiptek, Serpong, Tangerang 15320 , Indonesia*

Oxygenated water is water with increased concentration of physically dissolved oxygen, and can perform all the same functions as the oxygen absorbed through the lungs. Several structures of human organs participate in the absorption and transportation of the oxygen, including the villi and cells containing mitochondrion in the small intestine as well as the lymph system. The aim of this *in vivo* study were three folds, to validate the support of oxygenated water on viability of probiotic bacteria in the GUT, to suppress the fecal coliform, and to study the effect of oxygen concentration on the profile of fecal microbiota. There were one control group and three probiotic groups of 5 rats each based on strain of probiotic supplementation, control without probiotic (a_0), *Lactobacillus casei* commercial strain (a_1), *Lactobacillus* sp. IS-7257 (a_2) and *Lactobacillus* sp. IS-27560 (a_3). Each group was treated with three variable treatments, without oxygenated water supplementation (b_0), supplemented with oxygenated water at 50 ppm (b_1), and at 80 ppm (b_2). Fecal samples were collected before (c_0), after 3 days (c_1), 7 days (c_2) supplementation, followed by 3 days after returning back to normal diet (c_3), analysed by culture dependent analyses for viable fecal lactic, coliform and fecal anaerobic bacteria. Supplementation of oxygenated water at 50 ppm, significantly increase fecal lactic acid bacteria of all probiotic groups after 3 and 7 days ($P<0.05$); 80 ppm oxygenated water tends to lower the fecal coliform ($P<0.1$), while oxygenated water administration gives no effect on fecal anaerobic bacteria. As a conclusion, 50 ppm oxygenated water administration significantly increased viable fecal lactic acid bacteria in probiotic groups. On the other hand, 80 ppm oxygenated water administration tends to lower the fecal coliform bacteria. No effect of administration probiotic and/or oxygenated water on viability of fecal anaerobic bacteria.

Key words: oxygenated water, probiotic, *in vivo*, viable fecal microbiota, dadih

Professor A Pakdaman developed the first process to enrich water with oxygen to 60 mg O_2 L^{-1} in Germany in 1979, and introduced oral oxygen therapy into the clinical medicine and nutrition in 1988. Oxygen will be mostly absorbed by diffusion and osmosis through cells in the stomach and intestine and will enter the body's blood circulation system through the portal vein. The additional oxygen can perform all the same functions as the oxygen absorbed through the lungs. Several structures of our organs participate in the absorption and transportation of the oxygen, including the villi and cells containing mitochondrion in the small intestine as well as the lymph system (Drakhshan 1995).

Oxygenated water defines as water with increased concentration of physically dissolved oxygen, and improve oxygen availability of the body. However, increased oxygen concentrations can also lead to an increased production of reactive oxygen species (ROS). If antioxidant defences are not completely efficient, ROS can cause cell injury including DNA damage. Drinking oxygenated water has been proved not to increase DNA damage in peripheral blood cells of test subjects in *in vivo* and *in vitro* studies measured with the alkaline comet assay, a single cell gel electrophoresis (Speit *et al*. 2002), means did not provide evidence for a genotoxic effect of oxygenated water.

Oxygen-supersaturated table water is marketed by a rising number of companies in several countries, including Indonesia. Advocates of those waters attribute positive health and fitness effects to peroral oxygen uptake. (Nestle *et al*. 2004). Drinking of oxygenated water (i.e. water with increased concentration of physically dissolved oxygen) is said to improve oxygen availability of the body and will do the consumer good.

Several questions are raised and one of those questions is the stability of the oxygen-supersaturated water to out gassing in the mouth and the esophagus. Further questions are concerned with the possibility of oxygen uptake from the digestive tract and the small absolute amount of per orally administered oxygen compared to respiratory oxygen uptake.

Dadih is fermented buffalo milk in bamboo tubes by natural LAB, and the resulting product is thought to be beneficial to human health (Akuzawa and Surono 2002). The benefits may be a result of the indigenous LAB involved in dadih fermentation. Some strains of indigenous dadih LAB tolerate acid and bile, good adhesion properties to mucus, and have antimicrobial activity against pathogenic bacteria, and even have antimutagenic properties (Surono and Hosono 1996; Surono 2003; Dharmawan 2006; Surono *et al*. 2009). *Lactobacillus plantarum* IS-10506 was the best strain to adhere to intestinal mucus, and the most effective strain against the pathogens tested, including *E. coli* (Collado *et al.* 2007).

Azha *et al*. (2004) reported that the *in vitro* growth of *Lactobacillus casei* commercial strain in media enrich with 30 ppm oxygenated water showed higher viable counts as compared to the media without oxygenated water, and after 24 h, the growth was 1000 times higher than the media without oxygenated water.

In an *in vitro* preliminary study, the growth of *Lactobacillus plantarum* IS-10506 and *L. plantarum* IS-

*Coresponding author, Phone: +62-21-31902950,
Fax: +62-21-3913933, E-mail: gridsw@yahoo.com

27560 did not show any inhibition in the presence of oxygenated water at 20, 30 and 40 ppm in the enrich medium (Surono, unpublished).

Oxygenated water will have contact to the gut-associated lymphoid tissue (GALT), which is essential for the balance of intestinal microbiota and the entire immune system (Shao *et al.* 2001). There are remarkably few data available regarding the response of intestinal microbiota to altitude stress and/or hypoxia in human (Basnyat and Murdoch 2003)

The aim of this *in vivo* study were three folds, to validate the support of oxygenated water on viability of probiotic bacteria in the GUT, to suppress the fecal coliform, and to study the effect of oxygen concentration on the profile of fecal microbiota.

MATERIALS AND METHODS

Bacterial Cultures Preparation. The probiotic isolates used in this study were *Lactobacillus casei* commercial strain, indigenous lactic acid bacteria isolated from dadih, namely *L. plantarum* IS-10506 and *L. plantarum* IS-27560, cultured in deMan Rogosa Sharpe (MRS) broth (oxoid, Basingstoke, UK) for 48 h at 37°C, harvested by centrifugation, and freeze-dried. Viability and purity of each of probiotic bacteria was checked routinely before administration.

Animals. Sixty male Sprague Dawley rats (6 wk old) with an average initial bodyweight of 82.2-103.6 g (Veterinary Research Institute, Bogor, Indonesia), were placed in individual metabolic cages and housed in a room maintained at a constant temperature of 22 ± 2°C, and a 12-h light to dark cycle. Animal care was in accordance with the guidelines for Animal Experimentation of the Faculty of Veterinary Medicine, Bogor Agricultural University. All rats were initially adapted for 5 d to a commercially available basal diet.

The *in vivo* study was a pre-post treatment. Rats were divided into four groups of fifteen rats each which were further devided into 3 subgroups. The first group received basal diet only, treated as control on normal diet (a_0). Second group, each rat was administrated with lyophilized cells of *L. casei* commercial strain, final concentration 1.3 - 1.7 x 10^{10} cfu d^{-1} (a_1). In the third group, each rat was administered with *L. plantarum* IS-10506 (GenBank accession no. DQ860148) cells, final concentration 1.3 - 1.4 x 10^{10} cfu d^{-1} (a_2). The fourth group, each rat was given lyophilized *L. plantarum strain* IS-20506 (GenBank accession no. DC860149) cells, final concentration 1.2 - 1.6 x 10^{10} cfu d^{-1} (a_3). Each group was devided into 3 subgroups (5 rats each), administrated with water (b_0), 50 ppm oxygenated water (b_1) and 80 ppm oxygenated water (b_2) at the amount of 6.25 mL per day. Administration of probiotic strain and/or oxygenated water was carried out for 7 days, followed by normal diet for the next three consecutive days). The fecal samples were collected before, during, and after treatments, and the fecal microbiota was analyzed by culture dependent method at day 0 (c_0), 3 (c_1), 7 (c_2), and 10, after 3 days normal diet (c_3).

Rats were allowed to consume their diets and water ad libitum. The average daily consumption of food and water per rat was also recorded. The lyophilized cells were mixed with 2 g of normal diet and were fed each morning during the experimental period. The oxygenated water was administered twice, every morning and afternoon at 3.25 and 3.00 mL, respectively, by oro-gastric tube feeding. After ensuring the complete consumption of cells (approximately 2 h), additional portions of normal diet were given. The bodyweight of each rat was measured before separating the animals to individual cages followed by every 2 days measurement until completing the treatments.

Fecal Microbiota Analysis (Lactic Acid Bacteria, Anaerobic Bacteria and Coliform)

All fecal samples were collected fresh by gently squeezing the rectal area of the rat. The fecal pellets were immediately placed in sterile tubes kept in anaerobic jars and the analysis was carried out within 30 to 60 min of collection in triplicates. Anaerobic conditions were maintained as far as possible during the analysis. Following homogenization, a series of 10-fold dilutions of the specimens was made in a pre reduced sterile phosphate buffer. Triplicate plates were made of each sample in MRS agar (Oxoid, Basingstoke, UK) for fecal lactic acid bacteria, in plate count agar (Oxoid, Basingstoke, UK) for fecal anaerobic bacteria and in VRB agar for fecal coliform. Plates of fecal lactic acid and fecal anaerobic bacteria were incubated anaerobically in an anaerobic jar (BBL Gas Pak anaerobic jars, Becton Dickinson Co., Franklin Lakes, NJ) for 3 days at 37°C. Plates for the enumeration of coliforms were incubated at 37°C for 2 days.

Statistical Analysis. Results obtained were subjected to Statistica '99 edition. Statsoft. Inc.. Kernel release 5.5. Standard error and level of significance were calculated and compared to control animals or with the values of before administration (0 d) of the respective group.

RESULTS

The Effect of Probiotic Administration on Viable Fecal Lactic Acid Bacteria of Rats.
There were no significant differences in feed intake, water consumption, and weight gain among the groups to the control group (data not shown). Fig 1 shows the significant effect of the probiotic administration, namely *L. casei* commercial strain ($P<0.001$), *L. plantarum* IS-10506, GenBank accession no. Dq860148 ($P<0.00001$) or *L. plantarum* IS-20506, GenBank accession no. DC860149 ($P<0.001$) on the viable fecal lactic acid bacteria of the rats as compared to the normal diet without probiotic group of rats (control group), after 3 and 7 days of administration.

Administration of probiotic *L. casei* commercial strain, *L. plantarum* IS-10506, *L. plantarum* IS-20506 significantly increased by 1.4-1.6, 3.25-3.40 and 0.35-0.65 log cycles of viable fecal lactic acid bacteria of rats ($P<0.001$), respectively after 3 days, and maintained the increment of viable fecal lactic acid bacteria after 7 days administration by

1.4-1.6, 1.8-2.0, 2.1-2.3 log cycles ($P<0.001$), respectively. After administration was stopped and the diet shifted to normal feed for three days, a significant ($P<0.01$) decrease of viable fecal lactic acid bacteria was observed in all probiotic groups, even though the amount of viable fecal lactic acid bacteria were higher than before administration.

The Effect of Probiotic and Oxygenated Water Administration on the Viable Fecal Lactic Acid Bacteria of the Rats. Fig 2 shows that 50 ppm oxygenated water

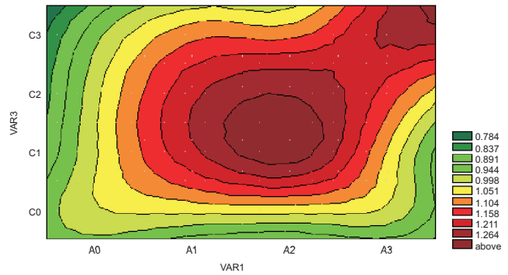

Fig 1 The effect of probiotic administration on viable fecal lactic acid bacteria of rat at different treatment periods. A, Probiotics: A0, control; A1, *Lactobacillus. casei*; A2, *L. plantarum* IS-10506; A3, *L. plantarum* IS-20506. C, Treatment period: C0, 0 day; C1, 3days; C2, 7 days; C4, 10 days.

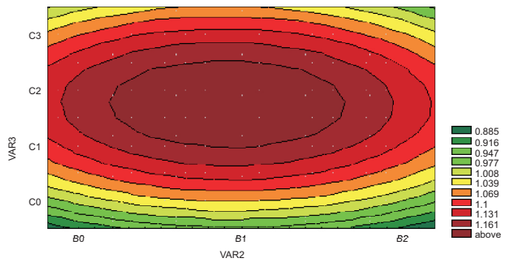

Fig 2 The effect oxygen concentration of oxygenated water on viable fecal lactic acid bacteria of rats at different treatment periods. B, Oxygenated water: B0, control (drinking water); B1, 50 ppm; B2, 80 ppm. C, Treatment period: C0, 0 day; C1, 3days; C2, 7 days; C4, 10 days.

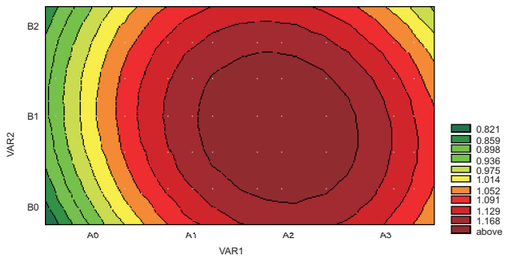

Fig 3 The effect of different concentration of oxygen in oxygenated water administrated on viable fecal lactic acid bacteria of rats at different probiotic groups. A, Probiotics: A0, control; A1, *Lactobacillus casei*; A2, *L. plantarum* IS-10506; A3, *L. plantarum* IS-20506. B, oxygenated water: B0, control (drinking water); B1, 50 ppm; B2, 80 ppm.

administration significantly increased viable fecal lactic acid bacteria of rats (cfu g^{-1}) after 3 and 7 days administration, ($P<0.03$) and ($P<0.01$), respectively, in the three probiotic groups as compared to the control group without oxygenated water and 80 ppm oxygenated water administration. *Lactobacillus plantarum strain* IS-10506 probiotic group showed the most possitive response (increased by 3.25 log cycles) after 3 days administration of 50 ppm oxygenated water, as compare to other probiotic groups, *L. casei* commercial strain (1.6 log cycles) and *L. plantarum* IS-20506 (0.65 log cycles) as shown in 3 dimension of contour plot in Fig 3.

The Effect of Probiotic Administration on Viable Fecal Coliform of Rats. Administration of probiotic for 3 days significantly ($P<0.03$) decreased the viable fecal coliform of rats in each of probiotic group, namely *L. casei* commercial strain, *L. plantarum IS-10506 and L. plantarum IS-20506* as compared to the control group, by 1.46, 1.38 and 1.35 log cycles respectively. And after 7 days, the viable fecal coliform was slightly increased by 0.22, 0.5 and 0.5 log cycles, respectively, but remain lower than before administration. This result is in agreement with the study on dadih lactic acid bacteria in competing and reducing *E. coli* K-2 adhesion (Collado *et al*. 2007).

After administration was stopped and the diet shifted to normal feed for three days, a significant ($P<0.01$) decreased of viable fecal coliform was still continued in *L. plantarum* IS-10506 and *L. plantarum* IS-20506 probiotic groups, by 1.64 and 1.15 log cycles, respectively, as compared to based line, while *L. casei* commercial strain group maintained the viable fecal coliform, as shown in Fig 4.

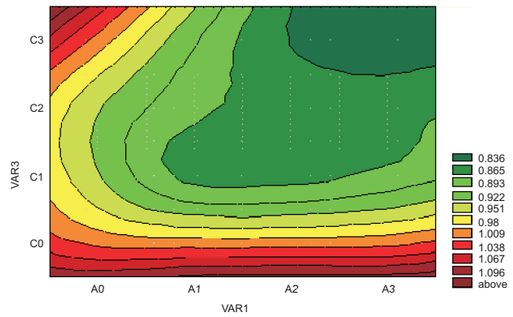

Fig 4 Effect of probiotic administration on viable fecal coliform at different treatment periods. A, Probiotics: A0, control; A1, *L. casei*, A2, *L. plantarum* IS-10506; A3, *L. plantarum* IS-20506, C, Treatment period: C0, 0 day; C1, 3 days; C2, 7 days; C4, 10 days.

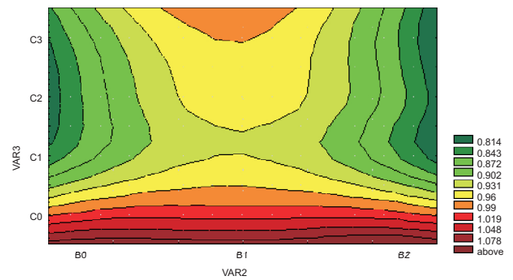

Fig 5 The effect of oxygenated water administration on viable fecal coliform at different treatment periods. B, Oxygenated water: B0, control (drinking water); B1, 50 ppm; B2, 80 ppm. C, Treatment period: C0, 0 day; C1, 3days; C2, 7 days; C4, 10 days.

The Effect of Oxygenated Water Administration on Viable Fecal Coliform of Rats. Figure 5 shows that administration of 80 ppm oxygenated water in combination with each of probiotic significantly decreased viable fecal coliform of rats after 3 and 7 days administration (P<0.02) and (P<0.001), respectively, and continued to decrease after three days normal diet, as compared to the control group without probiotic administration. The lowest viable fecal coliform bacteria was found in *L. plantarum* IS-20506 group.

Supplementation of 80 ppm oxygenated water only, in control group of rats significantly decreased viable fecal coliform of rats after 3 and 7 days administration (P<0.02 and P<0.001), respectively, as compared to rats administrated with drinking water.

Administration of oxygenated water at 50 or 80 ppm together with each of probiotic bacteria did not affect the viable fecal anaerobic bacteria of rats.

DISCUSSION

A significant increased of viable fecal lactic acid bacterial was observed, which is due to the viable probiotic administrated were excreted viable or because each strain colonizes the intestinal tract. Two to three log cyles (cfu) increased of lactic acid bacterial counts were observed (*P*<0.01). Collado *et al.* (2007) reported that some dadih strains have good adhesion capacity on human intestinal mucus.

The consumption of *probiotic* has been reported to change the fecal microbiota of humans (Plant *et al*. 2003). The three probiotic strains might have colonized the intestine and influenced the intestinal microbiota of rats, increasing the viable fecal lactic acid bacteria of rats.

Normal drinking water contains approximately 5-7 mg and fresh fountain water 10-12 mg of oxygen dissolved per liter. The additional oxygen in oxygenated water can perform all the same functions as the oxygen absorbed through the lungs. Several structures of human organs participate in the absorption and transportation of the oxygen, including the villi and cells containing mitochondrion in the small intestine (Drakhshan 1995). Probiotic bacteria administered to the rats are homofermentative and facultative anaerobic or microaerophilic bacteria. In anaerobic condition, lactic acid bacteria metabolite 1 mole of glucose into 2 moles of ATP by glycolysis pathway. The presence of oxygen in the GUT serve as acceptor electron, so that the production of pyruvic acid from glucose continue to respiration process produced more ATP, which in turn accelerate growth of lactic acid bacteria, including probiotics (Axelsson 2004). In the presence of oxygen, aerobic metabolism convert 1 mole of glucose into 38 ATP.

Administration of 80 ppm oxygenated water to rats might have supplied oxygen too much for microaerophilic probiotic bacteria. Hence, the excessive oxygen supplies suppress the growth of lactic acid bacteria in intestinal tract. This phenomenon confirmed that the dissolved oxygen can reach the intestinal tract. Excessive oxygen supply in the presence of glucose will produce excessive lactic acid and lowering the pH, which is not suitable for the coliform to grow and survive. However, there was no adverse effect observed during the study periods on the rats.

After treatment was stopped and the diet shifted to normal feed for three days, a significant (P < 0.01) decreased of viable fecal coliform bacteria was still observed in *L. plantarum* IS-10506 as well as *L. plantarum* IS-20506, which might be also due to the effect of exclusion of coliform bacteria by probiotic bacteria in the intestinal tract of rats.

Hypoxia may be related to ischemic symptoms owing to the insufficient oxygen supply to tissue such as the brain, heart and GI tract, which in turn may contribute directly or indirectly to the alteration in bacterial composition in GI tract. Likewise, it may alter immunological responses (Kleessen *et al*. 2005). This study validated the significant effect of oxygen supply in the form of 50 ppm oxygenated water in supporting the growth of beneficial bacteria such as lactic acid bacteria. Moreover, further studies are needed to observe the immunological response especially humoral immune response.

Administration of 50 ppm oxygenated water significantly (P<0.000) increased viable fecal lactic acid bacteria of rats in each of probiotic groups, after 3 and 7 days administration. Administration of probiotic significantly decreased fecal coliform of rats, and supplementation of 80 ppm oxygenated water only tends to decrease viable fecal coliform of rats after 3 days and significantly decreased viable fecal coliform of rats after 7 days administration (P<0.001), as compared to rats administrated with drinking water. Administration of probiotic and/or oxygenated water did not show significant effect on viable fecal anaerobic bacteria of rats. Administration of 80 ppm oxygenated water with probiotic *L. casei* commercial strain tend to increase viable fecal lactic acid bacteria, decrease viable fecal coliform, as well as viable fecal anaerobic bacteria of rats (P<0.015, P<0.01, P<0.008), respectively. Administration of 50 ppm oxygenated water in combination with probiotic strain either *L. casei* commercial strain, or *L. plantarum* IS-10506 or *L. plantarum* IS-20506 significantly increased the viable fecal lactic acid bacteria after 3 and 7 days. Safety on administration of oxygenated water as well as probiotics has been validated in this *in vivo* study. Taken together, it is challenging to validate the effect of probiotic and oxygenated water administration on humoral immune response *in vivo* and the effect of oxygenated water and probiotic on human health in human study.

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