



## Significance of fermented rice beverage on management of antibiotic associated diarrhea (AAD) on Wistar rats

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Antibiotic associated diarrhoea (AAD) is caused mostly by disruption of the physiological gut microflora. One potential strategy to prevent this is the concurrent use of probiotic bacteria or yeast. Tribal populations of West Garo Hill region of Meghalaya use locally available unique microflora to prepare fermented food products which serve as a potential source of novel probiotic organisms and bioactive compounds. In this context, here, we have evaluated the potentiality of laboratory made functional fermented rice beverage in mitigating AAD on animal models viz. Wistar rats. The animal models were administered with fermented rice beverage (test group A1), combination of indigenous *Lactobacillus* isolates (test group A2), a yeast isolate (test group A3), loperamide (test group STD), normal control (test group NC) and disease control (test group DC). Furthermore, various diarrhea assessment parameters were checked from each group followed by analysis of fecal microbiome, haematological parameters, histopathology of colon, liver and cecal short chain fatty acids (SCFAs) determination. NC and A1 was least affected by AAD induction with a faecal consistency score of 1 on the final day of the study. After day 10, a significant reduction ( $P < 0.05$ ) in the faecal water content was observed in A1, A2 and STD till day 15. Compared to the NC, a slight decrease in body weight was found in the rest of five test groups at day 5, 10 and 15. Except NC, the remaining five test groups showed a significant decrease of lactobacilli and yeast counts in faecal microbiota at 5<sup>th</sup> day. An increase in the enterococci and coliform counts indicated severe diarrheal condition but A1 reported with significant increase ( $P < 0.05$ ) in the population of *Lactobacillus* at day 15. An increase in red blood corpuscles, haemoglobin, packed cell volume, mean cell haemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration was observed. During the histopathology analysis of colon and liver, normal layers of mucosa, submucosa, muscularis and serous with absence of any abnormal changes or lesions was seen in A1. The cecal concentrations of lactate, acetate and propionate were significantly higher in A1 as compared to the other test groups. Therefore, fermented rice beverage possesses potential to be used in inhibition of antibiotic associated diarrhea with further clinical investigations.

**Keywords:** Cecum, *Chubitchi*, Colon, SCFAs, Garo Hills, Goatweed, Gut microflora, Heaptoprotective, Licorice weed, Liver, *Menil*, Sweet-broom, Traditional food

Indigenous fermented beverages (*Chubitchi*) brewed from a red variety of sticky rice (*Menil*) is considered as an integral part of the rich tribal diet and culture in the parts of West Garo Hills, Meghalaya, India. The beverage is fermented by utilizing locally available varieties of rice and traditional rice starter cultures containing a mixed population of indigenous microbes and topical medicinal plants<sup>1</sup>. Additionally, fermented rice beverages have also been considered as 'probiotic' due to the presence of many strains of lactic acid bacteria (LAB) found in it, which are often

perceived to carry probiotic potentials<sup>2</sup>. Likewise, various positive health claims have been attributed to fermented rice beverages which happen to be derived from added medicinal plants and herb that apparently improve the antioxidative and antidiarrheal property of the beverage<sup>3</sup>.

Since, antibiotic associated diarrhea (AAD) develops from the disturbance in the commensal gut microbiota due to excessive antibiotic therapy, administration of probiotic-based foods/beverages is a logical therapeutic approach to regulate/restore the gut microbiota<sup>4,5</sup>. *Lactobacillus* infused fermented foods are reported to prevent the reduction of number of beneficial bacteria and infection caused by antibiotic resistant bacteria<sup>6</sup> and can decrease the

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possibilities of occurrence of antibiotic associated diarrhea<sup>7,8</sup>. *Lactobacillus* strains in a combined form with compatible nature amongst one another can possibly prevent the risk of antibiotic-associated diarrhea (~57%), traveller's diarrhea (~8%), acute diarrhea (~34%), diarrhea among adults (~26%), and acute diarrhea in youngsters (~57%)<sup>5</sup>.

Not only lactic acid bacteria but yeasts, specifically, *Saccharomyces* are also used to enhance absorption of chloride in the jejunum and descending colon which has a positive effect on the volume of AAD<sup>9</sup>. Previous data also indicates that the mode of action of *Saccharomyces* includes the nitric oxide (a modulator of intestinal water and electrolyte transport) pathway to prevent AAD<sup>10</sup>. Antidiarrheal agents viz. loperamide and diphenoxylate hydrochloride with atropine sulfate are also used often for combating AAD, but as per few reports these compounds have been involved with severe intestinal obstruction and toxic abnormal expansion of colon<sup>11</sup>. Scientific reports claim that those probiotics may inhibit AAD by hindering either of the likely processes: by maintenance of the gut flora and ongoing carbohydrate fermentation; and/or by competitively preventing the growth of pathogens. The precise mechanism of action is as yet not known and may differ between strains of bacteria and yeasts<sup>8</sup>. In this study, we examined the therapeutic effects of the fermented rice beverage on AAD and explored the underlying mechanism associated with it by employing animal models.

## Materials and methods

### Preparation of functional fermented rice beverage

The indigenous rice beverage generally called as 'Chubitchi' in the region of West Garo Hills of Meghalaya forms an integral part of the tribal festivals and ceremonies. The rural ethnic tribal population bear an impregnable sense of traditional knowledge and conventional culture of which traditionally brewed rice beverage has been an indispensable part economically, culturally, and spiritually and assumes a very eminent role in their sociocultural activities. With respect to the traditional beliefs of the Garo tribal folks, the fermented rice beverage was improvised in laboratory conditions which could provide them with a defined starter culture having health beneficial attributes as well as standardized protocol for the preparation of rice beverage of Meghalaya. The formulation and

development of defined starter cultures (Fig. 1) and fermented rice beverage preparation (Fig. 2) were executed in methods of Liu *et al.*<sup>12</sup> and Mehra *et al.*<sup>3</sup> with requisite modifications.

### Initiation of the study on animal model and maintenance

The animals were procured from Sun Pharma Advance Research Center, Vadodara, India after receiving permission from Institutional Animal Ethics Committee (IAEC), Ramanbhai Patel College of Pharmacy, Changa, as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Govt. of India (Permit No. RPCP/IAEC/2019-20/R5). A total of 36 Wistar rats of 4-6 months weighing 200-250 g were used for the study. The animals were housed in polypropylene mouse/rat cages (3 animals were housed per cage per sex). Rice husk was used as the bedding material. The animals were further acclimatized to the laboratory conditions for a minimum period of 5 days prior to commencement of treatment with antibiotic mixture for inducing AAD. The laboratory conditions with: temperature at 22±3°C, humidity at 30-70% and 12 h light/dark was maintained throughout study. The diet provided was free from intestinal flora modifiers, such as antibiotics and probiotics. The test animals (n=36) were fed with normal diet composed as follows: crude protein 21.85%, crude fat 4.85%, crude fiber 3.15%, calcium 1.10%, phosphorus 0.5%, total ash 5.80% and carbohydrates 65%. Composition of normal pellet diet was supplied by VRN Nutrition Ltd., India. The feed consumption rate for each animal was 20-25 g per day.

### Experimental design and the AAD model

Thirty-six male Wistar rats were divided into 6 different groups viz. NC, DC, STD, A1, A2, A3, each group containing 6 rats. The untreated animals served as the normal control (NC) test group. Disease control (DC) group was administered with antibiotic mixture (combination of 50 mg/mL clindamycin, 55.5 mg/mL ampicillin, and 27.75 mg/mL streptomycin)<sup>13</sup>. Standard group (STD) was administered with loperamide (5 mg/kg). Test A1 group was administered with fermented rice beverage infused with well characterized strains of *Lactiplantibacillus plantarum* KGL3A, *L. fermentum* KGL4<sup>14</sup>, *Saccharomyces cerevisiae* WTS1A and the plant extract of *Scoparia dulcis*, commonly called as

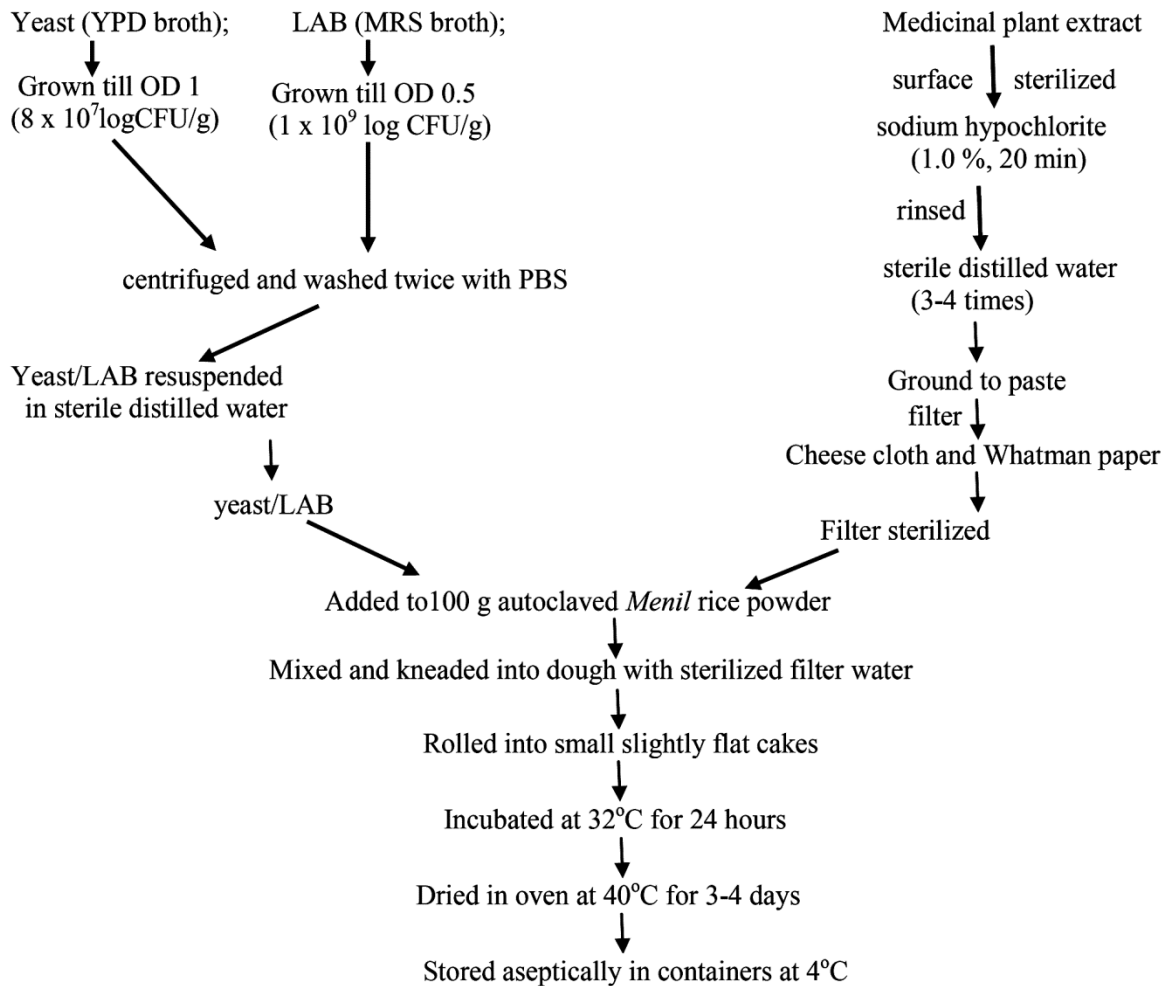


Fig. 1 — Flow diagram for development of defined starter culture. [LAB, *Lactiplantibacillus plantarum* KGL3A (GI: MG722814) and *L. fermentum* KGL4 (GI: MF951099); Yeast, *Saccharomyces cerevisiae* WTS1A (GI: MG183699); and Medicinal plant extract, *Scoporia dulcis* (2 mg/mL)]

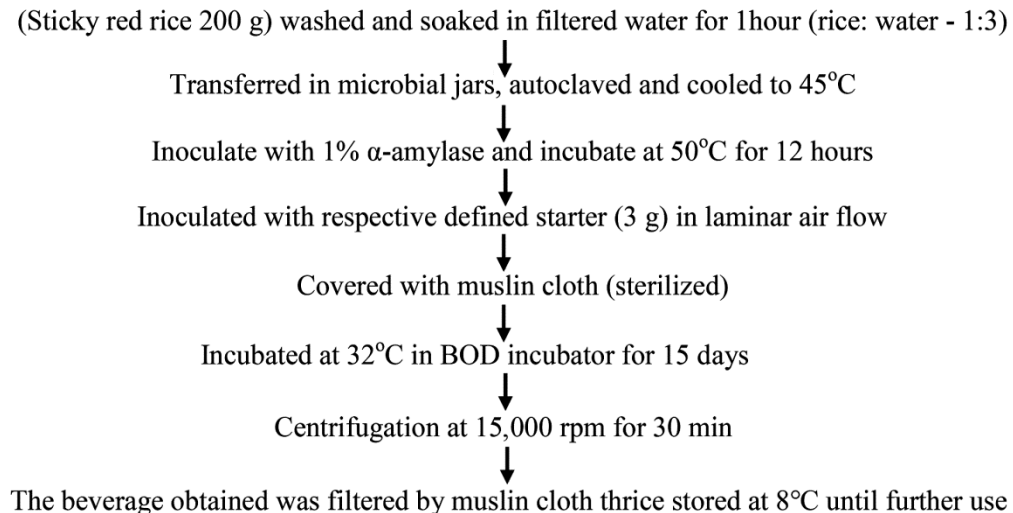


Fig. 2 — Flow diagram of brewing functional fermented rice beverage.

licorice weed, goatweed, scoparia-weed or sweet-broom Test A2 group administered with the combination of *L. plantarum* KGL3A and *L. fermentum* KGL4 [1:1 ratio in phosphate buffered saline (PBS) and diluted accordingly to adjust the cell counts to  $10^8$  CFU/mL]. Test A3 group was administered with *S. cerevisiae* WTS1A (cells were suspended in PBS and diluted accordingly to adjust the cell counts to  $10^7$  CFU/mL in final volume). Following pre-challenge with the antibiotic mixture for 7 days, during the treatment period, AAD rats from each test group were orally gavaged daily till day 15 with 1 mL of each of the above-mentioned sample viz. DC, STD, A1, A2, A3 twice a day (morning and evening).

The presence of diarrhoea was measured every 5<sup>th</sup> day during the experimental study up to 15 days. The faecal samples from all the 6 test groups were collected every 5 days up to 15 days for microflora analysis. On day 16, half of the rats in each group were euthanized and 2cm long samples of colon tissue – 3cm distal to the anus and liver tissue were collected for general histopathological analysis. Blood samples were collected for haematological analysis and presence of SCFAs in cecal cells of each test group was determined by HPLC analysis.

#### Diarrhea assessment

The antibiotic associated diarrheal symptoms were assessed using three parameters: changes in body weight, fecal consistency and fecal water content which were measured every fifth day during the experimental period<sup>15</sup>. Fecal consistency was classified based on the following visual grading scale: (i) formed, fecal maintains its shape, brown, score 1; (ii) semi-formed or soft, does not pour, yellow, score 2; and (iii) liquid, pours more easily, yellow, score 3. The faecal water content was calculated as follows: faecal water content =  $1 - (\text{dried solid content})/(\text{total faecal content})$ .

#### Microbial analysis of fecal samples

Freshly feces from each of the test groups were obtained at day 0, 5, 10 and 15. The faecal samples were homogenized (10% w/v) and were serially diluted in 1X phosphate buffered saline (PBS). Aliquots were then spread onto different selective agar plates ( $10^{-3}$  to  $10^{-7}$  dilutions). To determine viable lactobacilli counts, De Man, Rogosa and Sharpe (MRS agar; HiMedia, Mumbai, India) was

used (incubated for 48 h at 37°C). For detecting coliforms, violet red bile agar (VRBA; HiMedia, Mumbai, India) was used (incubated for 48 h at 37°C). KF streptococcal agar base (HiMedia, Mumbai, India) was employed for measuring viable *Enterococcus* (incubated for 48h at 37°C) and yeast potato dextrose agar (YPDA; HiMedia, Mumbai, India) was used to check fecal yeast counts (incubated for 72 h at 32°C)<sup>13</sup>.

#### Haematological analysis

Twenty-four hours after the last treatment was given, blood from the rats of each test groups was withdrawn from retro orbital plexus under light ether anaesthesia into EDTA treated screw-cap sample bottles<sup>16</sup>. The anticoagulated blood samples were used for haematological analyses which were carried out within 24 h of sample collection. The erythrocytes and the related parameters viz. RBC, Red blood corpuscles; HGB, Haemoglobin; PCV/HCT, Packed Cell Volume/Hematocrit; MCH, Mean cell haemoglobin; MCV, Mean cell/corpuscular volume; and MCHC Mean corpuscular hemoglobin concentration were estimated using the Abacus Junior Vet 5 Haematology Analyzer, Diatron, Budapest, Hungary.

#### Histopathological analysis

After the final day of the study, on day 16, the rats from each of the 6 test groups were euthanized after keeping them fasting overnight. For histological examination, liver and colon tissues were dissected properly and was cleaned with normal saline solution for removal of fecal matter if any. The tissues were dehydrated and embedded in paraffin as per the protocol suggested by Dong *et al.*<sup>17</sup>. Specific sections (5 µm) were stained with hematoxylin & eosin and histomorphologically examined under the microscope (Olympus, India).

#### Cecal short chain fatty acids (SCFAs) quantification

The SCFAs (lactate, acetate, and propionate) were extracted from the cecal samples from the six test groups after euthanization of the rats based on the procedure reported by Morales-Ferré *et al.*<sup>18</sup>. 0.2 g sample of cecal contents from each test group (NC, DC, STD, A1, A2 and A3) were added to 1.8 mL of 0.05 [N] sulfuric acid and homogenized. The mixture was centrifuged at 6000 ×g for 12 min. The supernatant (20 µL) was used for HPLC analysis after being filtered through a 0.22 µm syringe filter (Millipore, Bengaluru, India). The SCFAs were separated and quantified in a HPLC system (model no. RID-10A;

Shimadzu, Tokyo, Japan) equipped with wavelength detector (SPD-20A), ultraviolet (UV-260 nm) detector, Shimadzu LC-20 manual injector with 20 mL loop and SeQuant® ZIC®-cHILIC PEEK coated HPLC column (250×4.6 mm, 3 µm with 100 Å pore size; Merck, Darmstadt, Germany) with 0.012 [N] H<sub>2</sub>SO<sub>4</sub> (mobile phase) elution at 45°C. The flow rate was maintained at 0.4 mL/min and the sensitivity of detection was 1.0 µg/mL. Before connecting the mobile phase to the HPLC system, it was filtered through a 0.45-µm membrane filter (Millipore, Bengaluru, India) followed by degassing in an ultrasonic water bath (model no. LMUC-9; Labman, Chennai, India) at 37°C for 35 min.

#### Statistical analysis

All the data presented here are the average of three independent assays and the results obtained were expressed as mean ± standard deviation (M±SD). One way analysis of variance (ANOVA) was applied and comparison was made through Bonferroni's test with the least significant difference of  $P \leq 0.05$  using the IBM SPSS Statistical program (Ver. 20). For graphical presentation and data analysis, OriginPro (version 9.0) was used.

## Results and Discussions

### Changes in body weight, faecal consistency and faecal water content

For investigating the metabolic changes after inducing diarrhea through antibiotic mixture, a comparison was made amongst the 6 test groups of the rat models with respect to the alterations in the body weight. Contrary to the normal control group (without any supplementation), the rest of the five test groups were devoid of any significant difference ( $P$

$\geq 0.05$ ) between body wt./intake of diet after 0, 5, 10 and 15 days of the study (Fig. 3A). The body weight of the normal control group showed no basic alteration till 15<sup>th</sup> day of the study with 363.33 g (day 0), 366.66 g (day 5), 373.33 g (day 10) and 376.66 g (day 15). Slight decrease in weight was observed in rest of the test groups after 5<sup>th</sup> day (DC 363.33 g, STD 315 g, A1 335 g, A2 326.66 g and A3 335.66 g), 10<sup>th</sup> day (DC 360 g, STD 310 g, A1 350 g, A2 353.33 g and A3 343.33 g) and on the 15<sup>th</sup> day (DC 331.66 g, STD 318.33 g, A1 315 g, A2 348.33 g and A3 335 g) of the study. Our results are in agreement with Zhang *et al.*<sup>13</sup> where various doses of antibiotic mixture containing clindamycin, ampicillin and streptomycin were employed for inducing AAD in a rat model and no significant differences in body weight was reported between the AAD groups and the control group. In another study, the mixture of antibiotics (ampicillin, neomycin sulfate, metronidazole and vancomycin) was administered to mice for a period of 22 weeks which resulted in no significant change in the body weight of the treated mice relative to the mice in the control group<sup>19</sup>.

Faecal consistency primarily correlates with the major microbiome markers for assessing of diarrhea<sup>20</sup>. Based on the visual grading scale (Fig. 3B), it was observed that other than the rats in the NC test group, AAD was exhibited by other test groups as measured by faecal consistency. The faecal consistency was formed and compact in the rats of NC group till the 15<sup>th</sup> day of the study. Loose and semi solid fecales were observed in AAD induced rats in the test groups on day 5 with diarrheal symptoms lasting up to day 10. Out of all the test groups except NC, the test group A1 fed with

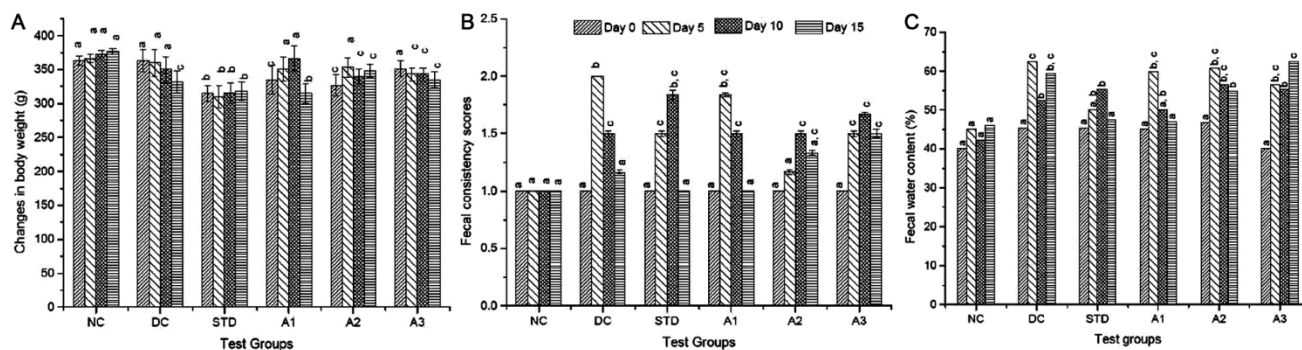


Fig. 3 — Diarrheal assessment parameters of the different AAD test groups till 15 days with an interval of 5 days. (A) Changes in body weight (g); (B) Faecal consistency scores; and (C) Faecal water content (%). [Test groups: NC, untreated animals; DC, administered with antibiotic mixture; STD, administered with loperamide; A1, administered with fermented rice beverage; A2, administered with the combination of *Lactiplantibacillus plantarum* KGL3A and *L. fermentum* KGL4 cells; and A3, administered with *Saccharomyces cerevisiae* WTS1A cells. Values are mean ± standard deviation of triplicate determinations (n=3). Level of significance  $P < 0.05$ ]

functional fermented rice beverage was comparatively least affected by AAD with a score of  $1.83 \pm 0.166$  on day 5,  $1.5 \pm 0.22361$  on day 10 and completely resolving the diarrhea on day 15 with formed fecal texture and with a fecal consistency score of 1. The rats in DC group were most affected with diarrhea induced by the antibiotic mixture with a fecal consistency score of 2 in day 5,  $1.5 \pm 0.22361$  at day 10 and  $1.166 \pm 0.166$  at day 15. On the final day of the study, the test groups, A1 and STD showed dry and normal consistency of feces with well-maintained shape and brown colour with a fecal consistency score of 1 presuming to derive that AAD was resolved (Fig. 3B). Based on the fecal consistency scores, a significant difference ( $P \leq 0.05$ ) was observed between the NC test group and the remaining five test groups. Our study is in agreement with Hu *et al.*<sup>21</sup> where rats with AAD exhibited antibiotic induced diarrhea and the fecal consistency was measured similarly on the basis of visual grading scale. With correlation to our study, Zhang *et al.*<sup>13</sup> reported fecal consistency of AAD rats with liquid feces on day 4 with diarrheal symptoms on days 7 and 8, and lasting up to day 14.

In addition to the above results, the fecal water content substantially increased in all the AAD test groups from day 0 to day 10 (Fig. 3C). However, after day 10, a significant reduction ( $P \leq 0.05$ ) in the fecal water content was observed in A1, A2 and STD up to day 15 while the faecal water content of other groups viz. NC, DC and A3 were not significantly reduced after day 10. Similarly, Zhang *et al.*<sup>13</sup> also reported the increase in faecal water content from all the AAD groups in their study, from day 4 to day 14 with significant decrease in the fecal water content in the AAD group treated with *Bacteroides fragilis* ZY-312 from day 12 onwards. Our study is also in agreement with Hagihara *et al.*<sup>22</sup> where fecal consistency score and faecal water content was examined on every alternate day to observe inhibitory effect by probiotic strain *Clostridium butyricum* MIYAIRI 588 against Wistar rats induced with AAD. These results indicated that longterm gavage of antibiotics caused diarrhea in rats, although the severity of symptoms was not dose-dependent. Therefore, treatment with fermented rice beverage (A1) followed by test group A2 (*L. planatarum*:*L. fermentum*) and STD (containing loperamide) appeared to have a protective effect in AAD rats, which showed the positive indication for further investigating the mechanism underlying these effects.

#### Microbial analysis of the fecal samples

Characterization of the bacterial diversity and abundance in the faecal microbiota between the six AAD test groups were determined up to day 15 with an interval of 5 days as represented in Fig. 4. During analysis of fecal microbiota on 5<sup>th</sup> day, other than the NC test group, rest of the five test groups showed a significant decline ( $P \leq 0.05$ ) in lactobacilli and yeast cells. A significant increase ( $P \leq 0.05$ ) in the enterococci (Fig. 4C) and coliform counts (Fig. 4D) might derive that diarrhea was severe on the 5<sup>th</sup> day of the study. Comparatively, test group A1 showed lactobacilli count of 7.87 log CFU/mL (Fig. 4A) and yeast cell count of 3.96 log CFU/mL (Fig. 4B) at day 5 that was approximately at par with NC test group fed with normal diet. Highest enterococcal (5.62 log CFU/mL) and coliform (7.54 log CFU/mL) count were observed in DC test group on day 5. From 10<sup>th</sup> day onwards, increase in lactobacilli (8.28 log CFU/mL) and yeast cell count (4.04 log CFU/mL) were found in test group A1. Test group DC was the most affected with decrease in lactobacilli (7.35 log CFU/mL) as well as yeast cells (2.43 log CFU/mL) from day 5 to day 15 and increase in enterococcal (5.60 log CFU/mL) and coliform (7.40 log CFU/mL) counts. The only test group after A1, where improved growth was observed was the group treated with loperamide (test group STD) from 10<sup>th</sup> day till the final day of the study. After 15 days study, a significant increase was observed in the population of *Lactobacillus* (8.323 log CFU/g) in the test group administered with the fermented rice beverage, therefore suggesting that the microorganisms might persist in the faecal microbiota during this period.

Zhang *et al.*<sup>13</sup> reported with the overall composition of the fecal microbial community of AAD rats which significantly differed from normal control group on day 11, but the difference between the groups appeared to be smaller on day 17 as compared to day 11. Their results confirmed that *B. fragilis* ZY-312 bearing rich probiotic potentiality could aid in modulating particular elements of the fecal microbiota in AAD rats. On the other hand, Stecher<sup>23</sup> reported increased cell count of *Enterococcus* spp. in mice administered with a soy beverage fermented with *E. faecium* CRL 183 and *L. helveticus* 416. However, the animals fed with the fermented soy beverage showed an increase in *Enterococcus* spp., interpreting that the fermentation

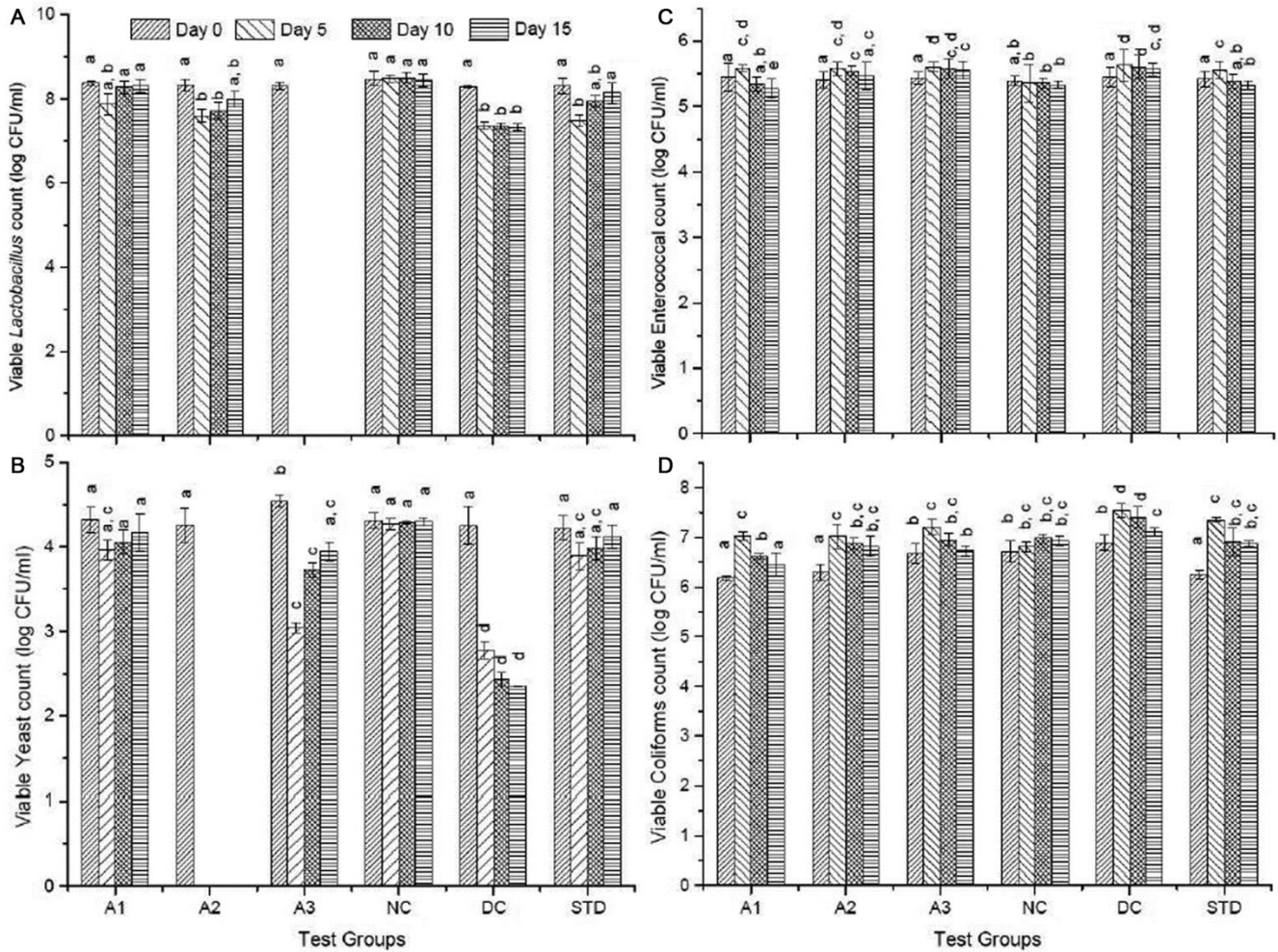


Fig. 4 — Microbial diversity of pooled feces from different AAD test groups till 15 days with an interval of 5 days. (A) Viable *Lactobacillus* count; (B) Viable yeast count; (C) Viable enterococcal count; and (D) viable coliforms count. [Test groups: NC, untreated animals; DC, administered with antibiotic mixture; STD, administered with loperamide; A1, administered with fermented rice beverage; A2, administered with the combination of *Lactiplantibacillus plantarum* KGL3A and *L. fermentum* KGL4 cells; and A3, administered with *Saccharomyces cerevisiae* WTS1A cells. Values are mean  $\pm$  standard deviation of triplicate determinations (n=3). Level of significance  $P < 0.05$ ]

process exhibits specific metabolites that might trigger the population of this genus of fecal microorganisms. Although, the genus *Enterococcus* spp. consists of broader species of microorganisms, and few of which are even being considered to be pathogenic<sup>24</sup>, and hence the observed alterations from the studies does not necessarily intend to state the decrease of the probiotic microorganisms. Kayasaki *et al.*<sup>25</sup> reported the impact of *Lactobacillus fermentum* 5716 administrations on the gut microbiota of rats with colitis induced by trinitrobenzenesulfonic acid. The colitis induced animals that was fed with the probiotic strain showed a higher cell count of *Lactobacillus* spp. than the colitis-induced animal group without the treatment.

#### Haematological analysis

The assessment of erythrocytes and related parameters indicates the adverse effects of foreign compounds on the blood constituents of an animal<sup>26</sup>. A reduction in number of red cells in the blood could be due to the stimulation of lipid peroxidative system by the diarrheal toxin resulting in lipid peroxides production which might hemolyse the RBC's in AAD animals<sup>27</sup>. As depicted in Table 1, lowest RBC content ( $P < 0.05$ ) was observed in DC test group ( $7.45, 10^6 \mu\text{L}$ ) that might be caused due to the administration of the antibiotic mixture for inducing diarrhea. With contrast to the NC test group ( $10.19, 10^6 \mu\text{L}$ ), test group A1 ( $8.95, 10^6 \mu\text{L}$ ) followed by STD ( $8.92, 10^6 \mu\text{L}$ ) were considered to be better in

Table 1 — Haematological analysis of antibiotic associated diarrhea (AAD) rats from different test groups

Test Groups	Erythrocytes and related indices					
	RBC (10 <sup>6</sup> μL)	HGB (g/dL)	HCT/PC V (%)	MCHC (g/dL)	MCV (fl)	MCH (pg)
DC	7.45*	13.9*	40.56*	31.2*	48*	16.2*
NC	10.19	16.4	55.87	34.0	55	18.7
STD	8.92	15.4	48.43	33.6	55	18.4
A1	8.95	18.2	49.01	36.9	60	19.7
A2	8.85	14.3	44.41	31.8*	54	17.2
A3	7.60*	15.0	42.08	32.6	53	17.9

[\*differ significantly ( $P \leq 0.05$ ). Test groups: NC, untreated animals; DC, administered with antibiotic mixture; STD, administered with loperamide; A1, administered with fermented rice beverage; A2, administered with the combination of *Lactiplantibacillus plantarum* KGL3A and *L. fermentum* KGL4 cells; and A3, administered with *Saccharomyces cerevisiae* WTS1A cells. Erythrocyte parameters: RBC, Red blood corpuscles; HGB, Haemoglobin; PCV/HCT, Packed cell volume/Hematocrit; MCH, Mean cell haemoglobin; MCV, Mean cell/corpuscular volume; and MCH, Mean corpuscular hemoglobin concentration]

terms of RBC content than the rest of the groups viz. A2 and A3. Low levels of hemoglobin arise as a result of loss of blood (hemorrhage) or accelerated blood cell disruption that might be due to the AAD<sup>28</sup>. Lowest HGB (13.9 g/dL) was showed by DC test group that was administered with antibiotic mixture only. On contrary, the test group A1 was reported that was infested with the functional fermented rice beverage showed increased levels of HGB content (18.2 g/dL). The NC test group showed 16.4 g/dL followed by STD, A3 and A2 test groups. Hematocrit represents the percentage of red blood cell volume of whole blood volume (called packed cell volume (PCV) in animals<sup>29</sup>. Lower percentage of PCV was shown by DC test group (40.56%) followed by test group A3 and A2 mainly which may be due to a decrease in the number of erythrocytes, a decrease in the amount of hemoglobin in each erythrocyte, or both. However, group A1 showed a PCV% of 49.01 which was the nearer most to the NC test group (55.87%) than the rest of the groups. The decreased levels MCHC (Mean corpuscular hemoglobin concentration) values might act as an indicator of abnormal haemoglobin synthesis, blood osmoregulation failure and plasma osmolarity<sup>30</sup>. Test group DC (31.2 g/dL) and A2 (31.8 g/dL) showed lower levels of MCHC values. Higher MCHC value was reported by A1 (36.9 g/dL) followed by NC, STD, A3 and A2 test groups which provides the information of average concentration of hemoglobin inside a single red blood cell.

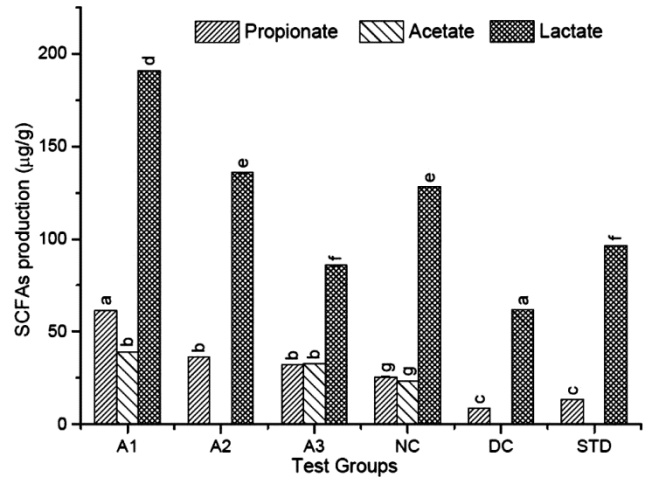


Fig. 5 — Cecal short chain fatty acids (SCFAs) determination. (A) Cecal content from each of the AAD test group; (B) Production of SCFAs (propionate, acetate, lactate) in the cecal content of the rats of different AAD test groups. [Test groups: NC, untreated animals; DC, administered with antibiotic mixture; STD, administered with loperamide; A1, administered with fermented rice beverage; A2, administered with the combination of *Lactiplantibacillus plantarum* KGL3A and *L. fermentum* KGL4 cells; and A3, administered with *Saccharomyces cerevisiae* WTS1A cells. Values are mean  $\pm$  standard deviation of triplicate determinations ( $n=3$ ). Level of significance  $P < 0.05$ ]

Mean cell/corpuscular haemoglobin (MCH) is the average amount of hemoglobin inside a single red blood cell and the higher MCH value was reported in test group A1 with 19.7 pg (pictograms), followed by NC, STD, A3 and A2. Due to the effect of antibiotic mixture, lowest MCH (16.2 pg) was found in DC group. Mean cell/corpuscular volume (MCV) is a measure of the average volume or size of a red blood cell and the highest MCV was observed in test group A1 with 60 fl per red cell followed by NC, STD, A3 and A2 test groups. Lowest MCV was also observed in DC test group (48 fl per red cell) which summarizes the impact of test group A1 in inhibition of AAD.

#### Cecal short chain fatty acids (SCFAs) quantification

Generally, the cecum is generally considered as the primary site for microbial fermentation<sup>31</sup>. To determine the impact of antibiotic treatment on cecal SCFA levels, rats from each of the test groups were euthanized after the final day of the study. Concentrations of SCFA viz. lactate, acetate and propionate in the cecal cells were determined which varied significantly ( $P \leq 0.05$ ) in all of the test groups viz. NC, DC, STD, A1, A2, A3 (Fig. 5). Test group A1 showed highest lactate production with 190.96  $\mu\text{g/g}$  of the cecum followed by test groups A2 (135.75  $\mu\text{g/g}$ ), NC (128.23  $\mu\text{g/g}$ ), STD (96.55  $\mu\text{g/g}$ ),



A3 (85.74  $\mu\text{g/g}$ ). The lowest production was reported by test group DC with 61.79  $\mu\text{g/g}$  of the cecum. Carbohydrate fermentation by LAB can lead to various short chain fatty acid productions<sup>32</sup>. Even in carbon depletion and anaerobic condition, lactic acid can be further converted into acetic acid by several LAB<sup>33</sup>. The results showed higher acetate production by test group A1 (39.12  $\mu\text{g/g}$ ) followed by test groups A3 (32.66  $\mu\text{g/g}$ ) and NC (23.63  $\mu\text{g/g}$ ). Acetic acid production was not detected in test groups viz. A2, DC and STD. In general, lactate and acetate are produced predominantly, followed by propionate production<sup>34</sup>. Test group A1 showed higher propionate production with 61.66  $\mu\text{g/g}$  of cecum followed by test groups A2 (36.50  $\mu\text{g/g}$ ), A3 (32.08  $\mu\text{g/g}$ ), NC (25.72  $\mu\text{g/g}$ ) and STD (13.23  $\mu\text{g/g}$ ). Due to the impact of antibiotic mixture and severe diarrhea, the lowest propionate production (8.68  $\mu\text{g/g}$ ) was detected in test group DC. Lactic acid is the major end product of lactate-producing lactobacilli<sup>35</sup>, and our results showed that the production of organic acids was dominated by lactic acid. Thus, the high production of lactic acid could indicate the possible antimicrobial capability on pathogenic microorganisms.

Short chain fatty acids (SCFAs) have emerged as key regulators of gut homeostasis for colonization resistance against enteric pathogens<sup>36</sup>. SCFAs including lactate, acetate and propionate are produced as a result of bacterial fermentation in the cecum<sup>37</sup>. Lactate and acetate are considered as the predominant SCFA present in the cecum, followed by butyrate and propionate<sup>38</sup>. Above findings in the cecal concentrations of SCFA might be associated to some extent with blood markers of the lipids profile in the rats. SCFA absorption in the cecum and the colon is a

process involving both passive diffusion and carrier-mediated transport through sodium dependent monocarboxylate transport (SMCT) and proton-coupled monocarboxylate transporters (MCT)<sup>39</sup>. Reportedly, Hino *et al.*<sup>40</sup> also detected acetate and propionate and butyrate from rat cecum and interpreted absorption of each of the major SCFA was increased in proportion to the rise in the level of both luminal SCFA concentrations and in volume/weight of cecum through fermentation. It was also reported that oral administration of cephalosporin antibiotic cefoperazone induced cecum microbiota disturbance in mice models 6 weeks after antibiotic withdrawal<sup>41</sup>. Metabolic disturbance of the colonic microbiota was accompanied by reduced antioxidant enzymes (SOD and catalase) activity with simultaneous rise in the intensity of lipid peroxidation (MDA level) in colonic mucosa after ceftriaxone administration. Thus, antibiotics can lead to long-term oxidative disturbance in the colonic mucosa of rats<sup>42</sup> that could be possibly reduced by administration of food products fermented by lactic acid bacteria and yeasts with rich probiotic potentiality.

#### Histopathological analysis

Histological assessment is commonly used in the diagnosis of gastrointestinal diseases<sup>43</sup>. The gut microbiota in the intestinal mucosa serves a crucial role in the development and integrity of the mucosal epithelium<sup>44</sup>. The histopathology study of colonic tissues (Fig. 6), showed rats from the NC test group with normal layers of mucosa, submucosa, muscularis and serous with the absence of any abnormal changes. While DC test group had shown typical changes of diarrhoeal condition like inflammatory cell infiltration in colonic sub mucosa, edema in colonic sub mucosa

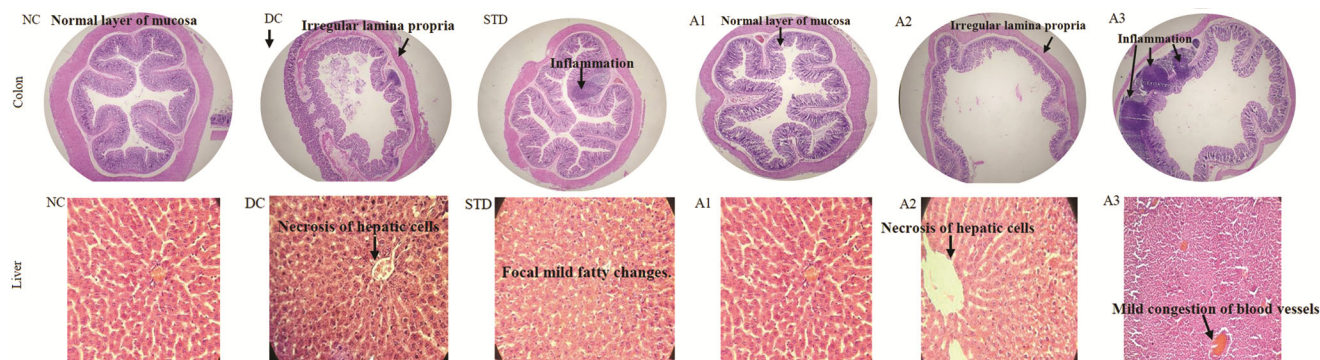


Fig. 6 — Histological sections of colon and liver tissues from different AAD test groups of Wistar rats. [Test groups: NC, untreated animals; DC, administered with antibiotic mixture; STD, administered with loperamide; A1, administered with fermented rice beverage; A2, administered with the combination of *Lactiplantibacillus plantarum* KGL3A and *L. fermentum* KGL4 cells; and A3, administered with *Saccharomyces cerevisiae* WTS1A cells]

and irregular lamina propria and epithelial damage. STD test group has shown aggregation of inflammatory cell. Test group A1 did not show any abnormal changes and was at par with the results of NC test group that confirms healing efficacy against AAD. Test group A2 showed irregular lamina propria and edema in submucosa part of GIT. Test group A3 resulted with typical abnormal changes with similar characteristics from the test group DC with inflammatory cell infiltration in the regions of colonic sub mucosa. Reports from previous study stated the investigation of histological sections of intestinal tissue from diarrhea mice revealing damaged surface epithelium with inflammatory infiltrates in the lamina propria<sup>45</sup>.

The histology investigations of the liver sections (Fig. 6) from each of the test group was analyzed. NC test group did not reveal any specific lesions. Swollen hepatic cells with granular cytoplasm were seen in the liver sections of DC test group indicating parenchymatous degeneration. STD test group revealed focal mild fatty changes. A1 test group did not reveal any specific lesions and was at par with the NC test group. Test group A2 exhibited necrosis of hepatic cells at periphery of hepatic lobules and congestion of blood vessels. The liver sections from test group A3 revealed mild congestion of blood vessels and mild degenerative changes. The results of test group DC, A2, A3 and STD showed the typical signs of infectious diarrhea<sup>46</sup>. The signs of similarity between test group A1 and NC shows that the treatment with fermented rice beverage (test group A1) may contribute in the maintenance of the structure of the rat mucosal microbiota.

### Conclusion

The fermented rice beverage fed to test group A1 could contribute to the maintenance of the structure of the rat mucosal microbiota compared to the normal group and disease control group. From the test groups in the study, NC and A1 was least affected by AAD induction with a fecal consistency score of 1 on the final day of the study. After day 10, a significant reduction in the fecal water content was observed in A1, A2 and STD till day 15. Slight decrease in weight was observed in rest of the test groups after 15<sup>th</sup> day of the study. During the microbial analysis of the fecal samples from 10<sup>th</sup> day onwards, increase in lactobacilli and yeast cell count were found in test group A1. Also, test group A1 followed by STD were

considered to be better in terms of RBC content than the rest of the groups. A1 showed increased levels of haemoglobin content and PCV% which was nearest to the NC test group. Apart from these, test group A1 had higher Mean cell/corpuscular hemoglobin concentration (MCHC) and Mean cell/corpuscular volume (MCV) value; and also highest lactate, acetate and butyrate production in the rat cecum. Results from the histopathological analysis (colon and liver), hematological analysis and short chain fatty acids production from the rat cecal content proves that test group A1 fed with fermented rice beverage to be an efficient product in inhibition of antibiotic associated diarrhea. With more exploration on clinical subjects, the fermented rice beverage made with well-characterized lactobacilli and yeast strains could be considered as a potential for mitigating antibiotic associated diarrhea.

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### Conflict of Interest

Authors declare no competing interests.

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