

Research Article

Fate of heavy metals in Swarna rice after traditional cooking and submerged fermentation linked to bacterial interactions

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ABSTRACT

This research aimed to assess the concentrations of As, Cd, Cr, Pb, and Zn in Swarna rice (NR) collected from Kaharole, Biral, and Sadar Upazilla of Dinajpur and their reduction by cooking associated submerged fermentation. The levels of As, Cd, Cr, and Zn were lower than the individual metal's safe limit, but the (Pb) concentrations were found at 0.2582, 0.9028, 0.9164, 0.7303, 0.8574, 0.6440, 0.6622 mg/kg in SR02, SR04, SR05, SR06, SR07, SR08, and SR09 of NR samples, respectively, which were higher than the (Pb) safe limit. Notably, the reduced hazard index (HI) was found from the standard limit 1 for all fermented rice (FR) samples and all boiled rice (BR) samples except SR05. Six different *Streptococcus* sp. strains were identified in FR that suggested *Streptococcal* involvement in leaching of HMs during submerged-fermentation. Consequently, compared to NR, hazard index and cumulative carcinogenic risk were declined by several folds in FR.

Introduction

Swarna rice variety is locally cultivated in approximately 46% of the land of Dinajpur in the Aman season (August to November) (Hossain et al., 2013). According to the Bangladesh Bureau of Statistics (BBS), in 2011, Bangladeshi people consumed an average of 416 gm of rice daily (BBS, 2011). Though life expectancy is gradually improving the percentage of people with non-communicable diseases increases yearly. According to the International Diabetes Federation (IDF) report in 2017, 6,926,300 diabetic patients were identified in Bangladesh, of which about 6.9% were adults (IDF, 2017). Over the last decade, the number of diabetic patients has been increasing in the rural areas of Dinajpur, even though they do not carry proper risk factors such as obesity, smoking, genetics, and other factors. Therefore, it is a surprising and common question how and why

diabetes is more frequent in this area? Recently, it has been reported that heavy metals (HMs) are involved in the risk factor of type-2 diabetes (Zheng et al., 2018).

Heavy metals (HMs) are accumulated in the human body through drinking water, dermal contact, HMs-contaminated foods, and exposure to dust particles (Zheng et al., 2018). In this regard, cooking rice with a large volume of water (6:1; water: rice) has been documented to reduce inorganic As compared to the general cooking of rice (Raab et al., 2009). It is reported that the rinse cooking of rice with a large volume of water reduced HMs concentrations (Sharafia et al., 2019), but Kathe cooking does not need steaming of rice. Rinse cooking was practiced for a couple of decades, which has changed to Kathe cooking due to technological development. In the last two decades, the mass media have suggested using

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the optimum doses of fertilizers and pesticides in the rice fields. Unfortunately, it is yet to be practically executed by the farmers. Consequently, HMs and toxic chemicals are gradually accumulating in the rice. Additionally, local people are ignorant about the rinse cooking of rice.

Panta-Bhat is semi-fermented boiled rice immersed in water overnight (about 12-15 h). It is generally softened due to partial fermentation, which is served to eat in the morning. Although HMs associated with hazardous risks of rice in America, India, Pakistan, Thailand, and China have been reported (Shraim, 2014; Roya and Ali, 2017; Morekian et al., 2013; Naseri et al., 2015), a little research has been conducted on it in some rice cultivars in Bangladesh (Proshad et al., 2019). Until now, HMs-associated hazardous risk in Bangladeshi Swarna rice (Normal rice, NR) has not been studied. Assessing the health risk of highly consumed rice, especially Swarna rice, is imperative exploring a cost-effective cooking approach to reduce HMs from rice is also essential. The investigation of the concentration levels of other HMs in rice grown in the northern part of Bangladesh is highly demanded. The toxicity levels of HMs in cooked rice and HMs abundance in fermented rice are still to be explored.

The study aimed to screen the HMs associated health risk in Swarna rice and to explore a strategy for reducing HMs from rice. First, this study reported the reduction strategy of HMs by rinsed associated Panta-Bhat (submerged fermented rice) preparation, which provides valuable information to rice consumer to be free of hazards from rice.

Materials and Methods

Rice sample collection and preparation

The rice sample was collected during the Aman season (November-January, 2017) from Sundarpur, Poyesh, Dhonigram at Kaharol (25° 47' 4.942" N, 88° 35' 46.593" E), Tegra, Kajihat, Kanchan at Biral (25° 37' 48.139" N, 88° 33' 20.75" E, and Gabura, Kawgaon, Pulhat at Sadar (25° 36' 36.814"

N, 88° 38' 59.403" E) Upazila's in Dinajpur, Bangladesh. Briefly, 250 gm of rice from each nine samples were sun-dried for 24 h and then de-husked manually. The de-husked rice grains were boiled (rice: distilled water; 1:10) in a water-containing steel saucepan set on an electric cooker. The temperature of the electric cooker was fixed at 160 °C, and cooking was continued for 45 min. To flash out of HMs from Swarna rice, grains were stirred smoothly by the wood spoon every 5 min so that HMs could separate and leach out properly. One-fourth of the boiled water was drained from the saucepan after 15 min of cooking; subsequently, an equal amount of distilled water was added and boiled again. The aforementioned process was repeated three times during the whole cooking. This boiled rice (BR) was allowed for cooling at room temperature; subsequently, 10 gm of rice was collected and stored in the refrigerator for further analysis. The rest of the BR samples were weighed and transferred to a pot containing normal distilled water. The rice was immersed in water (1:2) for 15 h for fermentation at room temperature. This short-time fermented rice is locally called Panta-Bhat. The water liquor from the Panta-Bhat samples was discarded, and then the FR was collected and stored in the refrigerator.

Flame atomic absorption spectrometry (FAAS)

One gram of rice powder from each group (NR, BR, and FR) was digested using a 15 mL ternary solution (HNO₃/H₂SO₄/ HClO₄, 5:1:1 v/v) at 80 °C to make the solution transparent. As, Cd, Cr, Pb, and Zn in digested solution were quantified using a flame atomic absorption spectrophotometer (FAAS; 240FS, Agilent, Australia). Before quantifying HMs, the FAAS was calibrated using a standard of the specific metals. The wavelength (λ) was set at 193.7, 228.8, 357.9, 283.3, and 213.9 nm for the standard As, Cd, Cr, Pb, and Zn solutions. The Zn, Cr, and As were analyzed using a hollow cathode lamp (HCL). In contrast, Cd and Pb were analyzed using an electrodeless discharge lamp

(EDL) in the flame atomizer AAS (Tchounwou et al., 2003). The blank reagent and metal-specific standards were considered for each sample in a separate batch to verify the accuracy and precision of the digestion procedure. Briefly, 250 μ L of standard solutions of each element were prepared from the stock standard solution (1000 mg/L) in a plastic volumetric flask (25 mL) and made up to the mark with 0.5 N HCL solutions, thus making the intermediate standard solution (10 mg/L). A suitable working solution (control) was prepared for As, Cd, Cr, Pb, Zn as 0.1583, 0.1686, 0.1400, 0.5226, 0.3103 ppm for control 1 and 0.6395, 0.5791, 0.46114, 1.7800, 0.65232 ppm for control 2, respectively and the absorbance of each sample solution was taken.

Statistical analysis

The HMs abundance results were statistically analyzed using variance analysis (ANOVA) and Duncan's multiple range test (DMRT) to determine the significant ($p < 0.05$) differences among the group means.

Estimation of daily intake (EDI)

The estimated daily intake (EDI) of As, Pb, Cd, Cr, and Zn from rice consumption was calculated by using the following equation (Hertzberg, 2000):

$$EDI = EF \times ED \times FIR \times C_m / WAB \times T_a$$

Where, EDI=Estimated daily intake (mg/kg/day) of a specific metal; EF=Exposure frequency (365 days/year); ED= Exposure duration (equivalent to a mean lifetime of the Bangladeshi people, i.e., 72 years); FIR=Ingestion rate (g/person/day) of rice (Based on the HIES, FIR was 367); C_m = Metals concentration (mg/kg, dry weight); and T_a = (EF \times ED) is the mean exposure time that is 72 years lifetime (Bamuwanye et al., 2015).

Incremental lifetime cancer risk (ILCR) and cumulative carcinogenic risk (CCR)

The probability of cancer risk of heavy metals (As, Pb, Cd, Cr, Zn) from rice consumption was estimated

after considering the incremental lifetime cancer risk (ILCR) by using the following equation (Sultana et al., 2017):

$$ILCR = EDI \times CSF$$

Where, EDI represents estimated daily intake (mg/kg/day), and CSF represents cancer slope factor 20. The CSF for As, Pb, Cd, Cr, and Zn is 1.5, 0.0085, 0.38, 0.5, and 0.0 (mg/Kg-day)⁻¹, respectively (OEHHA, 2009). The cumulative carcinogenic risk (CCR) is the sum of ILCR of As, Pb, Cd, Cr, and Zn that was calculated by using the following equation:

$$CCR = ILCR_{Pb} + ILCR_{As} + ILCR_{Cd} + ILCR_{Cr} + ILCR_{Zn}$$

Target hazard quotients (THQ)

The THQ was calculated by the following formula established by the EPA 19:

$$THQ = EF \times FD \times EDI / RfD \times W \times T,$$

Where EF represents the frequency of exposure of a particular HM =365 days/year for rice; FD is the duration of exposure = 72 years, EDI is the estimated daily ingestion (mg/person/day), and RfD is the oral reference dose (mg/Kg/day); W is the average body weight=58 Kg and T is the average exposure time for non-carcinogens (365 days year⁻¹ \times 72). The oral approval doses (RfD) were considered as 0.003, 0.0003, 0.001, 0.004 and 0.3 mg/kg for Cr, As, Cd, Pb, and Zn, respectively.

Isolation and biochemical characterization of lactic acid bacteria

The liquid portion of fermented rice (Panta vhat) was collected to evaluate the metal scavenging bacteria. The liquid portion was serially diluted using a 10-fold dilution method, and 30-50 μ L of diluted sample was spread on the sterilized De Man, Rogosa, and Sharpe agar (MRS) plates. The agar plates were incubated at 30 °C for 48 h in an incubator. The distinct bacterial colonies were picked up from the

MRS agar plates and streaked on MRS agar plates for further purification. A set of purified isolates was stored in 80% glycerol for further analysis. The isolates were biochemically characterized according to the results of oxidase, catalase, methyl red (MR), Voges-Proskauer (VP), triple sugar iron (TSI), citrate utilization (CU), indole, motility indole urease (MIU), and carbohydrates (lactose, maltose, sucrose and dextrose) fermentation tests in aseptic condition (Abdullah-Al-Mamun et al., 2022; Haque et al., 2015a). The activities of the extracellular amylase, pectinase, xylanase, and protease of the isolates were conducted by spreading the bacterial samples with a sterile loop in starch, pectin, xylan, and casein medium that served as the sole carbon source in an aseptic condition as described (Das et al., 2022; Haque et al., 2021). The agar plates were streaked by picking off a loopful colony of 24 h pure culture of the isolates. The culture plates were incubated at 37 °C for 24 h. Finally, the plates (starch, pectin, xylan, casein) were washed with Congo red followed by distilled water, and the hydrolytic zone was observed as described (Das et al., 2022).

Molecular characterization of lactic acid bacteria

The molecular characterization of the isolated bacterial strains was performed based on 16S rRNA gene sequencing analyses. The PCR amplification was carried out in a 200 µL PCR tube containing 25 µL of 2X PCR Master mix solution (Promega Corporation, USA), 27F (5' - AGA GTT TGT TGA TGG CTC AG - 3') as the forward primer (5.0 µL), and 1492R (5' -GGT TAC CTT GTT ACG ACT T- 3') as the reverse primer (5.0 µL), template DNA 2.0 µL, and a nuclease-free water 13.0 µL. The following PCR steps were programmed, visualized, purified, and sequenced for gene amplification, (Haque et al., 2021; Das et al., 2022). The query sequence was used to construct a phylogenetic tree using MEGA 7.0 software with the neighbor-joining method (bootstrap 1000x).

Results and Discussion

Level of heavy metal in normal Swarna rice (NR) grain

The FAAS technique determined the levels of free As, Cd, Cr, Pb, and Zn in NR grain, and the results are presented in Table 1. The analysis of five HMs indicated a significant ($p < 0.05$) difference between nine sample' highest and lowest levels. The accumulations of HMs in rice grain were ranged from (0.0129–0.01866) mg/kg, (0.0037–0.01723) mg/kg, (0.0043–0.2018) mg/kg, (0.01901–0.9164) mg/kg, (0.02893–0.3542) mg/kg for As, Cd, Cr, Pb and Zn, respectively. The levels of As were 1.26 to 1.47-fold higher in SR02, SR04, SR05, SR06, SR07, SR08, and SR09 samples compared to the SR01 sample, whereas no significant ($p < 0.05$) differences were observed in As accumulation among the rice samples of SR04, SR06, SR07, and SR09. In contrast, relatively higher levels of As (0.314 mg/kg) were observed in TajMahal rice samples of India (Roya and Ali, 2017). The levels of As should not be exceeded 0.050 mg/kg for a rice consumer who eats 200 g of rice per day, which is equivalent to an exposure of As in drinking water at 10 µg/L (Zhu et al., 2008). In this study, the rice samples SR04 and SR07 accumulated 0.01866 mg/kg As, representing 0.0093 mg/kg of inorganic As (Heikens, 2006). The highest level of As 0.01866 mg/kg was obtained from the SR06 sample, which was approximately 10.7-fold lower than the safe limit of 0.2 (WHO, FAO) and 8.03-fold lower compared to the safe limit of 0.15 mg/kg (National standards of Iran/China). One of the highest levels of As (0.23 mg/kg) in rice samples of the Southwestern part of Bangladesh has been reported by (Rahman et al., 2010), which is almost 12.3-fold higher than the Swarna rice samples SR04, SR06, and SR07. These results suggested that Swarna rice grown in Dinajpur district contains a lower level of As than in the South-western district of Bangladesh.

Table 1. Average concentrations (mean±SEM) mg/kg of heavy metals present in rice were determined using a flame atomic absorption spectrophotometer (FAAS).

HM (mg/kg)	SR01	SR02	SR03	SR04	SR05	SR06	SR07	SR08	SR09
As	0.0129 ^e ±5.7×10 ⁻⁵	0.0176 ^c ±2.88×10 ⁻⁴	0.0163 ^d ±1.73×10 ⁻⁴	0.0186 ^a ±2.40×10 ⁻⁴	0.0171 ^c ±8.68×10 ⁻⁵	0.0184 ^a ±1.49×10 ⁻⁴	0.0186 ^a ±2.88×10 ⁻⁴	0.0177 ^{bc} ±1.73×10 ⁻⁴	0.0182 ^{ab} ±2.18×10 ⁻⁴
Cd	0.0120 ^e ±2.90×10 ⁻⁴	0.0141 ^b ±2.05×10 ⁻⁴	0.0073 ^d ±1.87×10 ⁻⁴	0.0074 ^d ±1.47×10 ⁻⁴	0.0039 ^e ±8.54×10 ⁻⁵	0.0074 ^d ±1.84×10 ⁻⁴	0.0037 ^e ±2.27×10 ⁻⁴	0.0137 ^b ±3.18×10 ⁻⁴	0.0172 ^a ±2.05×10 ⁻⁴
Cr	0.0045 ^e ±2.51×10 ⁻⁴	0.0580 ^e ±4.04×10 ⁻³	0.0193 ^f ±2.40×10 ⁻⁴	0.0945 ^e ±4.84×10 ⁻⁴	0.0043 ^e ±4.04×10 ⁻⁴	0.2018 ^a ±2.75×10 ⁻³	0.0730 ^d ±2.64×10 ⁻³	0.1696 ^b ±7.22×10 ⁻⁴	0.1675 ^b ±1.72×10 ⁻³
Pb	0.01901 ^e ±6.01×10 ⁻⁵	0.2582 ^e ±7.26×10 ⁻⁴	0.0675 ^f ±7.55×10 ⁻⁴	0.9028 ^a ±9.13×10 ⁻³	0.9164 ^a ±5.69×10 ⁻³	0.7303 ^c ±2.90×10 ⁻³	0.8574 ^b ±1.62×10 ⁻²	0.6440 ^d ±6.38×10 ⁻³	0.6622 ^d ±2.18×10 ⁻³
Zn	0.0451 ^d ±3.46×10 ⁻⁴	0.0806 ^c ±6.12×10 ⁻⁴	0.1119 ^b ±9.93×10 ⁻⁴	0.0716 ^c ±6.08×10 ⁻⁴	0.0289 ^e ±3.93×10 ⁻⁴	0.3542 ^a ±1.14×10 ⁻²	0.0470 ^d ±8.54×10 ⁻⁴	0.0565 ^d ±5.68×10 ⁻⁴	0.0692 ^c ±3.18×10 ⁻⁴

Data were presented as mean values ± standard deviation of three independent experiments. According to Duncan's multiple range test, values with different letters are significantly different ($p \leq 0.05$). SR01: Sundarpur (Kaharole); SR02: Poyesh (Kaharole); SR03: Dhonigram (Kaharole); SR04: Teghra (Biral); SR05: Kajihat (Biral); SR06: Kanchan (Biral); SR 07: Gabura (Sadar); SR08: Kawgaon (Sadar); SR09: Pulhat (Sadar).

Table 1 shows the Cd levels ranged from 0.0037 to 0.17233 mg/kg. The difference between the highest level of Cd in SR09 and the lowest level in SR05 was 4.65-fold. Similar Cd concentrations were found for SR02 and SR08 samples that were about 1.2-fold lower than SR09. The differences among Cd concentrations in SR03, SR04, and SR06 samples were insignificant. It has been noticed that the variations of Cd level depend on the type of agricultural soil, application of phosphate fertilizer, and use of groundwater (Jorhem, 2000). This study also showed the variation in Cd levels which did not exceed the current safe limit of 0.1 mg/kg (FAO/WHO 2011; ISIRI, 2010). The Cd level has been screened in rice in some Asian countries, e.g., Thailand, China, and South Korea, and they were 0.150, 0.0345, and 0.021 mg/kg, respectively. But the higher levels of Cd were found in rice of Koshan-2015 (0.64 mg/kg), Mazandaran-2013 (0.0193 mg/kg), and Shiraz-2010 (0.34 mg/kg) in Iran (Jafari, 2018).

The concentration of Cr in rice samples SR01, SR02, SR03, SR04, SR05, and SR06 were significantly varied except for SR08 and SR09. Rice samples' Cr levels were screened between 0.0045-0.20183 mg/kg. The lowest content of Cr was found in SR01, almost 44.8-fold and 37.5-fold lower than that of samples SR06, and SR08, respectively. In a related study, the Cr levels were determined to be 0.24 mg/kg in Mazandaran-2013 and 0.39 mg/kg in Shiraz-2010 Iranian rice cultivars (Jafari, 2018). In addition, Indian rice contains 0.184 mg/kg Cr, which is lower than the minimum concentration limit (MCL) of 0.2 mg/kg 10. However, the levels of Cr in SR01, SR02, SR03, SR04, SR05, and SR07 Swarna rice samples were lower than in the MCL.

The maximum and minimum concentrations of Pb were statistically significant ($p < 0.05$) (Table 1). The highest level of Pb was determined in sample SR05, while the lowest was observed in sample SR01. No significant ($p < 0.05$) difference has been found in Pb accumulation between the samples SR08 and SR09. The Pb level of samples SR01 and SR03 was less than the safe limit (0.2 mg/kg), but the remaining samples SR02, SR04, SR05, SR06, SR07, SR08, and SR09 crossed the Pb safe limit. Therefore, these results revealed that most of the Swarna rice samples exceeded the maximum contaminant level of Pb. The Pb is highly accumulated in rice, and its accumulation level

depends explicitly on the plants' cultivar and ecological and regional distribution. For example, the concentration of Pb (0.64 mg/kg) in some Iranian rice was higher than that of MCLs 13, and similar results have been obtained in (Shraim, 2014) imported rice varieties (Roya and Ali, 2017). In contrast, a moderate level of Pb (0.254 mg/kg) was observed in the Indian rice sample that was higher than the maximum allowable level (Morekian, 2013).

The highest Zn level was obtained in sample SR06, whereas the lowest was in SR05. In Table 1, no significant difference was observed in Zn accumulation among the samples SR02, SR04, and SR09, and between the samples SR07 and SR08. In contrast, a significant difference has been found between the SR01 and other samples. Several reports have provided comparisons regarding the Zn level found in local rice cultivars (Kashian and Fathivand, 2015). In contrast, the highest level of Zn 290 mg/kg and 21.8 mg/kg has been determined in Taiwanese and Thai, respectively (Parengam et al., 2010), higher than the Zn levels in Swarna rice of Bangladesh. In a related study, Proshad et al. (2019) found the higher concentrations of HMs in rice samples cultivated in industrially polluted areas in Tangail district, Bangladesh. Therefore, we assumed that the difference in HMs levels in rice in South-Asian countries could be associated with spatial distribution, application of agrochemicals, and industrial activities.

Table 2. Correlation matrix (Pearson) for heavy metals in Swarna rice grown in Dinajpur, Bangladesh.

The right upper part is a significant level, and the left lower is the correlation coefficient. *Correlation is significant at the 0.05 level (2-tailed)

Table 2. Correlation matrix (Pearson) for heavy metals in Swarna rice grown in Dinajpur, Bangladesh.

	As	Cd	Cr	Pb	Zn
As		0.69	0.081	0.017	0.49
Cd	-0.152		0.32	0.29	0.77
Cr	0.609	0.369		0.25	0.11
Pb	0.759*	-0.39	0.42		0.88
Zn	0.264	-0.112	0.56	0.05	

A correlation (Pearson) of HMs found in Swarna rice was analyzed and presented in Table 2.

The regional distributions, along with HMs influences, were raised by a PCA biplot analysis. In the correlation biplot, the variables very close to each other and located in the same quadrant indicated a strong positive correlation. The F1 and F2 showed a 76.43% variation, in which F1 contributed 28.46% and F2 contributed 47.97% (Fig. 1).

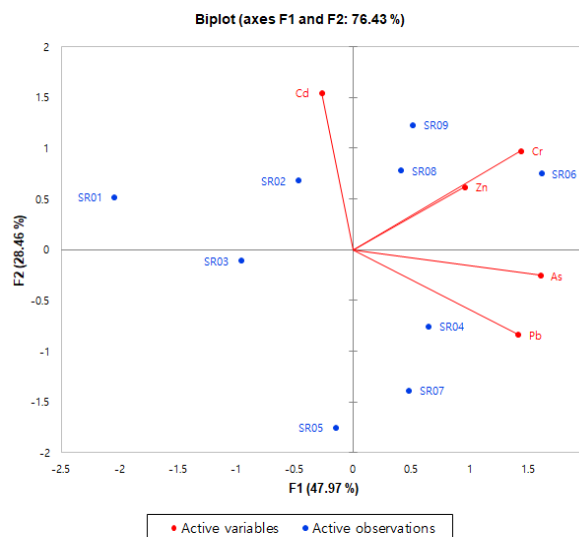


Fig. 1. Principal component analysis of heavy metals in Swarna rice. The biplot show the correlations among the five heavy metals (As, Cd, Cr, Pb, and Zn) and nine studied areas (SR01 to SR09).

A significant $\rho < 0.05$ positive correlation existed between the As and Pb but was negative in the case of Cd. Such spatial correlation among the HMs was not surprising because of its association with the variation of soil pH and organic matters. However, the selected variables with spatial correlations may help divide the zones for different agricultural management.

Leaching of HMs from rice in different processing conditions

A comparative study was performed to determine the HM content in regular Swarna rice (NR), boiled rice (BR), and fermented rice (FR). The As, Cd, Cr, Pb, and Zn levels in three conditions, NR, BR, and FR, were measured and presented in Fig. 3. Five samples (SR01 to SR05) for each condition were considered representative studies. The results dealt with a significant $\rho < 0.05$ reduction of As in samples SR01 to SR05 of BR and FR (Fig. 2A). In particular, compared to NR samples, As

levels were reduced by 7.16-, 2.31-, 6.27-, 4.53-, and 7.43-fold in BR, whereas 11.7-, 7.33-, 9.05-, 5.8-, and 10.0-fold in FR samples. As seen in Fig. 2B, the level of Cd was significantly reduced in BR and FR of SR01, SR02, and SR03 samples. However, the reduction of Cd was more prominent in FR than in BR. The Cr levels declined 3.75-, 4.86-, 6.63-, 1.94-, and 1.05-fold for BR, whereas 5.45-, 7.42-, 9.12-, 2.11-, and 3.54-fold for FR compared to NR. The level of Cr was significantly reduced for FR in the case of all five rice samples, as seen in Fig. 2C. No significant reduction of Cr has been found in samples SR02, SR03, and SR05 for FR and BR. But, the Cr level declined by 0.94-, 1.036-, 6.89-, and 1.95-fold for BR and 1.25-, 17.6-, 8.39-, and 2.7-fold for FR of SR01, SR02, SR03, and SR05 samples, respectively.

In Fig. 2D, the level of Pb was significantly declined in all samples of BR and FR compared to NR. The level of Pb was downturned by 2.97-, 40.3-, 25.0-, 2.14-, 1.12-folds in BR, and 14.6-, 58.61-, 45-, 2.5-, 1.78-folds in FR of the above five samples (SR01 to SR05). Like Pb, an almost similar trend of reduction of Zn level was found in SR01 to SR05 samples (Fig. 2E). The above results clarify that HMs in rice samples were substantially purged by boiling and fermentation.

Several studies have indicated the influence of cooking on HMs concentrations. For instance, As level declined from 3.5 to 6.0% in cooked rice compared to uncooked rice (Zhuang et al., 2016). It is reported that the reduction of inorganic arsenic is positively influenced by the volume of water, amount of rice, and flash out of boiled water during rice cooking (Carey et al., 2015). Most of the current rice cooking systems (electronic-based) are not up to the mark regarding his removal. In this auto-cooking system, a significant amount of total water is absorbed by the rice, and a small amount is evaporated (Sengupta et al., 2006; Torres-escribano et al., 2008). As a result, a marginal amount of HMs is removed from rice. Consequently, the concentrations of HMs did not decline in rice while microwave ovens were used for rice cooking (Zhuang et al., 2016). In contrast, traditional cooking

in South-East Asian countries is more convenient and effective for reducing HMs from rice. In manual cooking, flashing out of excess boiled water after a certain time was one of the key factors for reducing HMs from rice; local people were completely habituated to this cooking system, though they were not well-known concerning the HMs concentration reduction strategies. Several studies reported that inorganic As was reduced in cooked rice compared to raw rice (Torres-escribano et al., 2008; Jane and Shen, 1993), which is agreed with the present study results.

The cooking process led to the discharge of some molecules like amylose, amylopectin, protein, and fat (Yang et al., 2016). Carey et al. (2015) reported that leaching of HMs occurred during cooking rice with a greater volume of water. Therefore, we hypothesized that the free HMs linked with proteins due to the binding nature of HMs, allowing the leaching of HMs during continuous cooking concurrently. The manual draining of excess boiled water from the rice panel's upper layer helps reduce the HMs concentration.

Reduction of HMs by rice fermentation

Long time fermentation of rice leads to the breakdown of the original structure of rice. Therefore, fermentation of rice was performed for 15 h (short duration) at room temperature to maintain the eating suitability and native structure of rice. As seen in Fig. 3, the levels of HMs were significantly reduced after 15 h of fermentation of cooked rice. We tried to understand how HMs was purged during short time fermentation. Though no prior bacterial inoculation and sterile deionized water were used during the cooking and fermentation process, the rice became soft. As a consequence of this observation, including some additional microbial experiments, we identified six different strains of *Streptococcus* from the fermented rice liquor (Table 3). Molecular characterization of HSTU-1 to HSTU-8 bacterial strains was performed by 16S rRNA gene sequencing.

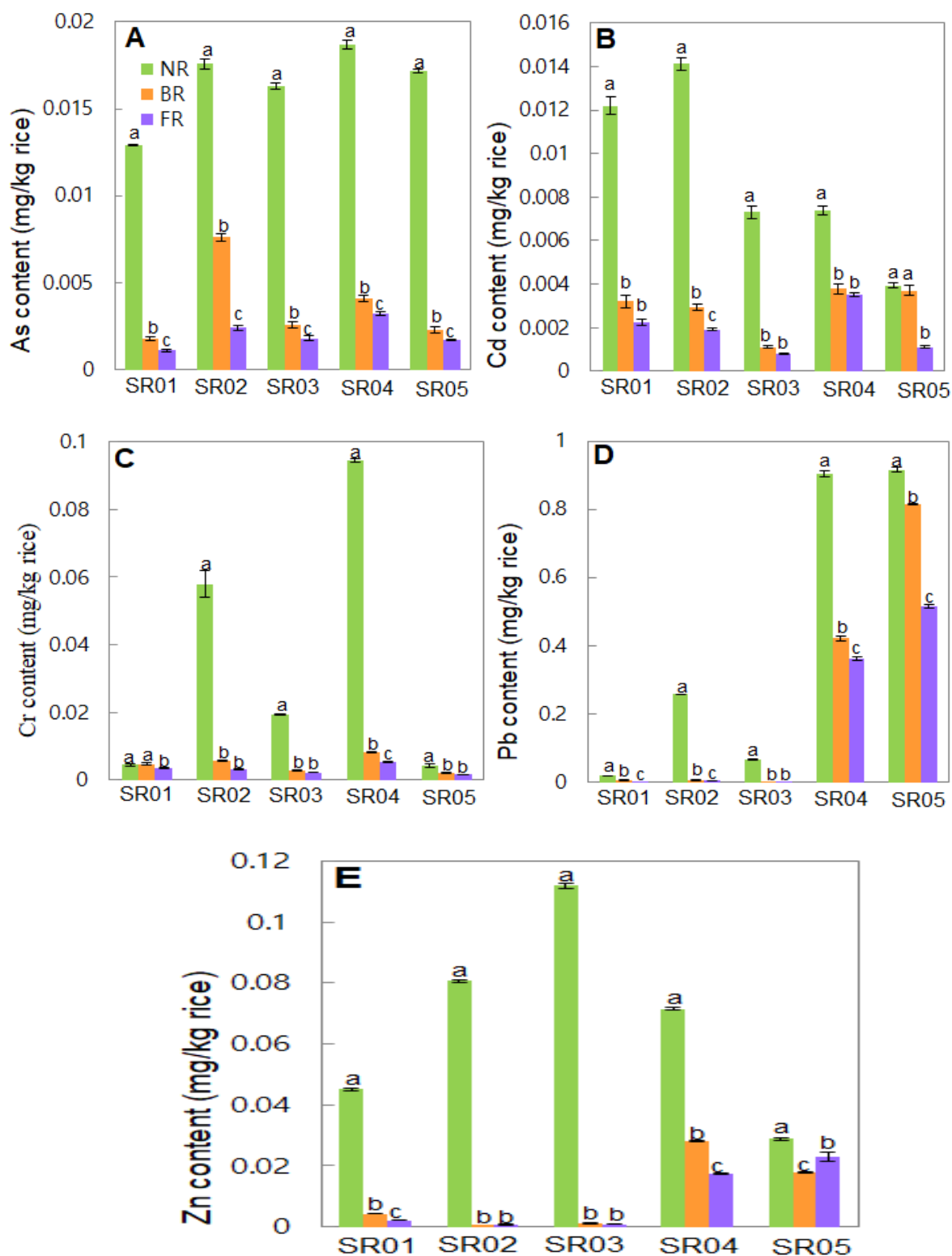


Fig. 2. A comparison of heavy metals in Swarna normal rice (NR), boiled rice (BR), and fermented rice (FR). According to Duncan's multiple range tests, different letters above the error bars indicate a statistically significant difference ($p \leq 0.05$).

Table 3. Biochemical characterization of six LAB isolates from fermented rice liquor.

Biochemical tests	<i>Streptococcus</i> sp. HSTU-1	<i>Streptococcus</i> sp. HSTU-3	<i>Streptococcus</i> sp. HSTU-4	<i>Streptococcus</i> sp. HSTU-6	<i>Streptococcus</i> sp. HSTU-7	<i>Streptococcus</i> sp. HSTU-8
Catalase test	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-
Methyl Red test	+	-	+	+	+	+
Voges-Proskauer (VP) test	-	-	-	-	-	-
Triple sugar iron test	+	+	+	+	+	+
Citrate utilization test	-	-	-	-	-	-
Motility indole urease test	+	+	+	+	+	+
Mortality	+	+	+	+	+	+
Indole test	-	-	-	-	-	-
Fermentation of maltose	-	+	+	-	-	-
Fermentation of sucrose	-	+	-	-	+	-
Fermentation of lactose	-	-	-	-	+	+
Fermentation of dextrose	+	-	-	-	-	-
Cellulase	-	-	-	-	-	-
Xylanase	+	+	+	+	+	+
Amylase	-	+	+	-	-	+
Pectinase	-	-	-	-	-	-
Protease	+	+	-	+	-	+

All strains showed xylanase activities but not cellulase and pectinase (Table 3). HSTU-3 and HSTU-4 showed amylase activity, and HSTU-1, HSTU-6, and HSTU-8 showed protease activity. The PCR bands of the 16S rRNA gene showed approximately 1.4 kb sizes in the agarose gel electrophoresis (Fig. 3A). Moreover, the DNA sequencing results of the 16S rRNA gene after nucleotide BLAST homology analysis revealed that the

strains were 99% related to *Streptococcus infantarius* and other species. Therefore, based on phylogeny, the isolates were named *Streptococcus* sp. strain HSTU-1 to *Streptococcus* sp. strain HSTU-8. The strains were different from each other according to their alignment results. The phylogenetic tree construction revealed that the strains belonged to lactic acid bacteria and especially were closer to the *Lactococcus* sp. (Fig. 3B).

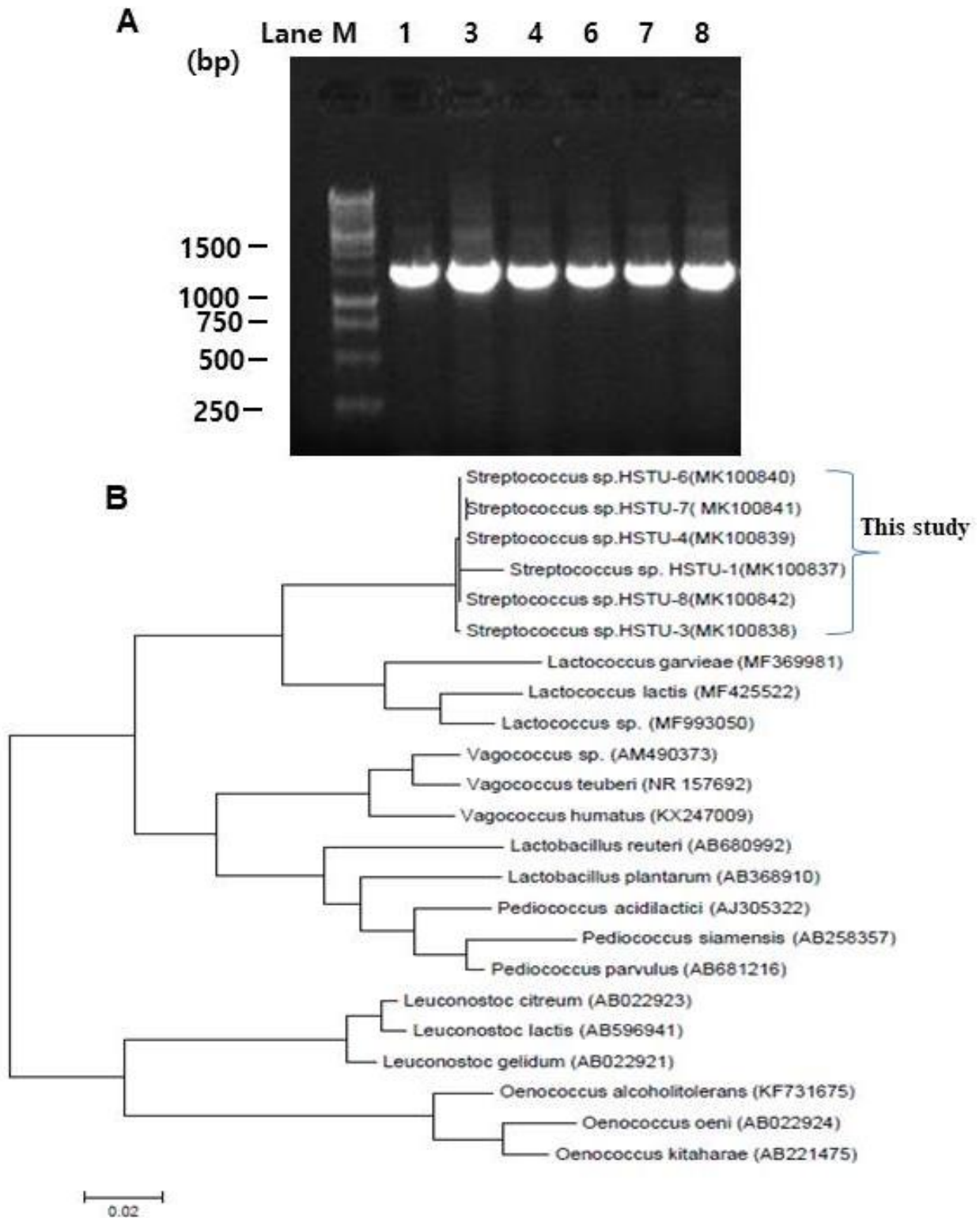


Fig. 3. Molecular identification of lactic acid bacteria (LAB) growing in fermented rice (FR). A) Polymerase chain reaction product bands of LAB isolates from the liquor of FR, B) Phylogenetic tree was constructed using a software MEGA8 using the neighbor-joining method.

The strains HSTU-3 and HSTU-7 were involved in sucrose fermentation, while the rest were not. In addition, the other sugars were randomly fermented by the strains. However, all six *Streptococcus sp.* together secrete xylanase, protease, and amylase enzymes (Table 3), which suggests that the rice was further softened and de-agglomerated during fermentation due to the unwinding of the helical structure of starch by the action of those enzymes. As a consequence, the HMs were leached out into the water. In addition, the *Streptococcus sp.* was capable of fermenting maltose, sucrose, lactose, and dextrose (Table 3), which suggested that the sugar residues were perfectly metabolized during fermentation. A group of Lactic acid bacteria, including *viridescens* MYU205, *L. Plantarum*, and *L. fermentum* ME3, is known to be involved in the removal of Pb and Cd (Haltunnen et al., 2007; Kinoshita et al., 2013). Moreover, a combined application of *L. Plantarum* and *P. pantosaceus* strains (2:1 v/v) ratio was used in the highly Cd impregnated (0.647 mg/kg) rice powder for the fermentation to reduce the level of Cd (Fu et al., 2015). Therefore, the above findings indicated that the generation of *Streptococcus sp.* HSTU strains were involved in rice fermentation along with reducing HMs from rice.

Target hazard quotient (THQ) and hazard index (HI)

The HM-specific THQ and the total THQ of five HMs were presented as HI of a particular rice sample (Fig. 5). The HI is an indicator that helps determine the potential exposure of HMs in our studied samples. The Pb level indicated its highest exposure, followed by As and Cr in SR04, SR05, SR06, SR07, SR08, and SR09 samples compared to all other metals (Fig. 4A). The HI level indicated that rice samples of Kaharole (SR01, SR02, and SR03) were free from hazardous risk, but the rice samples of Sadar (SR07, SR08, and SR09) and Biral (SR04, SR05, and SR06) were not free from risk of hazards. In this regard, we reduced the potential health risk by boiling and fermentation. Consequently, BR and FR samples SR01 to SR05 were provided a lower value of HI than the NR samples (Fig. 4B). In particular, the four samples (SR01 to SR04) showed HI lowers than 1, while the SR05 sample was greater than 1. It is important that the samples SR01,

SR02, and SR03 showed HI less than 0.2. The lower value of HI was found in both BR and FR samples, but a noticeable reduction (HI<0.1) was observed in samples FR samples (Fig. 4C).

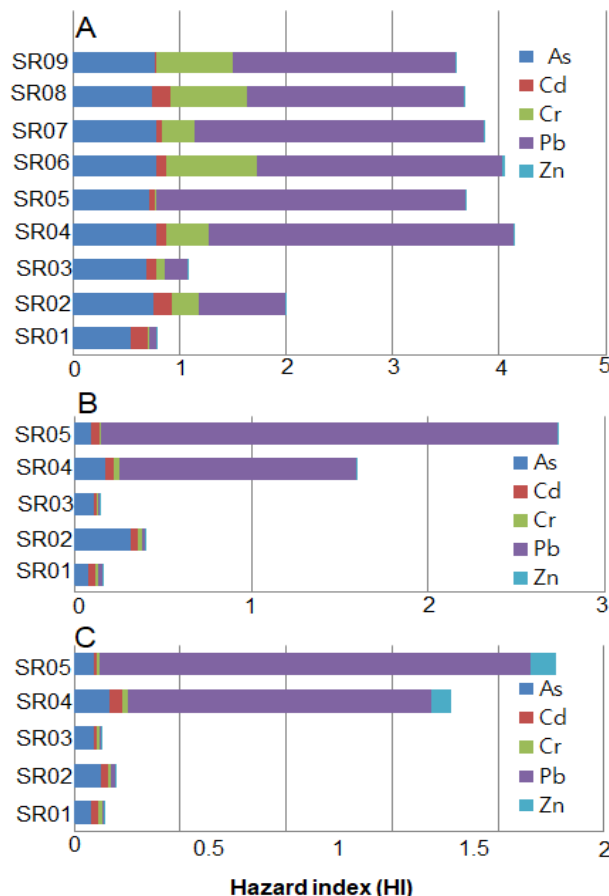


Fig. 4. The hazard index (HI) of Swarna rice samples of Dinajpur, Bangladesh. The numerically presented on each figure's left side indicates the sample number. The HI of the Swarna normal rice (A), boiled rice (B), and fermented rice (FR), respectively.

It could have happened due to the step-wise reduction of HMs during fermentation that caused the reduction of non-carcinogenic risk (HI) reduction in all five FR samples. A comparative heat map was constructed using the THQ results of rice samples. Heat map analysis revealed that the highest THQ was obtained from As, followed by Pb Cr, Cd, and Zn in NR samples (Fig. 5). The least THQ was observed for the Zn in almost all NR samples.

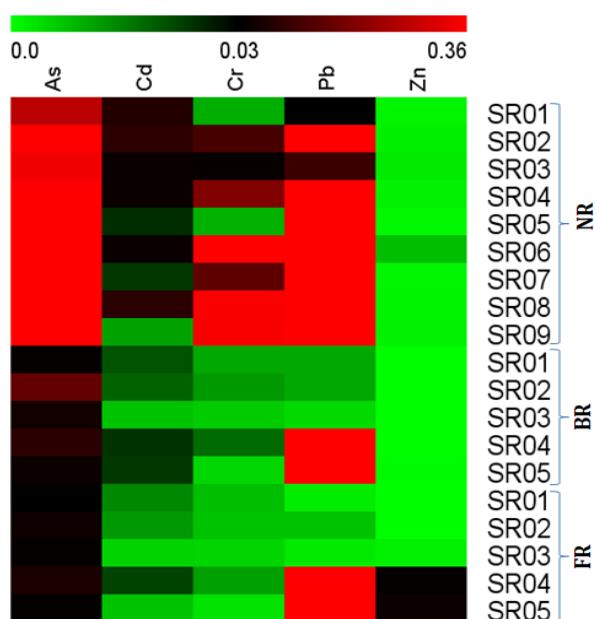


Fig. 5. Target hazard quotient (THQ) of heavy metal in rice, presented by hierarchical clustering analysis. The color ranges from green to red, indicating for the lowest to the highest heavy metal toxicity. Each row of colored boxes is representative of a single sample, and each metal is represented using a single column (indicated).

However, a considerable reduction in THQ level was observed for all HMs in BR samples, followed by FR. Although the Pb concentration was reduced in BR and FR, the highest THQ value was observed in heat map analysis due to the higher concentration of Pb compared to the other HMs. This result suggests that people of the Biral and Sadar area should return to their traditional rice-cooking system, which may provide better options for reducing HMs from rice. Suppose it is possible to consume boiling rice followed by partial fermentation from three meals per day. In that case, the carcinogenic hazard along with the risk associated with diabetes, pancreatitis, kidney disease, and hypertension might be avoided.

Incremental lifetime cancer risk (ILCR) and cumulative carcinogenic risk (CCR)

The ILCR and CCR for the NR, BR, and FR were presented in Table 4. The As, Cd, Cr, and Pb levels were higher than the safe limit (10^{-4} to 10^{-6}) of

ILCR and CCR for the NR sample recommended by USEPA (2002). In contrast, no carcinogenic risk was observed for Zn because the cancer slope factor (CSF) of Zn was constant at zero. Consequently, we calculated ILCR for BR and FR, where the ILCR values of HMs were declined in samples SR01 to SR05 compared to the NR. However, less ILCR has been observed in most BR and FR samples. The CCR of SR01, SR02, SR03, SR04, and SR05 was declined by 4.1-, 4.13-, 6.54-, 5.6-, 2.88-folds in BR, and 5.34, 10.54-, 9.10-, 7.22-, 4.55-folds in FR, respectively. ILCR reduction was due to the special cooking followed by fermentation of rice. Unfortunately, the over-consumption (367 gm/day) of rice was almost 3.33-fold higher than the Iranian people (Naseri et al., 2015), causing the greater risk levels of CCR in this study.

Conclusion

We have explored a straightforward and consumer-friendly cooking-based fermentation approach for reducing HMs-associated health risks for rice consumption. This study assessed HMs from rice samples in different conditions (NR, BR, and FR) and health risk-associated indices ILCR, CCR, and THQ. The results suggest that the rice grown in Kaharole contains a minor concentration of As, Cd, Cr, and Pb than the rice of Biral and Sadar Dinajpur, Bangladesh. In the case of all NR samples, the levels of As, Cd, Cr, and Zn were under the safe limit except Pb. However, the As, Cd, Cr, and Pb levels were dramatically reduced for BR and FR samples. The HI of the boiled rice followed by partial fermentation was below the safe limit 1. Our study provided the molecular-microbial evidence on rice fermentation and HMs reduction from rice. It also suggests that rice fermentation would be a practical approach for purging HMs from rice. This study hypothesized that HMs associated with hazardous risk could be reduced by:

1. Increasing the traditional rinsed rice cooking process instead of auto-rice cooking.
2. By reducing rice consumption per meal.
3. By motivating people to eat partially fermented rice e.g. Panta-Bhat.

Table 4. Incremental lifetime cancer risk (ILCR) and cumulative carcinogenic risk CCR) for normal, boiled, and fermented rice.**Normal rice**

Metals	SRO1	SRO2	SRO3	SRO4	SRO5	SRO6	SRO7	SRO8	SRO9
As	0.123	0.17	0.15	0.176	0.162	0.17	0.176	0.17	0.17
Cd	0.029	0.034	0.017	0.0178	0.0094	0.01778	0.00889	0.033	0.042
Cr	0.014	0.18	0.06	0.23	0.014	0.64	0.23	0.54	0.53
Pb	0.000462	0.01388	0.00363	0.0485	0.0493	0.0393	0.0462	0.0347	0.0356
Zn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CCR	0.1659540	0.400776	0.23683	0.54188	0.23453	0.87055	0.462554	0.772045	0.77950

Boiled rice

Metals	SRO1	SRO2	SRO3	SRO4	SRO5
As	0.0171	0.072	0.025	0.039	0.022
Cd	0.0077	0.0069	0.0026	0.009	0.0089
Cr	0.0152	0.0177	0.0088	0.026	0.0069
Pb	0.00034	0.00034	0.000145	0.0227	0.0438
Zn	0.0	0.0	0.0	0.0	0.0
CCR	0.040305	0.09713	0.03624	0.09664	0.08142

Fermented rice

As	0.0144	0.0228	0.0171	0.0304	0.016
Cd	0.0053	0.0046	0.0019	0.0084	0.0026
Cr	0.0114	0.01044	0.0073	0.0167	0.0050
Pb	0.000069	0.00024	0.000080	0.0195	0.028
Zn	0.0	0.0	0.0	0.0	0.0
CCR	0.031	0.038	0.026	0.075	0.0515

The level from 10^{-4} to 10^{-6} is acceptable/ permissible limits, higher than this value of ILCR, and CCR is carcinogenic.

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Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

Author's contribution

Haque MA: Conceptualization, fund acquisition, writing and editing, Rahman MA: Writing and editing,

analysis; Ferdousi J: experiments, writing; Halilu A: Metals Experiments and analysis; Rahman B: Microbial experiments.

References

Abdullah-Al-Mamun M, Hossain MS, Debnath GC, Sultana S, Rahman A, Hasan Z, Das SR, Ashik MA, Prodhan MY, Aktar S, Cho KM and Haque MA. Unveiling lignocellulolytic trait of a goat omasum inhabitant *Klebsiella variicola* strain HSTU-AAM51 in light of biochemical and genome analyses. *Braz. J. Microbiol.* 2022; 53(1): 99-130.

- Bamuwanye M, Ogwok P and Tumuhairwe V. Cancer and non-cancer risks associated with heavy metal exposures from street foods: evaluation of roasted meats in an urban setting. *Journal of Environment Pollution and Human Health*. 2015; 3(2): 24-30.
- BBS. Statistical Year Book of Bangladesh-2011. Bangladesh Bureau of Statistics. Statistics and Informatics Division. Ministry of Planning. 2011
- Carey M, Jiujin X, Farias JG and Meharg AA. Rethinking rice preparation for highly efficient removal of inorganic arsenic using percolating cooking water. *PLoS One*. 2015; 10(7): e0131608.
- Das SR, Haque MA, Akbor MA, Abdullah-Al-Mamun M, Debnath GC, Hossain MS, Hasan Z, Rahman A, Islam MA, Hossain MA, Yesmin S, Nahar MNN and Cho KM. Organophosphorus insecticides mineralizing endophytic and rhizospheric soil bacterial consortium influence eggplant growth-promotion. *Arch. Microbiol*. 2022; 204: 199.
- FAO/WHO: Joint FAO/WHO food standards programme, codex committee on contaminations in food (FAO/WHO), 2011, fifth session, Netherlands.
- Fu L, Liao L, Liu Y and Wu W. Optimization of fermentation process of removal of cadmium in rice powder using lactic acid bacteria. *Nongye Gongcheng Xuebao/Transaction of the Chinese Society of Agricultural Engineering*. 2015; 31: 319-326.
- Halttunen T, Salminen S and Tahvonen R. Rapid removal of lead and cadmium from water by specific lactic acid bacteria. *Int. J. Food. Microbiol*. 2007; 114(1) 30-35.
- Haque A, Hwan J and Man K. Endophytic bacterial diversity in Korean kimchi made of Chinese cabbage leaves and their antimicrobial activity against pathogens. *Food Control*. 2015a; 56: 24-33
- Haque A, Man K, Nath D, Keun M and Dae H. A potential cellulose microfibril swelling enzyme isolated from *Bacillus* sp. AY8 enhances cellulose hydrolysis. *Process Biochem*. 2015b; 50: 807-8015.
- Haque MA, Ashik MA, Aktar S, Akter MS, Halilu A, Haque MA, Islam MR, Abdullah-Al-Mamun M, Nahar MNN, Das SR, Das KC, Ahmed I, Manir MS, Islam MK and Shahadat MRB. Rapid deconstruction of cotton, coir, areca, and banana fibers recalcitrant structure using a bacterial consortium with enhanced saccharification. *Waste Biomass Valori*. 2021; 12(7): 4001-4018.
- Heikens A. Arsenic contamination of irrigation water, soil and crops in Bangladesh: Risk implications for sustainable agriculture and food safety in Asia. FAO. 2006.
- Hertzberg, R. Supplementary guidance for conducting health risk assessment of chemical mixtures. ESPEA. 2000.
- Hossain M, Jaim WMH, Alam MS and Rahman AM. Rice biodiversity in Bangladesh: Adoption, diffusion and disappearance of varieties. A statistical report from farm survey in 2005. BRAC Research and Evaluation Division, Dhaka, Bangladesh. 2013.
- ISIRI (Institute of Standards and Industrial Research of Iran: ISIRI 12968. Food and feed-maximum limit of heavy metals (1st edi.), Tehran, Iran, ISIRI Pres, 2010, p.210.
- IDF (International Diabetes Federation: IDF Diabetes Atlas, 8th edition, 2017.
- Jafari A, Kamarehie B, Ghaderpoori M, Khoshnamvand N, and Birjandi M. The concentration data of heavy metals in Iranian grown and imported rice and human health hazard assessment. *Data Br*. 2018;16: 453-459.
- Jane J and Shen JJ. Internal structure of the potato starch granule revealed by chemical gelatinization. *Carbohydr. Res*. 1993; 247: 279-290.
- Jorhem L. Determination of metals in foods by atomic absorption Spectrometry after dry ashing : NMKL collaborative study. *J. AOAC Int*. 2000; 83(5)
- Kashian S and Fathivand AA. Estimated daily intake of Fe, Cu, Ca and Zn through common cereals in Tehran, Iran. *Food Chem*. 2015; 176: 193-196.

- Kinoshita H, Sohma Y, Ohtake F, Ishida M, Kawai Y, Kitazawa H, Saito T and Kimura K. Biosorption of heavy metals by Lactic acid bacteria and identification of mercury binding protein. *Res. Microbiol.* 2013; 164: 701-709.
- Morekian R, Mirlohi M, Azadbakht L and Maracy MR. Heavy metal distribution frequency in Iranian and imported rice varieties marketed in central Iran, Yazd, 2012. *Int. J. Env. Health. Eng.* 2013; 2: 1-5.
- Naseri M, Vazirzadeh A, Kazemi R and Zaheri F. Concentration of some heavy metals in rice types available in Shiraz market and human health risk assesment. *Food Chem.* 2015; 175: 243-248.
- OEHHA, 2009. Air toxics hot spots program technical support document for cancer potencies. Appendix B. Chemical-specific Summaries of the Information Used to Derive Unit Risk and Cancer Potency Values. Updated 2011.
- Parengam M, Judprasong K, Srianujata S and Jittinandana S. Study of nutrients and toxic minerals in rice and legumes by instrumental neutron activation analysis and graphite furnace atomic absorption spectrophotometry. *J. Food Compos. Anal.* 2010; 23: 340-345.
- Proshad R, Kormoker T, Islam MS, and Chandra K (2019). Potential health risk of heavy metals via consumption of rice and vegetables grown in the industrial areas of Bangladesh, *Hum. Ecol. Risk Assess.* 2019; 26, 921-943.
- Raab A, Baskaran C and Meharg AA. Cooking rice in a high water to rice ratio reduces inorganic arsenic content. *J. Environ. Monit.* 2009; 11: 41-44.
- Rahman MS, Abdul M and Miah M. Arsenic concentrations in groundwater, soils, and irrigated rice in Southwestern Bangladesh. *Commun. Soil Sci. Plant Anal.* 2010; 41(16): 37-41.
- Roya AQ and Ali MS. Heavy metals in rice samples on the Torbat- Heidarieh market, Iran. *Food Addit. Contam. Part B Surveill.* 2017; 10: 59-63.
- Sengupta MK, Hossain MA, Mukherjee A, Ahamed S, Das B, Nayak B, Pal A and Chakrabarti D. Arsenic burden of cooked rice: Traditional and modern methods. *Food Chem. Toxicol.* 2006; 44(11): 1823-1829.
- Sharafia K, Yunesiana M, Nodehia RN, Mahvia AH, Pirsahab M and Nazmara S. The reduction of toxic metals of various rice types by different preparation and cooking processes-Human health risk assessment in Tehran households, Iran. *Food Chem.* 2019; 15(280): 294-302.
- Shraim, A. M. Rice is a potential dietary source of not only arsenic but also other toxic elements like lead and chromium. *Arab. J. Chem.* 2014; 10(2), S3434-S3444
- Sultana MS, Rana S, Yamazaki S, Aono T and Yoshida S. Health risk assessment for carcinogenic and non-carcinogenic heavy metal exposures from vegetables and fruits of Bangladesh. *Cogent Environ. Sci.* 2017; 166: 1-17.
- Tchounwou PB, Ayensu WK, Ninashvili N and Sutton D. Environmental exposure to mercury and its toxicopathologic implications for public health. *Environ Toxicol.* 2003; 149-175.
- Torres-escribano S, Leal M, Vélez D, Agroquímica Ide and Csic DA. Total and inorganic arsenic concentrations in rice sold in Spain, effect of cooking, and risk assessments. *Environ. Sci. Technol.* 2008, 3867-3872.
- Yang L, Sun YH, Liu Y, Mao Q, You LX, Hou JM and Ashraf MA. Effects of leached amylose and amylopectin in rice cooking liquid on texture and structure of cooked rice. *Braz. Arch. Biol. Technol.* 2016; 59: e16160504.
- Zheng T, Liu S, Bai Y, Cheng N, Buka S, Yang A, Shi K, Zhang X, Li Y, Xu S, Zhang B and Wise J. Current understanding of the relationship between metal exposures and risk of type 2 diabetes. *Curre. Res. Diabetes & Obes. J.* 2018; 7: 169-171.
- Zhuang P, Zhang C, Li Y, Zou B, Mo H, Wu K, Wu J and Li Z. Assessment of influences of cooking on cadmium and arsenic bioaccessibility in rice, using an in vitro physiologically-based extraction test. *Food Chem.* 2016; 15(213): 206-214.
- Zhu YG, Williams PN and Meharg AA. Exposure to inorganic arsenic from rice: a global health issue? *Environ. Pollut.* 2008; 154(2): 169-171