

Article

Organic Cultivation of Tomato in India with Recycled Slaughterhouse Wastes: Evaluation of Fertilizer and Fruit Safety

Malancha Roy ¹, Rimi Das ¹, Amit Kundu ², Sanmoy Karmakar ², Satadal Das ³, Pradip Kumar Sen ⁴, Anupam Debsarcar ⁵ and Joydeep Mukherjee ^{1,*}

¹ School of Environmental Studies, Jadavpur University, Kolkata 700 032, India; E-Mails: malanchadeymphil@gmail.com (M.R.); rimi.biotech@gmail.com (R.D.)

² Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700 032, India; E-Mails: amitjupcl@gmail.com (A.K.); sanmoykarmakar@gmail.com (S.K.)

³ Peerless Hospital and B K Roy Research Center, Kolkata 700094, India; E-Mail: drsatdas@hotmail.com

⁴ Department of Mathematics, Jadavpur University, Kolkata 700 032, India; E-Mail: pksen@math.jdvu.ac.in

⁵ Department of Civil Engineering, Jadavpur University, Kolkata 700 032, India; E-Mail: anupamju1972@gmail.com

* Author to whom correspondence should be addressed; E-Mail: joydeep_envstu@school.jdvu.ac.in; Tel.: +91-33-2414-6147; Fax: +91-33-2414-6414.

Academic Editor: Stephen R. Smith

Received: 29 June 2015 / Accepted: 6 September 2015 / Published: 16 September 2015

Abstract: Environmental and health safety of recycled slaughterhouse wastes-derived fertilizer and the produce obtained through its application is not well understood. Waste bovine blood and rumen digesta were mixed, cooked and sun-dried to obtain bovine-blood-and-rumen-digesta-mixture (BBRDM, NPK 30.36:1:5.75). $1.26 \pm 0.18 \log \text{CFU mL}^{-1}$ fecal coliforms were recovered in BBRDM. *E. coli* O157:H7, Mycobacteria, *Clostridium* sp., *Salmonella* sp., *Bacillus* sp. and *Brucella* sp. were absent. No re-growth of pathogens was observed after 60 days storage in sealed bags and in the open. However, prions and viruses were not evaluated. Heavy metals (Pb, Cr, Cd, Cu, Zn, As, Ni, Mn) concentrations in BBRDM were within internationally permissible limits. BBRDM was applied for field cultivation of tomato during 2012–2013 and 2013–2014. Lycopene and nitrate contents of BBRDM-grown tomatoes were higher than Diammonium

phosphate (DAP) + potash-grown tomatoes because BBRDM supplied 2.5 times more the amount of nitrogen than DAP (NPK 18:46:0) + potash (NPK 0:0:44). Heavy metals and nitrate/nitrite concentrations in tomatoes were within internationally acceptable limits. BBRDM-grown tomatoes showed no mutagenic activity in the Ames test. Sub-acute toxicity tests on Wistar rats fed with BBRDM-grown tomatoes did not show adverse clinical picture. Thus, no immediate environmental or health risks associated with BBRDM and the tomatoes produced were identified.

Keywords: Slaughterhouse; organic fertilizer; tomato; pathogen; lycopene; heavy metal; nitrate; nitrite; mutagen; sub-acute toxicity

1. Introduction

Disposal of vast amounts of animal wastes is a challenging problem to farmers [1]. On the other hand, land application of organic wastes is being considered as a promising and cost-effective method to confront this challenge. These wastes have been demonstrated to provide plant nutrients and organic matter to the soil required for crop production. However, direct application of untreated organic wastes to agriculture is improper because of their undetermined composition and their potential to harbor pathogens, toxic compounds, weed seeds, heavy metals and foul odours [2]. At the same time, consumer consciousness of the association between foods and health, attitudes towards taste, as well as environmental concerns have triggered an enhanced demand for organically produced foods grown with alternative cultivation practices that limit the use of chemical fertilizers and synthetic pesticides [3,4].

Notwithstanding the proclivity, organic farming remains controversial in its claim for healthier and safer foods. Lester and Saftner (2011) [5] observed that in majority of the reviews on investigations comparing organic and conventional cultivation, the variables common to organic as well as conventional practice during production, harvest, postharvest handling and storage were not applied. Consequentially, precise and meaningful conclusions comparing the nutritional quality of organic and conventional produce could not be attained. Authors [5] paired common production variables like the physical, biological and chemical/nutritional qualities of soils, the sources and quantity of irrigation applied, crop varieties, crop maturities and dates of harvest, pre- and postharvest processing, handling, and/or storage methods, individually as well as jointly, thus allowing greater intelligibility on how inputs distinctive to organic and conventional cultivations affected produce quality. Authors [5] concluded that organic crops tended to have more vitamin C, sugars, phenolics, dry matter and lesser nitrates, moisture as well as protein than conventionally-grown produce. Differences between organic and conventional foods in terms of heavy metals or specific minerals were not obvious. It was also deduced that the production system (organic or conventional) had trivial effect on the major sensory markers of several vegetables [5]. Furthermore, Seufert *et al.* (2012) [6] applied a comprehensive meta-analysis to examine the comparative yield performance of organic and conventional farming systems on a global scale. Overall, organic yields were demonstrated to be typically lower than conventional yields [6]. Moreover, organic fertilization is often alleged to be a high-risk practice.

Pagadala *et al.* (2015) [7] reported that manure amendment enhanced the possibility of the presence of *Salmonella* in the field up to a year following application. Use of dairy manure was also found to increase the risk of spinach contamination with generic *Escherichia coli*. Following a survey of Minnesota farms it was concluded that use of organic fertilizer increased the risk of *E. coli* contamination of the produce. In the United States, *Salmonella* and *E. coli* O157:H7 have been implicated in fresh produce-related outbreaks since 2005. Increased concern about *E. coli* O157:H7 from manures and green composts entering the food chain and its potential presence on vegetable products have been raised [8]. Application of untreated contaminated waste to land may aggravate the environmental spread of *E. coli* O157:H7 [9] and thus application methods of organic wastes play an important role in spread and survival of this pathogen [10]. Concerns were expressed on the microbial quality of the fertilizer products derived from slaughterhouse wastes as they may be contaminated with a large number of microorganisms including bacteria, viruses, prions, fungi, yeasts and their associated toxins [11]. Widespread application of abattoir waste (stomach and rumen contents) to land may introduce *E. coli* O157:H7 to the agricultural environment [12]. Limited information is available on the occurrence of pathogens in abattoir waste, their variations amongst different types of waste, presence in freshly processed, as well as stored waste, and their fate after land application [13]. On another aspect, heavy metal contamination of ecosystems through organic fertilization is also of apprehension. In fact, five of the six most frequently occurring heavy metals in Polish ecosystems were introduced through animal manure and not incorporated by the use of chemical fertilizers [14]. Likewise, presence of nitrates and nitrites in agricultural products have significant influence on human health and is an important indicator of fertilizer quality. Very recent literature [15] affirms that beneficial health effects (such as reduced risk of cardiovascular disease) are attainable by daily consumption of nitrate-rich vegetables. However, enhanced risk of cancer from ingested nitrate and nitrite cannot be ruled out.

In light of these opinions and concerns, it is desired that any new method of organic cultivation should not only aim to achieve yields comparable to conventional cultivation, but also ensure environmental and health safety of the organic fertilizer as well as the produce. In this regard appropriate tests (microbiological and chemical) should be carried out on the fertilizer and crops produced by applying the organic fertilizer. Regardless of a variety of probable human health impacts that could result, there are substantial data gaps that preclude thorough appraisal of human health risks associated with animal wastes [16], thus justifying the need for obtaining microbiological and chemical data.

There are about 3600 legal, licensed slaughterhouses [17] and comparably, abundant informal slaughterhouses in India [18]. Informal abattoirs do not have modern infrastructural facilities, do not follow hygienic practices, and lack organized system of waste disposal [19]. Direct application of untreated slaughterhouse waste is prohibited in the European Union (EU) by legislation (Commission Regulation EU No. 142/2011, dated 25 February 2011) [20] as well as in the US [9]. A variety of processes may be applied for treatment of slaughterhouse-derived wastes, for example, wastewater treatment plants, rendering, incineration, composting and land application [13]. Advanced, capital demanding technologies are impracticable in small rural slaughterhouses of less developed countries due to low volume of wastes generated and absence of infrastructural facilities. A more pragmatic approach would be to apply cheap, simple-technology based processes that would be financially and technically feasible as well as acceptable to the small abattoirs [21]. Accordingly, a cheap recycling

methodology for slaughterhouse wastes was developed where a combination of bovine blood and rumen digesta was converted to a dry powder termed bovine-blood-rumen-digesta-mixture (BBRDM) and applied as an organic fertilizer in pot cultivation of Solanaceous vegetables [22]. Tomato yields obtained with BBRDM and conventional inorganic fertilizers (Diammonium phosphate (DAP) + potash) in the agricultural field were compared. Although the partial factor productivity of DAP + potash was higher than BBRDM, the cost of BBRDM was anticipated to be much lower than DAP + potash as the organic fertilizer was produced through local entrepreneurship from slaughterhouse wastes. Furthermore, application of BBRDM eliminated the environmental cost of treating slaughterhouse effluents. Taking into account equal cost of applying fertilizers, greater yield with BBRDM should implicate higher potential revenue for the farmer compared to his/her earning by applying DAP + potash [23]. However, the safety of the fertilizer and the fruits should be evaluated before large-scale application of the organic fertilizer (BBRDM) is recommended.

Thus, the aim of the current investigation is to assess the safety of the fertilizer and the produce in terms of (1) presence/absence of microbial pathogens and heavy metals in BBRDM (2) presence/absence of heavy metals and nitrate/nitrite in tomatoes cultivated with BBRDM (3) determine the content of lycopene, the major antioxidant in tomatoes grown with BBRDM (4) mutagenic potential and sub-acute toxicity (on rats) of tomatoes produced with BBRDM.

2. Results and Discussion

2.1. Pathogenic Microorganisms in BBRDM

Slaughterhouse blood waste is a rich protein medium. So, mixing blood with other wastes (rumen digesta in this study) may afford beneficial conditions for pathogen growth [9,13]. Initially 5.65 ± 0.38 log CFU mL⁻¹ fecal coliforms were counted before processing of BBRDM. The number decreased to 1.26 ± 0.18 log CFU mL⁻¹ after processing, while 1.16 ± 0.08 log CFU mL⁻¹ and 1.72 ± 0.35 log CFU mL⁻¹ fecal coliforms were recovered after 60 days storage in sealed bags and similar stocking in the open respectively. The following counts of pathogens were recorded in BBRDM before processing (all units log CFU mL⁻¹): *E. coli* O157:H7 1.21 ± 0.35 ; Mycobacteria 1.67 ± 0.42 ; *Clostridium* sp. 1.58 ± 0.45 ; *Salmonella* sp. 1.47 ± 0.55 ; *Bacillus* sp. 2.51 ± 0.37 ; *Brucella* sp. 1.45 ± 0.69 . None of the pathogens were recovered following processing and storage for 60 days in sealed bags as well as in the open. Coliforms and *E. coli* are harmless [11] and not regarded as pathogens [24]. Fecal coliforms may be considered as useful indicators of organic fertilizer hygiene [8]. Furthermore, the number is within the EPA 503 limit (1000 MPN g⁻¹ fecal coliforms) for organic fertilizers [8]. Alike our results, non-pathogenic *E. coli* in mature composts were reported *in situ* where the composting process employed was not carried out for an adequate time period at a high enough temperature [11]. Non-pathogenic *E. coli* strains persist in some soils and animal wastes for extensive time periods [9]. Probably, the supplied heat did not penetrate the BBRDM mass properly to kill all (5.0 to 6.0 log CFU mL⁻¹) coliforms. However, the available heat was sufficient to eliminate the pathogens that were present in much lower numbers compared to fecal coliforms. Pasteurization-like treatment may be a suitable option for lowering pathogen loads in slaughterhouse wastes, so that land application may be possible with minimum

biological risk [13]. A variety of factors affect the thermal destruction rate of pathogens such as growth conditions (strain specificity, logarithmic or stationary growth phase and heat shock); the heating method applied (local temperature variations and open or closed heating systems); the heating medium (nature of proteinaceous matter, its fat, moisture and salt contents and pH of the medium) and finally the recovery conditions applied after heat treatment (pour or spread plate, media composition and aerobic or anaerobic incubation) [25]. Most likely, the possibility of blood waste contamination with *E. coli* O157:H7 is low. Digestive tract contents, however, may occasionally contain high numbers of enteric bacteria, including *E. coli* O157:H7 [13]. *E. coli* O157:H7 is not specifically heat-resistant [13] and heating to 60 °C or above for a short period of time eliminates *E. coli* O157:H7 from organic wastes [13]. Heat treatment probably alters the inherent physical and chemical properties of organic wastes that may cause them to be more or less favorable for re-growth of microbes [13]. Exposure of *E. coli* O157:H7 to high temperature produces stress due to enhanced unfolding/misfolding of molecular bonds leading to a heat-shock response, a defensive cellular response to tackle heat-induced protein damage. Although the usefulness of heat-shock response mechanism in *E. coli* O157:H7 is not fully understood, it influences the effects of heat-treatment on pathogen survival and recovery. *E. coli* O157:H7 cells subjected to heat-shock may show increased thermo-tolerance and virulence, thus emphasizing the importance of re-growth within heat-treated substrates [13]. Brinton *et al.* (2009) [8] quantified microorganisms in point-of-sale composts from 94 non-sludge facilities. One compost sample contained *Salmonella*, 28% presented fecal coliforms exceeding the EPA 503 sludge hygiene limit and 6% had detectable *E. coli* O157:H7. Similar to our observations bulk compost products that were processed in the open had significantly higher fecal coliforms than bagged products [8]. *Salmonella* has a very high probability of proliferation in the environment by animal slurry and may survive from 6 to 47 °C for more than 77 days [26]. *Salmonella* may re-colonize composts if the process did not attain sufficiently high temperatures but will not re-grow if the substrate is efficiently pasteurized (70 °C in 30 min) [26]. *Salmonella* in sludge cannot tolerate more than 5 min exposure at 70 °C and a pasteurization step should act as additional measure for *Salmonella* inactivation from slaughterhouse wastes [11]. In this study, heat treatment of BBRDM was adequate to prevent re-growth of *Salmonella* after 60 days of storage. Most organic fertilizers analyzed in the study of Miller *et al.* (2013) [24] went through some types of heat treatment, such as thermophilic composting, dry-heat and boiling. Therefore, the finished organic fertilizers had very low levels of potential pathogens. The survey of Miller *et al.* (2013) [24] found 28% of organic fertilizers sampled to be positive for *E. coli*. However, none of organic fertilizer samples analyzed by the authors [24] were positive for *Salmonella* and *E. coli* O157:H7. *Mycobacterium bovis* causes bovine tuberculosis, a chronic disease of cattle [11]. *Mycobacterium paratuberculosis*, which occurs worldwide, causes severe chronic enteritis in ruminants. This bacterium is extremely resistant to various environmental conditions [26]. However, Avery *et al.* (2012) [27] reported the inactivation of *Mycobacterium paratuberculosis* at 55–60 °C. Bacteria of the genus *Brucella* are responsible for causing the zoonotic disease brucellosis and humans infected by this organism develop a severe fever. *Brucella* has been reported to be very heat sensitive [11]. Vegetative cells of spore-forming bacteria such as *Bacillus spp.* and *Clostridia* are susceptible to elevated temperatures. However, spores are capable of withstanding thermal inactivation and spore germination may occur subsequently at lower temperatures [27]. In this investigation, however, no re-growth of *Bacillus* and *Clostridia* were

observed after storage of BBRDM for 60 days. In Sweden, slaughterhouse wastes were pasteurized at 70 °C prior to anaerobic digestion and digestates were used in agriculture. Spore-forming pathogens such as *Clostridium* and *Bacillus* were not reported. The sterile end product of alkaline hydrolysis of abattoir wastes may be used as a fertilizer [11]. Therefore, taking into account the results of the present investigation and the reports of previous workers, BBRDM application should not introduce pathogens (those tested) into the land. Enteric viruses harbored by farm animals or wild/domestic birds as well as other wildlife persists in the environment and could possibly enter animal wastes through accidental exposure of soil and or feces. However, viruses do not replicate outside the host cells but bacteria and fungi do [27]. Thus, virus growth should not occur during storage of BBRDM. The spinal cord and, to a lesser extent, the brain are the organs carrying the highest infectivity and designated as specified risk materials (SRM) for prion infection. As the worst-case scenario, de Motes *et al.* (2007) [28] suggested that 0.75 g of CNS per animal could drain through slaughterhouse outflows. BBRDM neither requires CNS tissue for its production nor is it associated with wastewater and therefore, the risk of prion contamination of BBRDM should be minimal. Assessment of the presence of pathogens in BBRDM, a new recycled product derived from slaughterhouse wastes is being undertaken for the first time. Therefore, to make health and environmental claims of absolute safety, additional microbiological assays are required and numerous batches of the BBRDM should be evaluated to account for batch-to-batch variations.

Abattoir wastes are potential reservoirs of bacterial, viral, prion and parasitic pathogens that may bring about disease in humans and animals and may enter the human food chain [29]. In order to be recycled, animal residues must be confirmed to be hygienically safe for both humans as well as animals. Else, new ways of transmission of pathogens between humans and animals would be established [26]. Among the different methods for the disposal of slaughterhouse wastes, concerns exist as to whether anaerobic digestion during the composting processes can inactivate pathogens. In contrast, alkaline hydrolysis is able to inactivate almost all known microorganisms [11]. Another process, rendering, comprises cooking of animal wastes, eliminating the moisture and dividing the materials into protein meals and fat products [29]. The animal tissues are transformed into a protein-rich substance appearing like sand or soil. Rendered products are much safer, more easily stored and less offensive compared to composted wastes. The temperature and duration of the cooking process kills or inactivates commonly known pathogens. Valuable and marketable protein and fat products have been derived from meat production waste, including dead animals through rendering. This process has recycled what would else have been considerable amounts of waste [29]. BBRDM can be considered as a cooked and rendered product. However, as per Commission Regulation (EU) No. 142/2011 dated 25 February 2011 [20], rendering requires pressure sterilization with saturated steam. According to the same regulations, the core temperature of animal byproducts should reach 100 to 133 °C during heating [20]. United States Department of Agriculture (USDA)'s recommended cooking temperature ranges from 71 to 77 °C (160 to 170 °F) for meat products suspected to be contaminated with manure. Temperatures during such treatment of manure or litter could reach 150 °C (300 °F) or higher depending on the process applied [1]. Thus, in consonance with the cited literature, low numbers of microbes were detected in BBRDM as the product was processed at a temperature higher than the inactivation temperatures of most disease-causing germs. However, it should be

considered that the inactivation kinetics of foodborne pathogens is closely linked to the medium in which they reside [25].

2.2. Lycopene Content in Cultivated Tomatoes

Ideally a plot without fertilizer addition should have been included in this field trial. However, such plot was not considered because importance was given to the local cultivation practice. Local farmers have applied chemical fertilizers since past 15–20 years. The intention of this study was to develop an organic fertilizer by utilizing the local abattoir waste as an alternative to the chemical fertilizer. Although this trial seems confounded through application of disparate N levels, several studies on tomato cultivation supplying different levels of N similar to our approach were undertaken as detailed in our recent publication [23]. On the other hand, some investigators compared the efficacy of organic and inorganic fertilizers during pot and field cultivation of tomatoes by applying equivalent levels of N in both systems. Although higher yield of tomato in pots upon application of organic fertilizer was attained, such enhanced yields were not obtained in field studies as described in our recent report [23]. DAP + potash supplied N, P and K while BBRDM provided the three macronutrients as well as a host of other undetermined organic micronutrients. Thus, micronutrient deficiencies may be present in the chemical fertilizer. The treatments are not truly comparable. From Table 1, it is evident that the lycopene contents of BBRDM-grown tomatoes were much higher than inorganic fertilizer-grown tomatoes. The lycopene content was low in the eighth week but increased with the progress of the cultivation. Lycopene values in field-grown tomatoes obtained by different investigators ranged from 36 to 119.4 mg·kg⁻¹ [30]. In the study of Hallmann *et al.* (2012) [31], the conventional farm received more nitrogen (N) than the organic farm. Conventional tomatoes had an appreciably higher content of lycopene compared to organic tomatoes. The increase in the lycopene level is related to increased N fertilization. Nitrogen, the main element which forms acetyl-CoA, in turn plays a pivotal role in the synthesis of carotenoid pigments and the conversion of β -carotene to lycopene. BBRDM supplied more N than DAP + potash (2.5 times) and was thus more effective in enhancing the lycopene content than DAP + potash. This logic is supported by Chassy *et al.* (2006) [4], who reported that organic and conventional fertilizer cultivation systems when provided with equivalent rates of N to vegetable crops showed little difference in the composition of flavonoids between the systems which was again supported by the carbon-nutrient-balance theory. In another study, lycopene content of conventionally grown tomatoes were found to be significantly lower compared to organically cultivated ones in India. However, the level of N fertilization was not reported [32]. In yet another experiment, organic tomatoes were richer in lycopene compared to conventional ones [33]. In conventional production, a synthetic fertilizer (Diamant) was applied every week, while in organic production an organic fertilizer NPK (Agrimartin) was incorporated into the soil, just before sowing. Nitrogen levels, however, were not reported [33]. The fruit lycopene content increased when the supplied N was enhanced in tomatoes grown in pots containing peaty loam soil [34]. Apart from N, the antioxidant level is dependent on genetic as well as environmental factors such as temperature, light, water and nutrient availability. Lycopene production was arrested by extreme sunlight and the most favorable conditions were adequately high temperatures along with sufficient dense foliage to shield the fruit from direct sunlight. The lycopene was probably formed more rapidly in fruits protected by crop foliage [34]. High

foliage density may have shielded tomato fruits from intense solar radiation and high temperatures reached during summer in Tunisia, thus promoting the synthesis of lycopene [30]. Understandably, the BBRDM-fertilized plants, which had more number of leaves and dense foliage (as counted during field studies, average number of leaves of BBRDM grown plants was 27 ± 1.43 and DAP + potash grown plants was 13 ± 1.9 ; according to the Student's *t*-test signifies that the difference of number of leaves between two treatments was highly significant at 1% level), contained more lycopene than the DAP + potash-fertilized ones that had lesser number of leaves and sparse foliage. In recent years efforts are being made to understand relationships between crop management and antioxidant micro constituents of fruits and vegetables as these foods are the principal sources of flavonoids in the Western diet [4]. Lycopene, which comprises about 80%–90% of the total carotenoids of red ripe tomatoes is the most effective antioxidant among all carotenoids through its quenching activity of singlet oxygen and scavenging of peroxy radicals [31,35,36]. Lycopene content is the most sought-after quality desired by the food industry and consumers [30]. The effect of organic fertilizers on the quality of field grown tomatoes has not been adequately studied [30] and therefore this study should be of significance.

Table 1. Lycopene contents of tomatoes grown with BBRDM and DAP + potash. Determination was done thrice in duplicate sets ($n = 6$), taking 10 randomly selected mature tomatoes in 2012–2013 (S¹) and 2013–2014 (S²).

Soil Treatment	Season	Lycopene Content (mg·kg ⁻¹)				
		Week 8	Week 9	Week 10	Week 11	Week 12
BBRDM	S ¹	34.32 ± 4.23 **	44.62 ± 6.35 **	41.65 ± 5.6 **	45.7 ± 2.24 **	44.46 ± 3.56 **
DAP + potash	S ¹	27.14 ± 5.32	24.34 ± 6.85	26.54 ± 5.48	21.32 ± 5.23	24.45 ± 3.34
BBRDM	S ²	32.29 ± 4.4 **	45 ± 6.03 **	41 ± 4.54 **	46 ± 2.23 **	45 ± 3.45 **
DAP + potash	S ²	29.03 ± 4.35	26 ± 4.56	27 ± 5.24	23 ± 4.25	25 ± 4.03

According to the Student's *t*-test ** signifies that the difference between two treatments are highly significant at 1% level and * signifies that the difference between two treatments are significant at 5% level and no star signifies that the difference between two treatments are not significant; error ranges indicate one standard deviation from the mean; S¹: season 1; S²: season.

2.3. Nitrate and Nitrite Content in Tomatoes

The nitrate contents calculated in BBRDM-cultivated tomatoes were 2.97 ± 0.10 mg·kg⁻¹ fresh weight (in season 1) and 2.74 ± 0.09 mg·kg⁻¹ fresh weight (in season 2) and the nitrite concentrations was below the detection limit. The nitrate concentration in DAP + potash-cultivated tomatoes was 0.15 ± 0.02 mg·kg⁻¹ fresh weight (in season 1) and 0.12 ± 0.01 mg·kg⁻¹ fresh weight (in season 2) and the nitrite level was below the detection limit. The nitrate level in BBRDM-cultivated tomatoes was higher than DAP + potash-grown tomatoes. As BBRDM supplied greater amount of N compared to DAP + potash, nitrate uptake in the BBRDM-fertilized fruits occurred to a greater extent in comparison to DAP + potash-fertilized tomatoes, although this concentration was within the maximum allowable limit. Similar to our conclusion, greater soil N availability was the probable reason for the highest fruit (tomato) N concentration found in conventional system compared the organic system [37]. The Scientific Committee on Food (SCF) established an acceptable daily intake (ADI) of

nitrate as $3.7 \text{ mg}\cdot\text{kg}^{-1}$ body weight day^{-1} (equivalent to 222 mg nitrate day^{-1} for a 60 kg adult) that was re-confirmed by the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) in 2002 [38]. The WHO standard for nitrate content in vegetables is $300 \text{ mg}\cdot\text{kg}^{-1}$ of fresh weight [39]. An earlier review [40] maintains that ingested nitrites and nitrates can induce methemoglobinemia in humans, especially in young infants. Additionally, nitrites and nitrates cause mutagenicity, teratogenicity, birth defects, influence insulin-dependent diabetes mellitus, development of thyroid hypertrophy and result in spontaneous abortions as well as respiratory tract infections. A recent review [15], however, asserts that nitrate is a normal constituent of the human diet. It is partially converted to nitrogen monoxide, which decreases blood pressure by inducing vasodilation. Dietary nitrate is also related with beneficial effects observed in gastric ulcer, renal failure and metabolic syndrome patients. Nitrate and nitrite are not carcinogenic *per se*, but following endogenous nitrosation, ingested nitrate and nitrite may increase cancer risk and may possibly be carcinogenic to humans [15]. Association of nitrate/nitrite with bladder cancer [41], renal cell carcinoma [42], esophageal squamous cell carcinoma [43], ovarian cancer [44] and gastric cancer [45] were recorded. On the other hand, no relationship of nitrate/nitrite exposure with colorectal cancer [46], non-Hodgkin lymphoma [47] and prostate cancer [48] was noted. Nitrite was related with the risk of thyroid cancer while nitrate was not [49]. In view of this divided opinion and from a conservative standpoint (as human life is concerned), we prefer that the connection of carcinogenicity with nitrate/nitrite ingestion be considered.

The concentration of nitrates and nitrites in agricultural products is effected by the type of the product, harvest time, application of N-containing fertilizers, irradiation, temperature, growth period and soil physico-chemical properties [50]. Maximum allowable levels of nitrates in the European Union are specified only for spinach and lettuce [38]. The highest average nitrate contents found in tomatoes cultivated in different countries were: Slovenia $4.3 \text{ mg}\cdot\text{kg}^{-1}$, Greece $62 \text{ mg}\cdot\text{kg}^{-1}$, Belgium $36 \text{ mg}\cdot\text{kg}^{-1}$, China $77.9 \text{ mg}\cdot\text{kg}^{-1}$, UK $1.3\text{--}1.6 \text{ mg}\cdot\text{kg}^{-1}$, Denmark $110\text{--}164 \text{ mg}\cdot\text{kg}^{-1}$, France $1\text{--}19 \text{ mg}\cdot\text{kg}^{-1}$ and Italy $10 \text{ mg}\cdot\text{kg}^{-1}$ [50]. The nitrate level in tomatoes grown in Romania ranged between 82.24 to $116.75 \text{ mg}\cdot\text{kg}^{-1}$ and these values were under the safe limit set by Romanian legislation [51]. Nitrate concentrations found in vegetables of Hong Kong averaged $57 \text{ mg}\cdot\text{kg}^{-1}$ and ranged from not detectable to $180 \text{ mg}\cdot\text{kg}^{-1}$ [52]. The content of nitrate in tomatoes cultivated in Turkey was $11.06 \text{ mg}\cdot\text{kg}^{-1}$ and that of nitrite was $0.36 \text{ mg}\cdot\text{kg}^{-1}$ and was of little apprehension [53]. The nitrate concentration in tomatoes ranged between $0.71\text{--}61.17 \text{ mg}\cdot\text{kg}^{-1}$ with an average of $10.89 \text{ mg}\cdot\text{kg}^{-1}$ in fruits collected in the Syrian market. On fresh weight basis, the nitrite levels in tomatoes and their derived products were very low, under $1.0 \text{ mg}\cdot\text{kg}^{-1}$ [54]. Thus, in light of the cited literature, it may be considered that the content of nitrate in tomatoes grown with BBRDM was within the levels recorded in different countries.

2.4. Heavy Metal Content in BBRDM and Cultivated Tomatoes

It is evident from Table 2 that the content of various heavy metals in BBRDM were within maximum permissible limits as prescribed by Indian and international agencies. The concentrations of heavy metals in BBRDM were very low, similar to other organic fertilizers. As an example, the concentrations of heavy metals (Cu, Zn, Mn, Pb, Cd, Cr) in fertilizer derived from the liquid residue

(LR) of lipopeptide biosurfactant production were less than $4 \text{ mg}\cdot\text{kg}^{-1}$ [55]. Authors suggested that the application of LR in field was feasible and safe. It is further apparent from Table 2 that heavy metals content in tomatoes cultivated with BBRDM and DAP + potash were within maximum allowable limits of Indian and international agencies. Tomatoes have been grown with organic fertilizers derived from various sources by previous workers. The fruits so obtained were safe in terms of their heavy metal contents. As examples, 20% municipal sewage sludge mixed with *Eichhornia crassipes* was composted using *Eisenia foetida* worms. The effect of this vermicompost on the growth and yield of two tomato cultivars as well as distribution of heavy metals in different organs of the fruits were studied. Heavy metal concentrations in all organs of the two varieties studied under diverse treatments were lower than the acceptable toxicity levels [56]. Leafy deposits of the seaweed *Posidonia oceanica* are disposed off as wastes and their unusual accumulation in the Sardinian sea beaches, as well as other countries of the Mediterranean, is an environmental problem. The effects of different nutrient media (composted and beached *P. oceanica*) on growth, yield and heavy metals concentrations in hydroponically grown tomatoes were studied in a greenhouse [57]. The use of composted as well as beached *P. oceanica* as fertilizers did not cause any metal accumulation in tomato fruits and plants. The quality of pot-grown tomatoes with field soil as the major medium and municipal solid waste compost as a fertility supplement was examined in another greenhouse experiment [58]. No heavy metal contamination was observed and authors concluded that municipal solid waste compost was a useful soil amendment in a greenhouse-growing medium for tomatoes. The effects of wastewater application on soil properties and tomato cultivation were investigated [59]. Wastewater, with or without inorganic fertilization, enhanced biomass production, distribution of nutrients to aerial plant parts as well as water use efficiency to significant levels without allowing adverse concentrations of heavy metals in tomatoes. The heavy metals (Cu, Zn, Pb and Cd) concentrations were within the allowable values of Indian standards in municipal sewage sludge vermicompost used as an organic fertilizer. Heavy metals in the leaves, roots and fruits of two varieties of tomatoes (Araka Saurabh and Pusa Ruby) were high but within permissible limit [56]. The main source of heavy metals found in Dutch and Polish agro-ecosystems came from the application of organic fertilizers. High contents of some heavy metals in animal feed caused high concentrations of metal pollutants in manure. Research showed that only a small amount of the heavy metals were retained by the animals and about 90% of the Cu and Zn were excreted into manure [14]. This report should be an important consideration when applying BBRDM as this product is prepared by using partially digested rumen contents.

2.5. Mutagenicity and Toxicity Study

Similar numbers of revertant colonies in tomato powder suspension (TPS, derived from BBRDM and DAP + potash-grown tomatoes) were correlated with the blank control indicating that no increasing frequency of mutation was caused by TPS. It is evident from Table 3, that increasing the amounts of TPS, from 0.1 to $0.2 \text{ }\mu\text{g}\cdot\text{plate}^{-1}$, did not show substantial increase in the number of revertants when compared with either of the blank plates. In contrast, the number of revertants was significantly increased by the known mutagen sodium azide. Thus, it was inferred that TPS showed no evidence of mutagenic activity in the tested bacterial system. It should, however, be relevant to consider the limitations of the Ames test. Compounds that are activated in a mammalian cell may not

be active in a bacterial cell as mammalian cell metabolism is different from bacterial cell metabolism. The number of common DNA sequences between mammals and bacteria is trivial. A large number of carcinogens have affinity for specific nucleotide sequences. Thus, many chemicals that cannot produce mutations in bacteria may have severe effect on mammals. Consequentially, the Ames test is of importance as an initial screening test, and its limitations should be realized [60].

Table 2. Heavy metal content of BBRDM (fertilizer) and tomatoes (grown with BBRDM and DAP + potash). Ten fresh tomatoes grown with BBRDM and an equal number cultivated with DAP + potash obtained at the end of the harvest (by combining all five harvests) were considered for this test. Determination was done thrice in duplicate sets ($n = 6$) for each harvest (a). Indian and international standards for heavy metal content in fertilizer and food (b).

Samples		Heavy Metals ($\text{mg}\cdot\text{kg}^{-1}$)							
		Pb	Cr	Cd	Cu	Zn	As	Ni	Mn
BBRDM	S ¹	0.0434	0.045	0.0041	0.2197	0.1659	BDL	0.0206	1.794
	S ²	0.0412	0.039	0.0038	0.1984	0.1725	BDL	0.0198	1.823
Tomato (grown with BBRDM)	S ¹	0.1341	0.216	0.0093	0.3544	2.1629	BDL	0.0503	0.812
	S ²	0.1265	0.198	0.0087	0.2965	2.3865	BDL	0.0498	0.796
Tomato (grown with DAP + potash)	S ¹	0.1256	0.185	0.0084	0.3865	2.4967	BDL	0.0462	0.836
	S ²	0.1149	0.243	0.0088	0.3218	2.0298	BDL	0.0532	0.827
Standards									
Maximum permissible limit as per Government of India, Ministry of Agriculture and Rural Development, The Fertilizer (Control) Order 1985 [61]		100	50	5	300	1000	10	50	NA
Association of American Plant Food Control Officials *		61	NA	10	NA	420	13	250	NA
California Department of Food and Agriculture *		20	NA	4	NA	NA	2	NA	NA
Oregon State Department of Agriculture *		43	NA	7.5	NA	NA	9	175	NA
The Prevention of Food Adulteration Act and Rules as on 1 October 2004, Government of India [62]		1	NA	1.5	100	5	0.2	1.5	NA

Results indicate mean of six independent measurements; error ranges (not shown) were within one standard deviation of the mean; S¹: season 1; S²: season 2; BDL: Below detection limit; NA: Not available; * source: please see reference [63].

Table 3. Dose–response relationship between tomato powder suspension (TPS) and the number of revertants observed in the Ames (bacterial reverse mutation) assay.

Test Substance	Season	Dose ($\mu\text{g}\cdot\text{plate}^{-1}$)	Number of Revertants (TA98)	Dose ($\mu\text{g}\cdot\text{plate}^{-1}$)	Number of Revertants (TA98)
Blank control	S ¹	0	4 ± 2	0	4 ± 2
	S ²	0	5 ± 2	0	4 ± 2
Positive control (sodium azide)	S ¹	0.1	42 ± 10	0.2	68 ± 14
	S ²	0.1	48 ± 10	0.2	72 ± 12
TPS derived applying DAP + potash	S ¹	0.1	4 ± 2	0.2	5 ± 2
	S ²	0.1	5 ± 2	0.2	4 ± 2
TPS derived applying BBRDM	S ¹	0.1	4 ± 2	0.2	4 ± 2
	S ²	0.1	4 ± 2	0.2	5 ± 2

Twenty fresh tomatoes grown with BBRDM and an equal number cultivated with DAP + potash obtained at the end of the harvest (by combining all five harvests) were considered; determination was done thrice in duplicate sets ($n = 6$); S¹: season 1; S²: season 2.

Toxicity testing was not done with DAP + potash-grown tomatoes and not repeated with season 2 tomatoes to prevent unnecessary killing of experimental animals. During the study on sub-acute toxicity, all animals increased in weight and looked normal and lively. No deaths or obvious signs of disease were observed in any group throughout the experimental period. Administration of TPS did not affect body weight gain as shown in Figure 1. Food intake increased in accordance with body weight and such observation was similar amongst all groups (Figure 2). No meaningful differences in food consumption between control rats and animals fed with TPS were noted during the four-week treatment. Absolute and relative organ weights in female as well as male rats were not influenced by the 4-week feeding of TPS as evident from Table 4. Administration of TPS did not cause any major change in the hematological profile. TPS did not cause any appreciable change in blood glucose, blood urea and serum creatinine (Table 5). Systolic blood pressure in animals of all four groups ranged from 115.9 ± 0.91 to 120.6 ± 0.74 . Statistically significant alteration in blood pressure of the test animals were not observed when compared with their self-control values. Vital organs appeared normal after macroscopic examination. Figures 3 and 4 show that there was no change in histological appearance of the stomach, which showed normal mucous, submucous, muscular and serous coats. Renal glomeruli, Bowman's capsule and tubules were absolutely normal. Liver showed normal lobular pattern with normal portal tracts. Cardiac muscles showed normal architectural appearance. There was no evidence of any inflammation, degeneration or necrosis in any of the histological sections studied in the experimental animals.

There is a large body of evidence supporting rats' ability to sense toxicants and essential nutrients in their food and stay away from foods that containing toxic substances. This capacity was applied to judge whether the two major agronomic factors, soil fertility management and crop protection had an effect on the food preferences of laboratory rats [64]. Authors recorded a positive influence of organic fertilization on food choice. In all cases, wheat produced with organic fertilizer and organic crop protection was preferred to the wheat produced conventionally using inorganic fertilizers. Another study [65], reported surplus of tomatoes were abandoned due to their failure to satisfy customer standards. To allow value additions as well as to ensure effective re-use of surplus tomatoes, tomato

vinegar (TV) containing phytochemicals was developed [65] and its anti-obesity effects *in vitro* and *in vivo* was evaluated using Sprague-Dawley rats. Visceral fat and lipid accumulation in adipocyte and obese rats were suppressed by TV, thus preventing obesity. Therefore, our animal experiments supported by the conclusions [64,65] establish that BBRDM-produced tomatoes are absolutely safe for consumption, considering the rat model as a surrogate for the human physiological system. To the best of our knowledge, this study represents the first animal-model food safety evaluation of tomatoes cultivated with an experimental organic fertilizer.

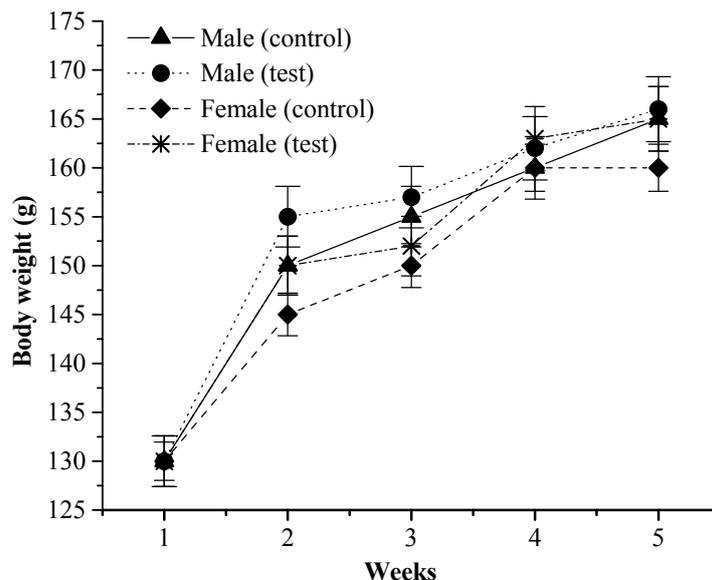


Figure 1. Mean body weight of rats administered with tomato powder suspension (TPS) for 4 weeks. Each point represents mean of six determinations. Error ranges denote one standard deviation from the mean ($n = 6$).

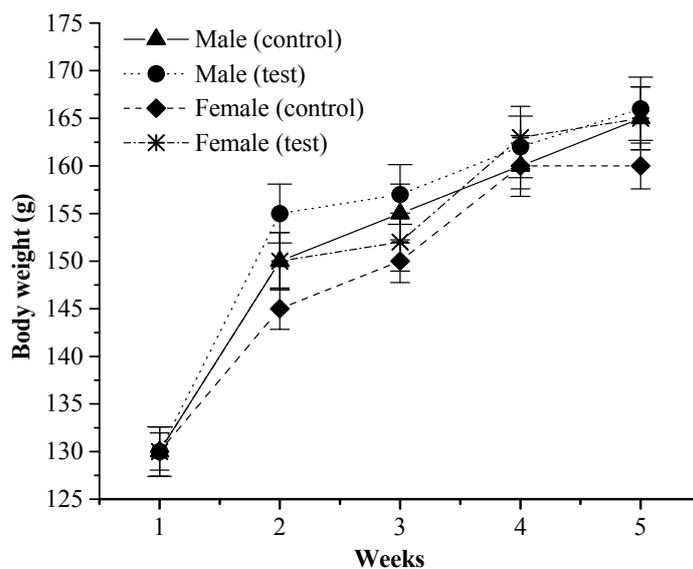


Figure 2. Mean food consumption in rats administered with tomato powder suspension (TPS) for 4 weeks. Each point represents mean of six determinations. Error ranges denote one standard deviation from the mean ($n = 6$).

Table 4. Absolute (g) and relative (%) organ weights of Wistar rats administered with tomato powder suspension for 28 consecutive days.

Organs		Male	Male	Female	Female
		(Control)	(Treated)	(Control)	(Treated)
Liver	A	5.4 ± 0.74	5.8 ± 0.72	5.3 ± 0.69	5.9 ± 0.71
	R	3.06 ± 0.11	3.31 ± 0.09	3.03 ± 0.08	3.37 ± 0.085
Kidney	A	0.7 ± 0.03	0.9 ± 0.02	0.6 ± 0.02	0.8 ± 0.03
	R	0.4 ± 0.01	0.52 ± 0.02	0.34 ± 0.01	0.45 ± 0.02
Heart	A	0.6 ± 0.02	0.8 ± 0.03	0.5 ± 0.04	0.7 ± 0.03
	R	0.34 ± 0.01	0.46 ± 0.02	0.29 ± 0.01	0.4 ± 0.02
Stomach	A	6.2 ± 2.48	7 ± 2.56	6.4 ± 2.3	7.3 ± 1.19
	R	3.54 ± 1.12	4 ± 1.35	3.65 ± 1.26	4.17 ± 1.43

24 male and female rats were used; rats of both sexes were randomly assigned to four groups of the same sex; two control groups and two treatment groups ($n = 6$); error ranges indicate one standard deviation from the mean; A: absolute organ weight (g); R: relative organ weight (%).

Table 5. Effect of oral administration of tomato powder suspension on haematological parameters of Wistar rats fed for 28 consecutive days.

Parameters	Male	Male	Female	Female	Normal
	(Control)	(Treated)	(Control)	(Treated)	Range
Haemoglobin (g·dL ⁻¹)	10.9 ± 0.1	13.5 ± 0.2	12.6 ± 0.2	12.9 ± 0.3	11.5–16.1 ¹
Total erythrocytes (10 ⁶ mm ⁻³)	6.55 ± 0.21	7.02 ± 0.01	7.02 ± 0.01	7.01 ± 0.01	6.76–9.75 ¹
Total leucocytes (10 ³ mm ⁻³)	6.8 ± 0.1	7.1 ± 0.1	7.2 ± 0.1	8.1 ± 0.1	6.6–12.6 ¹
Platelets count (10 ³ mL ⁻¹)	423 ± 3	440 ± 4	444 ± 4	443 ± 3	150–460 ¹
Neutrophils (10 ³ mm ⁻³)	2.1 ± 0.1	3.6 ± 0.1	4.5 ± 0.1	2 ± 0.5	1.77–3.38 ¹
Eosinophils (10 ³ mm ⁻³)	0.04 ± 0.01	0.04 ± 0.1	0.06 ± 0.01	0.06 ± 0.01	0.03–0.08 ¹
Basophils (10 ³ mm ⁻³)	0	0	0	0	0.00–0.03 ¹
Lymphocytes (10 ³ mm ⁻³)	7.5 ± 0.1	7.3 ± 0.1	8.6 ± 0.2	7.7 ± 0.1	4.78–9.12 ¹
Monocytes (10 ³ mm ⁻³)	0.02 ± 0.01	0.01 ± 0.002	0.02 ± 0.002	0.01 ± 0.002	0.01–0.04 ¹
PCV(%)	33.1 ± 0.2	33.1 ± 0.2	32.8 ± 0.2	38.8 ± 0.1	37.6–50.6 ¹
MCV(cu μ)	50.6 ± 0.3	50.6 ± 0.3	45.3 ± 0.1	51.4 ± 0.2	52 ²
MCH(μμ·gm)	16.3 ± 0.2	16.3 ± 0.2	16 ± 2	16.3 ± 0.1	17 ²
MCHC(%)	33.1 ± 0.1	33.1 ± 0.1	32.4 ± 0.2	33.4 ± 0.1	34 ²
Blood glucose (mg·dL ⁻¹)	115 ± 3	98 ± 2	97 ± 3	87 ± 2	50–135 ¹
Blood urea (mg·dL ⁻¹)	36 ± 2	26 ± 2	18 ± 2	29 ± 1	-
Serum creatinine (mg·dL ⁻¹)	0.64 ± 0.02	0.65 ± 0.01	0.71 ± 0.01	0.73 ± 0.01	0.2–0.8 ¹

24 male and female rats were used; rats of both sexes were randomly assigned to four groups of the same sex; two control groups and two treatment groups ($n = 6$); error ranges indicate one standard deviation from the mean¹ reference[66],² reference [67].

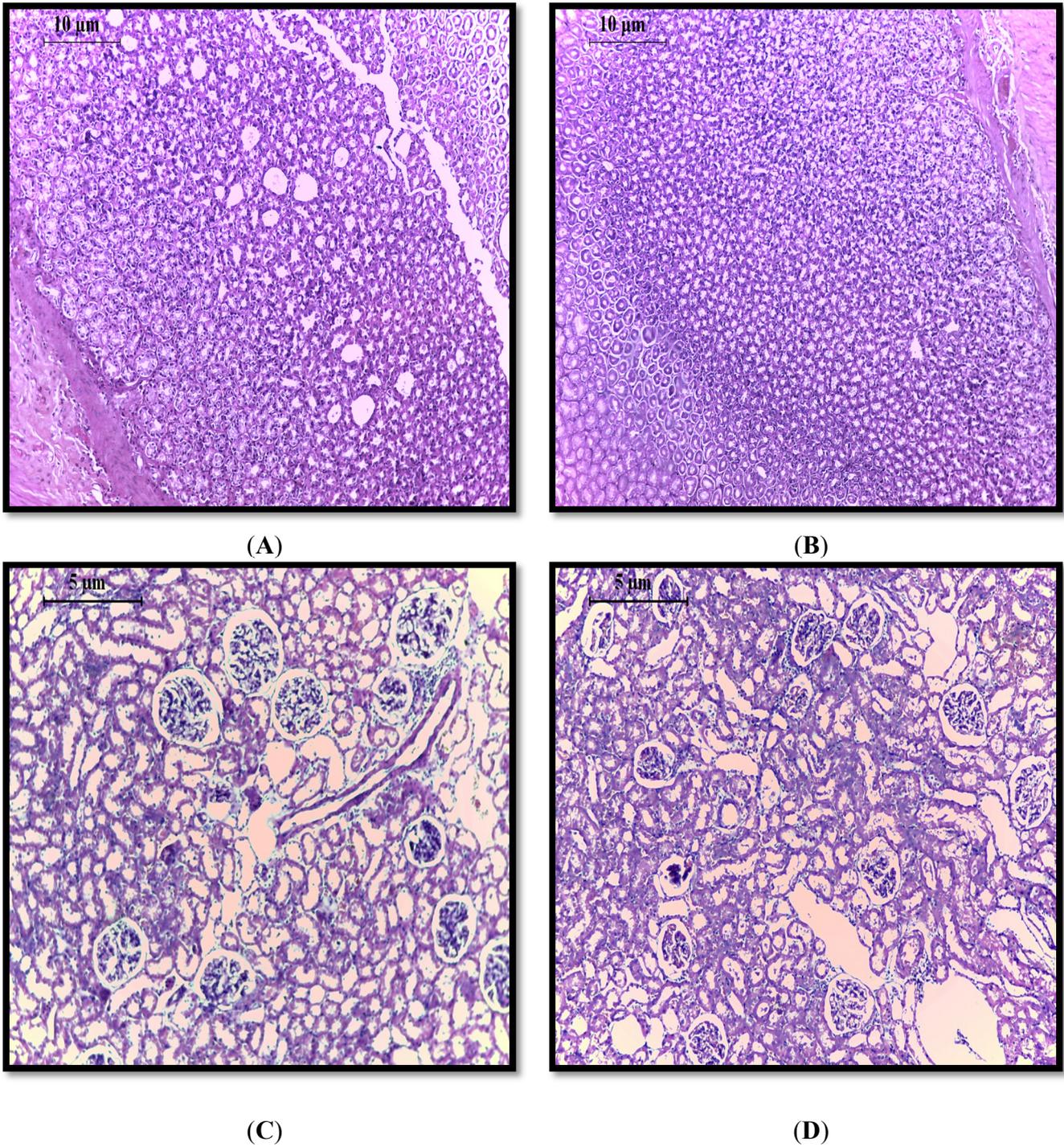


Figure 3. Histological appearance of tissue sections of various organs. (A) section of stomach of test animal; (B) section of stomach of control animal; (C) section of kidney of test animal; (D) section of kidney of control animal.

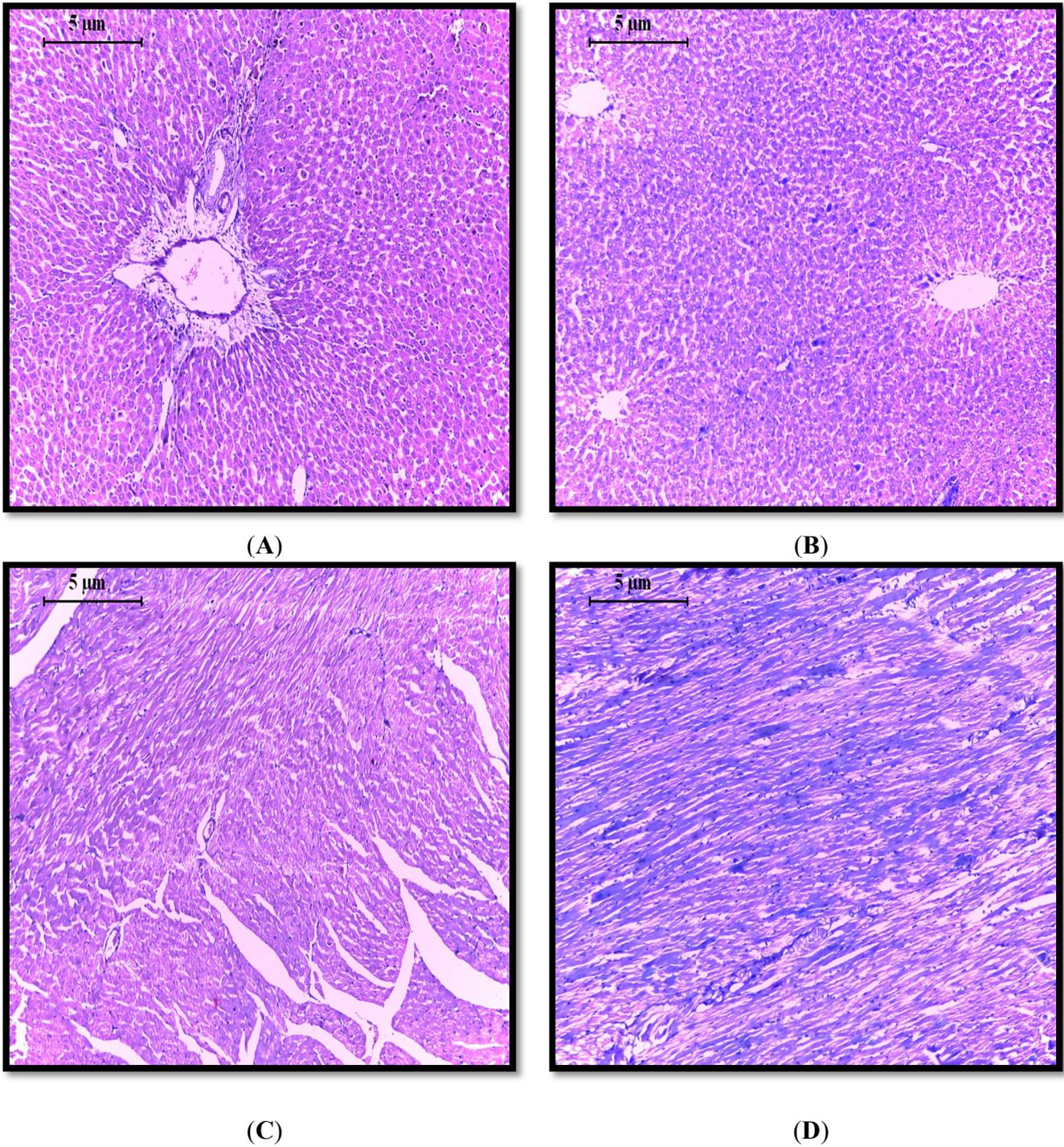


Figure 4. Histological appearance of tissue sections of various organs. (A) section of liver of test animal (B) section of liver of control animal; (C) section of heart of test animal; (D) section of heart of control animal.

3. Experimental Section

3.1. Preparation of BBRDM

Fresh blood was collected in containers immediately following killing of animals. Bovine blood and rumen digesta (ratio 3:1) were weighed and mixed. “G.1.3. INDOCERT Organic Standards for Non-EU Country Operators, Version 2, March 2012” [68] was followed to comply with the requirements of organic production. Therefore, anti-coagulant (sodium citrate) was not used. Mixture of blood and rumen digesta was boiled for 90 min by keeping the metallic container on a coal-fired earthen stove. Blood and rumen digesta mixture was continuously stirred until the mass was almost free of water and sun-dried for 3 days to finally obtain BBRDM (a dry, solid, deep brown-colored powder which is easily spread Figure A1, [23]). BBRDM was stored at ambient temperature during the period of investigation. Nitrogen content of BBRDM was ascertained by estimation of total Kjeldahl N (TKN) [69].

3.2. Detection of Pathogenic Microorganisms in BBRDM

Presence/absence of pathogens in BBRDM (before and after processing) was recorded. BBRDM was stored in sealed plastic bags and kept in the open for 60 days, following which re-growth was ascertained. Twenty grams of solid was suspended in 20 mL sterile water, homogenized in a mixer and suspensions of four forms of BBRDM were tested [70]. For each of the four forms of BBRDM examined, three samples from one batch were tested in duplicate sets ($n = 6$).

3.2.1. Presence/Absence of Fecal Coliforms

All BBRDM samples obtained after processing were enriched in Universal Pre-enrichment broth (Hi Media, Mumbai, India). BBRDM before processing was not enriched. One hundred microliters of undiluted BBRDM samples were spread on Mac Conkey agar (Hi Media, Mumbai, India). Plates were incubated at 44.5 ± 0.2 °C for 24 h [71]. *E. coli* ATCC 25922 cultured in Mac Conkey Broth (Hi Media, Mumbai, India) was used as the positive control. Detection limit of this test was 10 CFU mL⁻¹.

3.2.2. Presence/Absence of *E. coli* O157:H7

All BBRDM samples obtained after processing (but not before processing) were enriched in Universal Pre-enrichment broth (Hi Media, Mumbai, India). One hundred microliters of undiluted BBRDM samples were spread on Sorbitol-MacConkey (SMAC) HiCrome EC O157:H7 Selective Agar Base (Hi Media, Mumbai, India). Plates were incubated overnight at 36 °C for 24 h [71]. *E. coli* O157:H7 on SMAC medium was detected with 100% sensitivity of, 85% specificity and 86% accuracy [71]. *Pseudomonas aeruginosa* ATCC 27853 cultured in HiCrome EC O157:H7 Selective Agar Base was used as the positive control. Detection limit of this test was 10 CFU mL⁻¹.

3.2.3. Presence/Absence of Mycobacteria

Detection of Mycobacteria in BBRDM was performed through enrichment (of post-processed BBRDM samples only) in Universal Pre-enrichment broth (Hi Media, Mumbai, India) applying the

BacT/ALERT 3D system (BioMérieux Inc., Durham, NC, USA). Undiluted sample was decontaminated by *N*-acetyl-L-cysteine-sodium hydroxide method [72]. Decontaminated sample (500 µL) was aseptically injected under vacuum to the BacT/ALERT MP bottle which contained 10 mL of aqueous media (Middlebrook 7H9 broth, pancreatic digest of casein, bovine serum albumin and catalase) and incubated in the BacT/ALERT 3D Mycobacteria Detection System at 37 °C for 42 days. Positive detection through increase of reflectance units proportional to CO₂ produced by *Mycobacterium* growth was made by the gas-permeable colorimetric sensor installed in the bottom of each culture bottle. Colonies of positive samples were counted on Middlebrook 7H9 Broth Base. *Mycobacterium tuberculosis* H₃₇Ra ATCC 25177 in Middlebrook broth was used as the positive control.

3.2.4. Presence/Absence of *Clostridium* sp.

All BBRDM samples obtained after processing (but not before processing) were enriched in Universal Pre-enrichment broth (Hi Media, Mumbai, India). Cooked Meat medium (Hi Media, Mumbai, India) was used for enumeration of *Clostridia*. This medium detects saccharolytic as well as proteolytic species. Inoculation with undiluted samples was made in the bottom of the tube within the meat particles. Incubation at 35–37 °C for 40–48 h was done in anaerobic jars. Growth was indicated by increase of turbidity. *Clostridium perfringens* ATCC 12924 in Cooked Meat medium was used as positive control. Clostridia in positive samples were enumerated on Perfringens Agar Base (Hi Media, Mumbai, India). Limit of detection for this test was 10 CFU mL⁻¹.

3.2.5. Presence/Absence of *Salmonella* sp.

Universal Pre-enrichment broth (Hi Media, Mumbai, India) was used for enhancing numbers of suspected pathogens in all BBRDM samples obtained after processing. One hundred microliters of undiluted BBRDM samples were spread on xylose lysine deoxycholate (XLD) agar (Hi Media, Mumbai, India). Plates were incubated at 35 °C for 48 h. *Salmonella typhimurium* ATCC 14028 in XLD Broth (Hi Media, Mumbai, India) was used as the positive control. Detection limit of this test was 10 CFU mL⁻¹.

3.2.6. Presence/Absence of *Bacillus* sp.

All BBRDM samples after processing were enriched in Universal Pre-enrichment broth (Hi Media, Mumbai, India). One hundred microliters of undiluted BBRDM samples were spread on PLET Agar Base medium (Hi Media, Mumbai, India) to detect *Bacillus anthracis* and on Nutrient Agar with tyrosine to identify *Bacillus cereus*. Plates were incubated at 35–37 °C for 48 h. *Bacillus anthracis* ATCC 14578 (in PLET medium) and *Bacillus cereus* ATCC 10876 (in Nutrient Broth with tyrosine, Hi Media, Mumbai, India) were used as positive controls. Detection limit of this test was 10 CFU mL⁻¹.

3.2.7. Presence/Absence of *Brucella* sp.

Enumeration of *Brucella* sp. was done by the BacT/Alert FA plus microbial detection system (BioMérieux Inc., Hazelwood, MO, USA). Following enrichment as described before, undiluted sample was dissolved in Brucella broth (Hi Media, Mumbai, India) and placed in study bottles. Samples were incubated for 21 days at 37 °C and monitored for growth following the manufacturer's recommendations. Positive detection was achieved as described previously in the test for Mycobacteria. *Brucella abortus* ATCC 23452 grown in Brucella Agar Base (Hi Media, Mumbai, India) was used as positive control. Brucella present in positive samples were counted on Brucella Agar Base.

3.3. Cultivation Site and Plot Arrangement

The first field study (2012–2013) was done in Kuldiya Baganchi village of Magrahat block (latitude: 22°15'06.19" N, longitude: 88°20'52.02" E, altitude: 4 m) of South 24 Parganas district, West Bengal state, India [23]. The second field experiment (2013–2014) was carried out in Bankipur village of Magrahat block (latitude: 22°14'25.24" N, longitude: 88°22'42.09" E, altitude: 4 m) of South 24 Parganas district, West Bengal state. No previous cultivations were done in the experimental plots. The topography of the landscape was flat without any slope. In both years experiments were laid out as completely randomized block design with six replications for each treatment in a 500 m² (25 m × 20 m) plot which was divided into twelve sub-plots, each covering 16 m² soil surfaces. Arrangement of the sub-plots was random. Six of the sub-plots were treated with BBRDM and another six were treated with DAP + potash. Organic fertilizer (BBRDM) was considered as the test treatment while chemical fertilizer (DAP + potash) was regarded as control treatment. Local farmers usually apply the selected chemical fertilizers to tomato plants. Plantation was done on 14 November 2012 (season 1) and 28 November 2013 (season 2). Plant seedlings were arranged in four rows, eight plants in a row, maintaining 40 cm distance from each other along the single row in all sub-plots. In each single sub-plot, the distance between rows was 60 cm and the distance between sub-plots was 150 cm. There were 32 tomato plants in each sub-plot implying 20,000 plants ha⁻¹ [73–75].

3.4. Soil Characteristics, Crop, Time of Experiment and Weather

The soil of the experimental site in South 24 Parganas district of West Bengal state is characterized as saline-alkali soils of tidal origin [76]. Soil characteristics were previously described [23]. Pest and disease-tolerant tomato (*Lycopersicon esculentum*) plants local variety "Patharkuchi" was selected for the field study. The experiment was carried out in the winter season from 14 November 2012 to 28 February 2013 (total 14 weeks) and from 28 November 2013 to 15 March 2014 (total 14 weeks) [23]. The mean maximum temperatures during 2012–2013 and 2013–2014 were 27.1 °C ± 2.3 °C and 28.2 °C ± 1.3 °C, respectively whereas the mean minimum temperatures were 15.7 °C ± 2.2 °C and 16.2 °C ± 1.9 °C, respectively. The mean maximum humidity during 2012–2013 and 2013–2014 were 86.4% ± 3.0% and 85.7% ± 2.5%, respectively, while the mean minimum humidity were 40.8% ± 5.8% and 41.3% ± 4.6%, respectively. The total rainfall in 2012–2013 was 0.5 mm ± 0.1 mm and the same in 2013–2014 was 0.6 mm ± 0.1 mm [23].

3.5. Raising of Seedlings in Nursery

Tomato seedlings were developed in nursery beds near the main experimental plot. The soil was ploughed and the seedbed elevated. Seeds were sowed at 0.5 cm to 1 cm depth, in straight rows and 5 cm intervals allowing 2 cm gap between successive seeds and watered instantaneously. The seeds were lightly covered with top soil, shaded from direct sunlight and watered every alternate day [23].

3.6. Main Experimental Field Preparation, Seedling Transplantation and Staking

The experimental field was ploughed twice, plots were shaped and ridges made for growing tomato plants on the top of the bed. Furrows in between the plots were made to serve as irrigation and drainage channels. At the end of the fourth week following sowing, seedlings of roughly equal heights and about four or five leaf stage were transplanted to the main experimental plot in the late afternoon. The experimental plots were watered by furrow irrigation and sprinkle irrigation when required. All plots received equal amounts of water. The plants were staked with thin bamboo sticks of 2 m length at two weeks following transplantation. Pruning, weeding by hand pulling and other horticultural operations were done when required [23,77].

3.7. Fertilizer Application

BBRDM (NPK 30.36:1:5.75) fertilizer was applied to the plants designated in BBRDM plots. The control plot was fertilized with DAP (NPK 18:46:0) + potash (NPK 0:0:44). Organic BBRDM and inorganic DAP + potash (2:1) were applied in the equal amounts. $75 \text{ kg}\cdot\text{ha}^{-1}$ was applied as first dose (evenly distributed along the furrows of each sub-plot by hand) at two weeks after transplantation of the plants to the main experimental field $150 \text{ kg}\cdot\text{ha}^{-1}$ as second dose was applied at the eighth week after transplantation [74,77,78]. The annual fertilizer application rates were $68.31 \text{ kg}\cdot\text{N}\cdot\text{ha}^{-1}$, $2.25 \text{ kg}\cdot\text{P}\cdot\text{ha}^{-1}$, $12.9 \text{ kg}\cdot\text{K}\cdot\text{ha}^{-1}$ (BBRDM) and $27 \text{ kg}\cdot\text{N}\cdot\text{ha}^{-1}$, $69 \text{ kg}\cdot\text{P}\cdot\text{ha}^{-1}$, $33 \text{ kg}\cdot\text{K}\cdot\text{ha}^{-1}$ (DAP + potash) [23]. Tomato is a high N-demanding crop. Although higher P and K were applied in DAP + potash treatment, when N is limited, P and K responses are nominal.

3.8. Control of Plant Diseases

Organic pesticide (neem oil) was sprayed twice (third and ninth weeks after transplantation) over the tomato plants as a preventive measure [79]. During the study period common pests (aphids, bollworms, leaf miners, thrips, whiteflies, spider mites and nematodes) were not appreciably noticed. Wilts, blight, leaf spots and mildews were not notably observed during the cultivation. Removal of weeds, old leaves and branches as well as overshadowed lower leaves were done regularly by hand [23].

3.9. Harvesting

First harvest was carried out at the 10th week after transplantation. The mature red fruits were harvested from all plants of both treatments (BBRDM and DAP + potash). Successive harvests were

done four additional times at seven-day intervals, totaling five harvest events in each growing season [23].

3.10. Determination of Lycopene Content in Tomatoes Grown with BBRDM and DAP + potash

Lycopene content of tomatoes were determined in two seasons separately. Ten randomly selected BBRDM-grown mature tomatoes from each of the five harvests were taken for lycopene analysis. All the steps of sample preparation were done in minimal light at room temperature. The ten tomatoes were sliced into cubes, size ranges from 1.4–2.6 cm (length) approximately. The mass was mixed with deionized water 1:1 (w/v) and homogenized to form uniform slurry with particles smaller than 2×2 mm. An equal number of DAP + potash-grown tomatoes were also taken randomly for estimation of lycopene content [80]. Lycopene content in cultivated tomatoes was assayed spectrophotometrically according to the low or reduced volume hexane extraction method. Approximately 0.6 g samples were measured from each puree and taken in two 40 mL amber screw-top vials, which contained 5 mL of 0.05% (w/v) butylated hydroxytoluene in acetone, 5 mL of 95% ethanol and 10 mL hexane. Samples were collected while the purees were continually stirred on a magnetic stirrer. Samples were placed in an ice-bath and extracted by shaking at 180 rpm for 15 min in an orbital shaker. Three milliliters of deionized water was then added to each vial and the samples were shaken for further 5 min on ice. The vials were kept in ambient temperature for 5 min to allow phase separation. The absorbance of the upper hexane layer (containing lycopene) was recorded in a 1 cm path length quartz cuvette at 503 nm with hexane blank in a Perkin Elmer Lambda 25 UV/VIS spectrophotometer [81]. Determination was done thrice in duplicate sets ($n = 6$).

3.11. Determination of Nitrate and Nitrite Contents in Tomatoes Grown with BBRDM and DAP + potash

Ten randomly selected BBRDM and DAP + potash-grown mature tomatoes from each of the five harvests for both seasons separately were taken for nitrate and nitrite analyses. Determination was done spectrophotometrically [82]. For nitrite determination the test samples were quantitatively transferred to beakers and 5 mL of the sodium tetraborate solution and about 100 mL of hot (70 to 80 °C) water was added. Beakers were heated for 15 min in a boiling water bath. Two milliliters of potassium hexacyanoferrate (II) solution and 2 mL of the zinc acetate solution were added successively and shaken. After cooling the filtrate was quantitatively transferred to 200 mL volumetric flask, filtered and the filtrate collected for further analysis. Ten milliliters of filtrate was diluted with 30 mL of distilled water and the volume made up to 50 mL. Five milliliters of sulfanilamide chloride solution and 3 mL of hydrochloric acid were mixed. The resultant solution was left at ambient temperature protected from light. 1 mL of *N*-(1-naphthyl) ethylenediamine dichloride solution was added, mixed and left for 3 min at ambient temperature protected from light. After 15 min the absorbance was measured at 538 nm. For nitrate determination, 2 g of cadmium and 5 mL of buffer solution were added to the 10 mL of test sample and agitated for 5 min. After filtration the filtrate was collected and the same method for nitrite estimation was followed.

3.12. Assessment of Heavy Metal Contents in BBRDM and Tomatoes Grown with BBRDM

Determinations were performed separately in two seasons. Ten fresh tomatoes grown with BBRDM and an equal number cultivated with DAP + potash obtained at the end of the harvest (by combining all five harvests) were considered for this test. The test was done by dry ashing of the samples followed by acid dissolution of the ash. Dry ashing technique is applied for samples containing a significant amount of organic material as the matrix whereas nitric acid is mainly used for the preparation of inorganic samples. One gram of sample in a crucible was placed in a pre-heated muffle furnace at 200–250 °C for 30 min and ashed at 480 °C for 4 h. The sample was taken out from the furnace, cooled down, 2 mL of 5M HNO₃ added and dried by evaporation in a sand bath. The sample was again placed in a furnace, heated to 400 °C for 15 min, taken out from the furnace, cooled and moistened with few drops of distilled water. Two milliliters of concentrated HCl was then added, the sample dried by evaporation and 5 mL of 2 M HCl was added. Next, the solution was filtered through Whatman No. 42 filter paper, <0.45 µm Millipore filter paper, transferred quantitatively to a 25 mL volumetric flask and volume made up with distilled water. Heavy metal analysis was performed using atomic absorption spectrophotometer (Perkin Elmer, Model 1100, Paris, France), having a specific lamp of the particular metal and using appropriate drift blanks. This test assessed the levels of arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), manganese (Mn), lead (Pb), nickel (Ni) and zinc (Zn) concentration in the samples. Determination was done thrice in duplicate sets ($n = 6$) for each harvest.

3.13. Ames Test for Mutagenicity

Twenty fresh tomatoes grown with BBRDM and an equal number cultivated with DAP + potash obtained at the end of the harvest (by combining all five harvests) of the two seasons separately were considered for this test. Fresh tomatoes were properly homogenized in a laboratory homogenizer, the solid particle free juice collected and lyophilized. The liquid was cooled at –20 °C overnight and then at –80 °C for two days following which lyophilization was done to attain a powdered form. The tomato powder was stored at –20 °C until use [83]. Standard Ames test [84] with slight modifications was done using both BBRDM and DAP + potash-grown tomatoes. The assay was done according to the plate-incorporation procedure, using histidine-deficient (*his*[–]) *Salmonella typhimurium* strain TA98 (MTCC 1251) in the reverse mutation test including and excluding the lyophilized tomato samples [60]. The test strains were cultured overnight in modified E minimal broth for 12–14 h. The lyophilized tomato powder was mixed in sterile water to prepare a tomato powder suspension (TPS) which was used at 0.1 µg plate^{–1} and 0.2 µg plate^{–1} concentrations. Tomato suspension (0.5 mL) and 200 µL of bacterial culture were added to 3 mL of top agar, mixed well and poured into minimum agar plates. The plates were incubated at 37 °C for 48 h, following which *his*⁺ colonies (revertant cells having the ability to synthesize histidine) were counted. Standard mutagen (sodium azide) was used as positive control in each experiment. The mutagen was dissolved in water at the final concentration equal to that of the tomato suspension [60]. The solution was then added to 3 mL of top agar followed by addition of 200 µL of bacterial culture, mixed well and then poured into minimum agar plates. Bacterial plates without any test compound or mutagen was used as the negative control. Mutagenicity

of test sample was confirmed if the number of revertant colonies were at least twice the negative control. Determination was done thrice in duplicate sets.

3.14. Sub-Acute Toxicity Study of Tomatoes Grown with BBRDM

Sub-acute toxicity test was performed for assessment of the predictive safety of the BBRDM fertilizer grown tomatoes, which are intended for human consumption. The animal study was performed with approval of the Institutional Animal Ethics Committee (AEC) of Jadavpur University, Kolkata, India on 7 September 2012 (Project Identification Code: Utilization of Slaughterhouse Wastes as an Organic Fertilizer for the Cultivation of Tomatoes). Growth, hematological parameters, relative organ weights and histological parameters of Wistar rats fed with BBRDM-grown tomatoes for 28 days was compared with the results obtained from the control rats fed with water [85]. A total of 24 male and female Wistar rats of 2 months age (male, 130–150 g; female, 150–170 g) were used for the toxicological studies. The animals were acclimatized to the laboratory conditions (approximately 25 °C, 55% humidity, 12-h day/night cycle) for a week prior to the experiments [86]. During acclimatization, the cages were properly disinfected and two to three rats were kept in each polycarbonate cage and fed with normal diet and water. Rats of both sexes were randomly assigned to four groups of the same sex; two control groups and two treatment groups ($n = 6$). The lyophilized tomato powder was suspended in water and administered orally twice daily for 28 days keeping the total amount as 7.0 g (of tomato fruit weight) per day to the test groups corresponding to a daily human consumption of 1.0 kg. The control groups received similar volume of water (the vehicle for suspension of lyophilized tomato powder). The treated animals were starved for 3 h before and after administration of the feed. At the start of the sub-acute toxicity study, blood was collected from the retro-orbital plexus of the anesthetized animals. Hematological analysis was carried out using an automatic hematological analyzer (Sysmex XT-4000i, Sysmex America, Lincolnshire, IL, USA). The parameters measured were: red blood cell (RBC) count, leukocyte (WBC) count, haemoglobin (Hb), haematocrit (Hct), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelet count. Microscopic examination of stained blood smears was done to obtain differential leukocyte counts. Assays for blood glucose, urea and serum creatinine were performed using an automated biochemistry analyzer (Roche Cobas 6000, Roche Diagnostics, Indianapolis, IN, USA). Systolic blood pressure of rats in each group was recorded by the noninvasive tail cuff method (NIBP Biopac Systems Inc., Santa Barbara, CA, USA). During the course of the study, cage side observations included examination of skin, fur, eyes, respiratory and autonomic effects like salivation, urination as well as central nervous system effects such as tremors and convulsions, changes in the level of activity, gait and posture. The behavior of the rats was noted daily and their weights and mean food consumption were recorded once every week [85,87,88]. On the 28th day, blood pressures of all the rats were measured and on the 29th day after an overnight fast, blood from each animal was collected by retro-orbital bleeding for hematological and biochemical tests described before. After blood collection, all the animals were euthanized and the principal vital organs (liver, kidney, heart and stomach) were removed, cleaned with saline, weighed and macroscopically analyzed [88]. Vital organs were preserved for histological study. Organs were fixed in a 10% solution of buffered formalin, having pH 7.4 and enclosed in

paraffin. Five-micrometer tissue sections were colored with hematoxylin-eosin and observed microscopically [85,86].

3.15. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 20 software [89]. The Student's *t*-test evaluates the means of one variable (in this study lycopene content only) between two treatments. Lycopene content of tomatoes grown with BBRDM and those cultivated applying DAP + potash for both seasons were compared using Student's *t*-test. Highly significant difference of the parameter (lycopene) between two treatments at 1% level was denoted by double asterisk and significant difference at 5% level was indicated by single asterisk. Standard deviations were calculated for the mean values of all determinations and the “*n*” value is specified for each test in the relevant table/figure.

4. Conclusions

Through this investigation the product (BBRDM) has been proven to be safe in terms of pathogen (*E. coli* O157:H7, Mycobacteria, *Clostridium* sp., *Salmonella* sp., *Bacillus* sp. and *Brucella* sp.), as well as heavy metal content (Pb, Cr, Cd, Cu, Zn, As, Ni and Mn). Tomatoes cultivated using BBRDM as an organic fertilizer had permissible contents of heavy metals and acceptable levels of nitrate and nitrite. High lycopene content ensured superior nutritional quality of the BBRDM-grown tomatoes. Organically grown tomatoes were non-carcinogenic (Ames test) and non-toxic (rat model). There was no immediate safety or health risks associated with tomato fruits produced in this experiment. In light of the stringent regulations prevalent in the European Union and the United States of America against direct application of slaughterhouse wastes to land, the use of treated abattoir wastes (BBRDM) as an organic fertilizer may be considered as a practicable pollution mitigation measure. This study could be a starting point for designing additional and more extensive studies to evaluate fertilizer and food safety.

Acknowledgments

Malancha Roy is thankful for financial support through the DST Women Scientist B Scheme (SSD/SS/009/2011). Research funding from Jadavpur University, Kolkata, West Bengal, India is thankfully acknowledged. Authors are grateful to Anarul Gazi for supplying blood and rumen digesta. Authors express their gratitude to Joyashree Roy, Coordinator Global Change Programme and Professor of Economics, Jadavpur University for her helpful suggestions.

Author Contributions

Malancha Roy and Rimi Das—Field work and laboratory analyses, manuscript draft preparation; Amit Kundu, Sanmoy Karmakar and Satadal Das—Microbiological analyses and animal studies; Pradip Kumar Sen—Statistical analyses; Anupam Debsarcar and Joydeep Mukherjee—Conceptualization of the problem, preparation of manuscript and overall supervision.

Conflicts of Interest

The authors declare no conflict of interest.

Appendix



Figure A1. Bovine blood and rumen digesta mixture (BBRDM) used as organic fertilizer for cultivation of tomato.

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