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RESEARCH ARTICLE

Biofilm nutraceuticals shape gut microbiota better than diet-based interventions: a novel approach to next-generation medicine

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Abstract: *In-vitro*-developed microbial biofilms are reported to restore degraded agroecosystems via reinstating soil-plant-animal-microbial networks by supplying a mixture of diverse biochemicals that act as network components. Here we hypothesize that the same approach can be used to revitalize the gut microbiota altered due to modern lifestyle and dietary patterns. We tested biochemicals exuded by a developed fungal-bacterial biofilm (BFEx) on the dormancy-breaking of five test gut microbes. The growth and development of the microbes were evaluated in a simulated gut environment with eight different dietary patterns consisting of low and high levels of carbohydrates, proteins, lipids, and fiber. In addition, the BFEx was tested for cytotoxic activity. Results revealed that the BFEx promoted the growth and possibly dormancy-breaking of all the tested gut microbes. However, these observations were made only in mixed cultures suggesting that there is a need for the interaction of diverse microbes in order to achieve a beneficial outcome from the BFEx. Further, the BFEx showed no cytotoxicity. In conclusion, this biofilm-based method seems a better solution than that of diet-based interventions for achieving healthy gut microbiota as the latter option does not restrict peoples' dietary preferences. The next step would be to evaluate this microbial intervention in animals and humans.

Keywords: Biochemicals; Biofilms; Ecosystem restoration; Gut microbiota; Health

INTRODUCTION

The diversity of gut microbiota controls the host throughout homeostasis and sickness (Kho & Lal, 2018; Wu & Wu, 2012). Healthy host–microorganism equilibrium is important in maintaining the intestinal barrier and immune system functions, thus preventing disease development. Also, the latest advances in science have shown the importance of gut microbiota in connecting the emotional and cognitive centers of the brain with peripheral intestinal functions (i.e. gut-brain axis) (Carabotti et al., 2015). In the human body, dietary constituents are chemically transformed by the gut microbiota, and the gut-derived metabolites are dispersed to all organs, including the brain. Scientists revealed that the gut microbiota controls neurotransmitter production in the brain and gut, thus leading to behavioral alterations in mice (Needham et al., 2022). Nonetheless, the diversity of the gut microbiota in humans has diminished severely with the adoption of modern lifestyle and dietary habits including the use of processed food with low fiber content and antibiotics (Moossavi & Bishehsari, 2019). Likewise, it is a rising health concern, as it is strongly related to obesity and associated metabolic diseases. Currently, novel diet-based interventions are developing to maintain health and prevent diseases. However, they limit people's dietary preferences.

It is reported that the diversity of gut microbiota is a subset of the diversity of soil microbiota (Hirt, 2020). A close connection between the human gut microbiota and the soil microbiota has evolved throughout the evolution and is still developing (Blum et al., 2019). In soil, application of microbial biofilms developed *in-vitro* has been reported to restore lost microbial diversity, as their biofilm exudates [BFEx, i.e. biochemicals exuded as extracellular polymeric substances (EPS)] break the dormancy of microbial seed bank formed under stress (Herath et al., 2013; Seneviratne & Kulasooriya, 2013; Herath et al., 2017; Meepegamage et al., 2021). The most solid indication of this has come from a study where the application of a fungal-bacterial biofilm improved cyanobacterial diversity in an agroecosystem (Buddhika et al., 2013). In addition, it was found that the soil application of fungal-bacterial biofilms increased the abundance of endophytic microbes in the host (rice plant) and that improved the soil-plant-microbial network interactions in the degraded agroecosystem with chemical fertilizers alone application. This led to establishing a healthy agroecosystem consisting of better plant growth and yield with a higher abundance of endophytes. In undisturbed ecosystems, there is a delicate balance among the interacting counterparts, the center of which is represented by microbes (Seneviratne, 2015). Therefore, it is assumed that the same theory can be applied to recover unhealthy human body ecosystems caused by altered gut microbiota due to processed foods and modern lifestyles (Seneviratne & Premarathna, 2020). If succeed it would be a better option than the diet-based interventions that restrict people from choosing their dietary preferences. Hence, this study was designed to inspect the effect of BFEx on the human gut microbiota in a simulated gut environment using diverse dietary patterns containing high and low levels of carbohydrates, proteins, lipids, and fibers. Also, as a next step, the BFEx has been tested for cytotoxic effects using a brine shrimp lethality assay.

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MATERIALS AND METHODS

Biofilm formation and extraction of BFEx

A fungal-bacterial biofilm (FBB) of *Aspergillus niger* and *Stenotrophomonas maltophilia* was used to produce BFEx, based on the outcomes of the former studies i.e. high biochemical diversity (Premarathna et al., 2022).

The physicochemical method described by Dorina et al. (2020) and Chang et al. (2019) was performed after modifications to extract BFEx. The method consisted of adding NaCl to trigger the release of EPS from microbial cells (Chiba et al., 2015), ultra-sonication (using VWR USC 1700D sonicator) at 60 W to detach EPS from the cells, and centrifugation at 5000 rpm for 10 minutes to pellet cells.

Assessing the potential of biofilm exudates in dormancy breaking of gut microbes

Five commonly found gut microbes *viz. Bacillus clausii, Lactobacillus sporogenesis, Lactobacillus reuteri, B. subtilis,* and *A. niger* were used as the test microbes (Rinninella et al., 2019a; Hallen-Adams & Suhr, 2017). The microbes were separately inoculated to nutrient broth (NB) to produce monoculture solutions (MS).

After 24 hours, the MS were homogenized by vortexing them for 15 minutes, and 1 ml of each MS was added separately to 15 ml centrifuge tubes, each containing 9 ml of distilled water. Six replicates were prepared for each MS, and here they were introduced for nutrient starvation. The MS were then kept in the refrigerator at -10 ^{o}C for 48 hours to induce dormancy triggered by nutrient starvation (Gray et al., 2019; Jones & Lennon, 2010). After 48 hours, the MS were taken out from the refrigerator, and the initial assessment of live microbial cell concentrations (LMC) was done using "Invitrogen™ LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit" (Invitrogen Molecular Probes, Eugene, OR, USA) (Mona et al., 2021; Rosenberg et al., 2019; Vatansever & Turetgen, 2021). Then, the MS were divided into two groups, and one group was treated with 10 µl of BFEx. All the MS were incubated at 34 ^{o}C for 24 hours and were analyzed again for LMC.

In-vitro gut simulation study

The stomach hosts relatively very few microbes, while the small intestine (where food is digested) has some more microbes, and the large intestine (where food ferments after digestion) is home to the majority of microbes of the human microbiome (American Museum of Natural History, 2016). The large intestine is colonized by many bacteria approximately 10^{12} per gram of intestinal content (Anda-Flores et al., 2021). Therefore, the large intestine was selected to be simulated in the *in-vitro* experiment.

The aforementioned five test gut microbes were grown as mono and mixed cultures in a simulated gut environment using eight diverse dietary patterns, i.e. low-carbohydrate, high-carbohydrate, low-protein, high-protein, low-lipid, high-lipid, low-fiber and high-fiber. Here, the culture medium described by Parmanand et al. (2019) and Macfarlane et al. (1998) was used after modification. The culture medium contained (g/l); casein 3, yeast extract 2, NaCl 0.1, K_2HPO_4 0.04, KH_2PO_4 0.04, $MgSO_4.7H_2O$ 0.01, $CaCl_2.6H_2O$ 0.01, $NaHCO_3$ 2, Tween-80 2, glucose 10, vitamin K1 10, cysteine HCl 0.5, bile salts 0.5, Starch 10, Pectin 2. Casein, starch, pectin, and tween 80 were selected to represent protein, carbohydrate, fiber, and lipid, respectively. Eight diverse media were set to represent relatively low and high concentrations (i.e. 50% and 150% of the above concentrations of each dietary source as the low and high concentrations, respectively) of the diets in the gut.

A hundred microliters of each monoculture and mixed culture microbial solutions were separately added to eppendorf tubes containing the eight different media. The BFEx (10 µl each) was added into half of the eppendorf tubes. Each media with or without BFEx made two treatments. Each treatment had four replicates in a completely randomized design. The samples were kept under a pH of 6.2 - 6.6, and a temperature of 37 ^{o}C. After 24 and 48 hours, each tube was centrifuged at 8000 rpm for 10 minutes to pellet the cells, and the supernatant was removed. Then, 1 ml of sterilized distilled water was added into each tube and centrifuged again at 8000 rpm for 10 minutes to wash the pellet. Finally, 1 ml of sterilized distilled water was added again into each tube, and live microbial cell concentrations were measured by staining with SYTO® 9 and propidium iodide using LIVE/DEAD™*Bac*Light™ bacterial viability kit (Invitrogen Molecular Probes, Eugene, OR, USA) (Mona et al., 2021; Rosenberg et al., 2019; Vatansever & Turetgen, 2021).

Staining microbes

Equal volumes of SYTO® and propidium iodide were combined in a microfuge tube. Three microliters of the dye mixture were added to each milliliter of the microbial broths and incubated at room temperature in the dark for 15 minutes. Measurements (blue and far-red emission) were taken using Qubit™ 4 fluorometer (Invitrogen, MA USA).

Brine shrimp lethality assay for biofilm exudates

Brine shrimp (*Artemia salina* L.) eggs were hatched in a half-covered beaker (250 mL) containing artificial sea water and were allowed to stay for 2 days at room temperature (RT) (Krishnaraju et al., 2005). Throughout the hatching period, constant and continuous aeration was supplied through an oxygen pump, and lighting was supplied using a 20 W bulb. After 48 hours, 2nd larval stage (nauplii) of brine shrimps was identified towards the lightened side of the beaker. Test samples were prepared in artificial seawater having 0%, 5%, 10%, 15%, 20%, and 25% of BFEx. The samples were triplicated for each concentration. The test samples of 2 mL each were poured into a 24-well plate. To each sample, 10 brine shrimp nauplii were added and allowed to stay for another 48 hours under lightened conditions at RT. After 12, 24, 36, 48, and 72 hours, the number of survivals of brine shrimp nauplii was counted, and the percentage lethality was calculated.

Statistical analysis

Data were analyzed using the statistical package Minitab, version 17. Analysis of variance followed by Tukey's HSD test was performed to compare the means. The probability

of < 0.05 was used as the threshold for significance.

RESULTS AND DISCUSSION

The potential of biofilm exudates in dormancy breaking of gut microbes

The LMC of each tested gut microbe was observed to increase significantly with the application of BFEx (Figure 1). Here, the effect of BFEx as a nutrient supplement can be excluded because the application of 10 µl of BFEx is relatively a negligible amount when compared to the nutrient content in the media used. The increased LMC are suggestive of the dormancy breaking of gut microbes with the application of BFEx. This can be attributed to the diverse compounds of BFEx viz. low molecular weight sugars, amino acids, etc., which induce to break dormancy of gut microbes (Seneviratne & Kulasooriya, 2013).

Breaking dormancy of gut microbes: *in-vitro* gut simulation study

Similar to the previous study, higher LMC were observed in the mixed cultures of the gut microbes with the application of BFEx in all dietary patterns in the simulated gut environment (Figures 2 & 3). The high protein diet tends to increase LMC higher than the other dietary patterns suggesting a limitation of protein to grow or break the dormancy of the gut microbes (Figure 4). These results revealed that the BFEx promoted the growth and possibly the dormancy-breaking of tested gut microbes in the simulated gut environment. Further, the same action of BFEx was observed in all diverse dietary patterns, which consisted of both low and high amounts of nutrients.

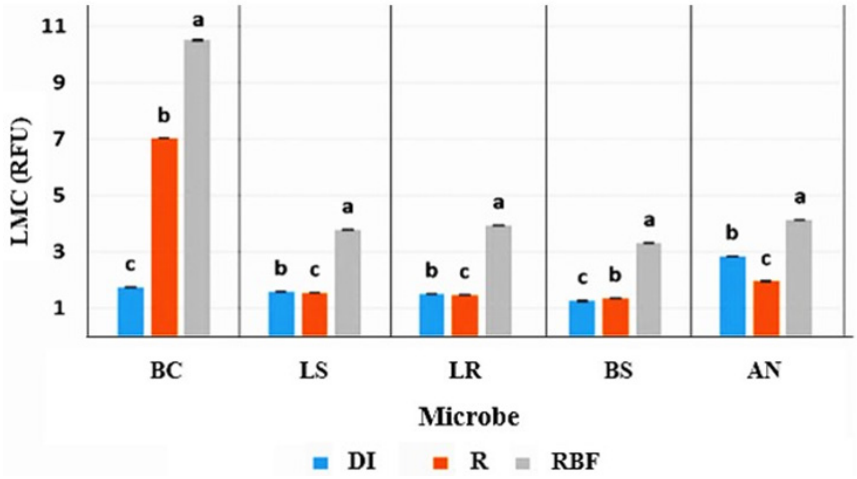

Figure 1: Live microbial cell concentrations (LMC) of different gut microbes after inducing dormancy by changing temperature and nutrients, and recovering cells using BFEx. *DI: dormancy-induced monocultures, R: Recovering monocultures incubated at 34 °C for 24 hours, RBF: Recovering monocultures treated with biofilm exudates (BFEx) and incubated at 34 °C for 24 hours. BC: Bacillus clausii, LS: Lactobacillus sporogenesis, LR: Lactobacillus reuteri, BS: Bacillus subtilis, AN: Aspergillus niger.* Significant differences are indicated using different lowercase letters.

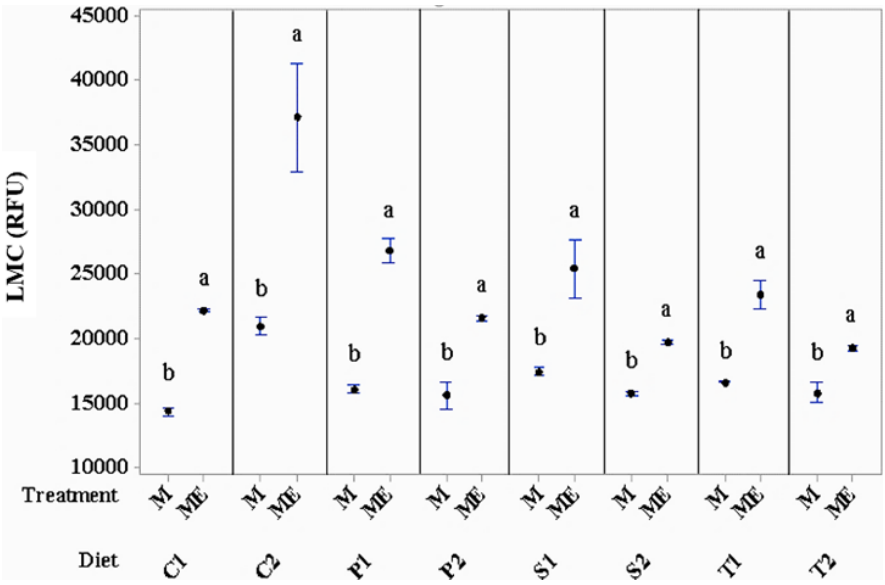

Figure 2: Change of live microbial cell concentrations (LMC) of mixed culture gut microbes with the application of biofilm exudates (BFEx) in different dietary patterns after 24 hours. RFU – relative fluorescent units, *M – without BFEx, ME – with BFEx, C1 – low protein, C2 – high protein, P1 – low fiber, P2 – high fiber, S1 – low carbohydrate, S2 – high carbohydrate, T1 – low lipid, T2 – high lipid.* Significant differences are indicated using different lowercase letters.

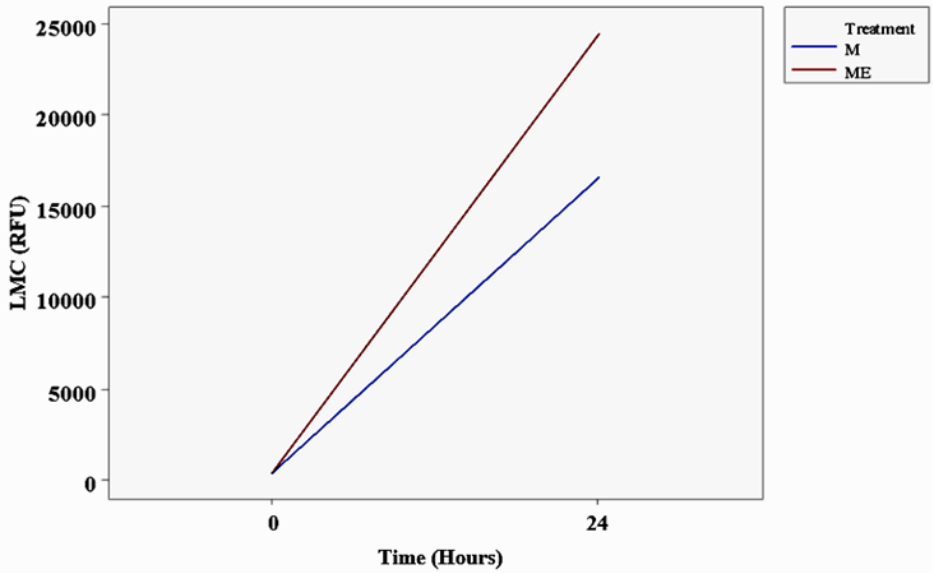

Figure 3: Increase of live microbial cell concentrations (LMC) after 24 hours with different treatments of mixed cultures. *M – without BFEx, ME – with BFEx.*

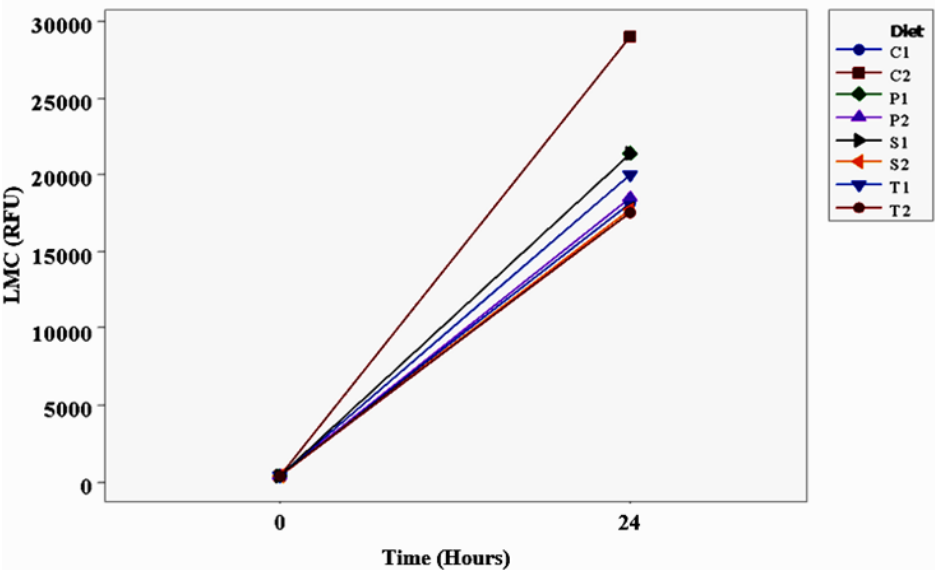

Figure 4: Increase of live microbial cell concentrations (LMC) within 24 hours in different dietary patterns. *C1 – low protein, C2 – high protein, P1 – low fiber, P2 – high fiber, S1 – low carbohydrate, S2 – high carbohydrate, T1 – low lipid, T2 – high lipid.*

However, all the tested gut microbes showed lower LMC with the application of BFEx when they were in monoculture form (Figure 5). Here, by-products produced by the monocultures per se might have suppressed the microbial growth in the presence of BFEx (McDonald et al., 2018).

The live microbial cell concentrations decreased after 24 hours in the monoculture treatments whereas it increased further in the mixed culture treatments possibly due to the metabolic corporation (Figures 6 & 7). Moreover, the rate of increase of LMC further increased in the mixed cultures after 24 hours with the application of BFEx (Figure 7) showing a possible increase in nutrient use efficiency in gut microbes with the application of BFEx. The different action of BFEx in monoculture and mixed culture environment is suggestive of a need for an interaction of diverse microbes to trigger the favorable mechanisms of BFEx. However, this needs further studies to understand the mechanism of BFEx via constructing interaction networks of the microbes.

In the soil, microbial biofilms developed *in-vitro* are reported to reinstate lost microbial diversity in degraded agroecosystems as explained above (Buddhika et al., 2013, and Figure 8), thus leading to restoration of network interactions for improved rice production in large-scale cultivations (Seneviratne and Kulasooriya, 2013; Premarathna et al., 2021). The same mechanism could have operated here to increase the LMC of the gut microbes because the gut microbial diversity is a subset of soil microbial diversity (Hirt, 2020), and the action of BFEx in triggering ecosystem intelligence is effective to any ecosystem alike (Seneviratne & Premarathna, 2023).

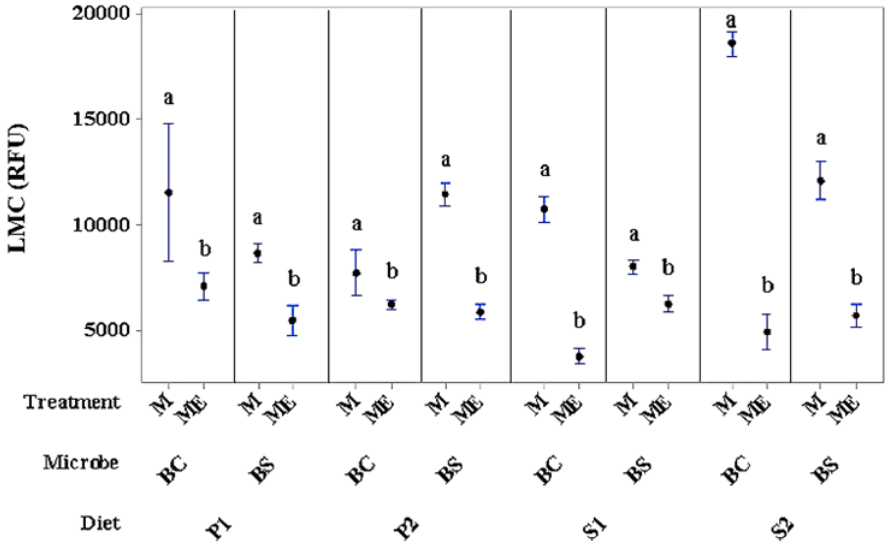

Figure 5: Change of live microbial cell concentrations (LMC) of mixed culture gut microbes with the application of biofilm exudates (BFEx) in different dietary patterns after 24 hours. *M – without BFEx, ME – with BFEx, P1 – low fiber, P2 – high fiber, S1 – low carbohydrate, S2 – high carbohydrate.*

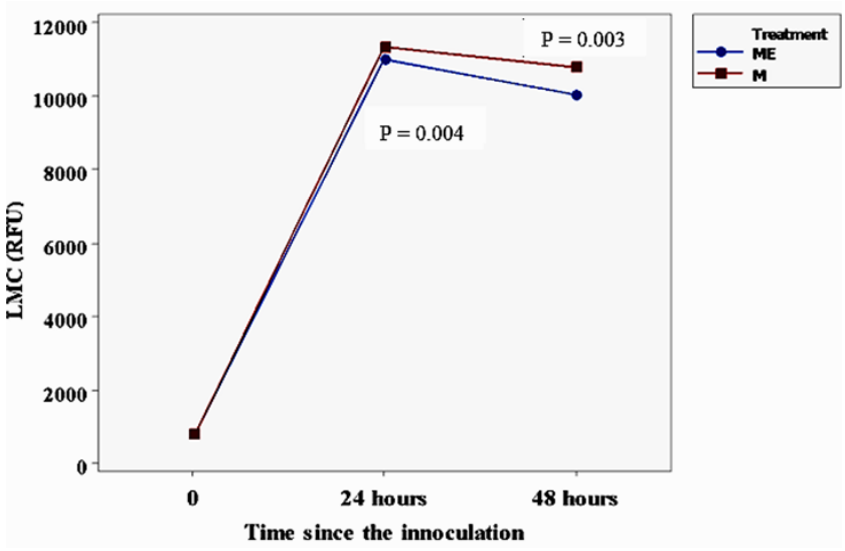

Figure 6: Variation of live microbial cell concentrations (LMC) with the time in mono-culture of *Bacillus clausii*, in high carbohydrate dietary pattern. *M – without BFEx, ME – with BFEx.*

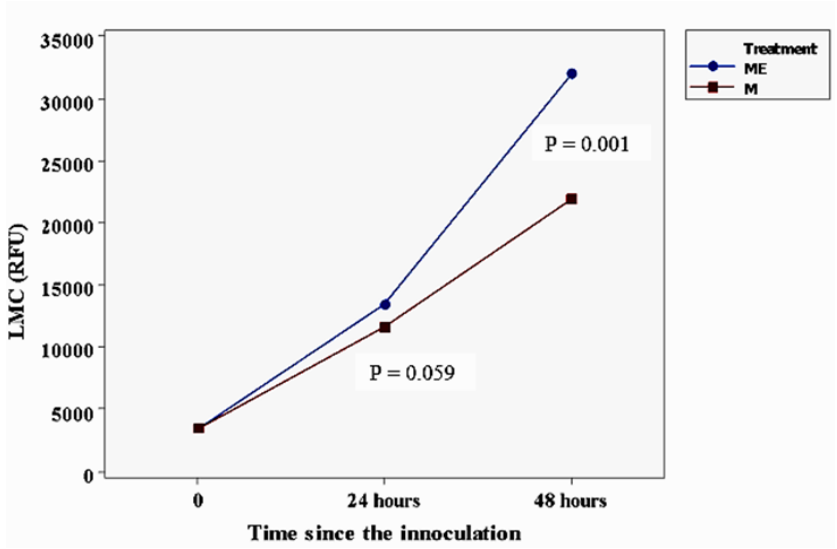

Figure 7: Variation of live microbial cell concentrations (LMC) with the time in mixed-culture of gut microbes in high carbohydrate dietary pattern. *M – without BFEx, ME – with BFEx.*

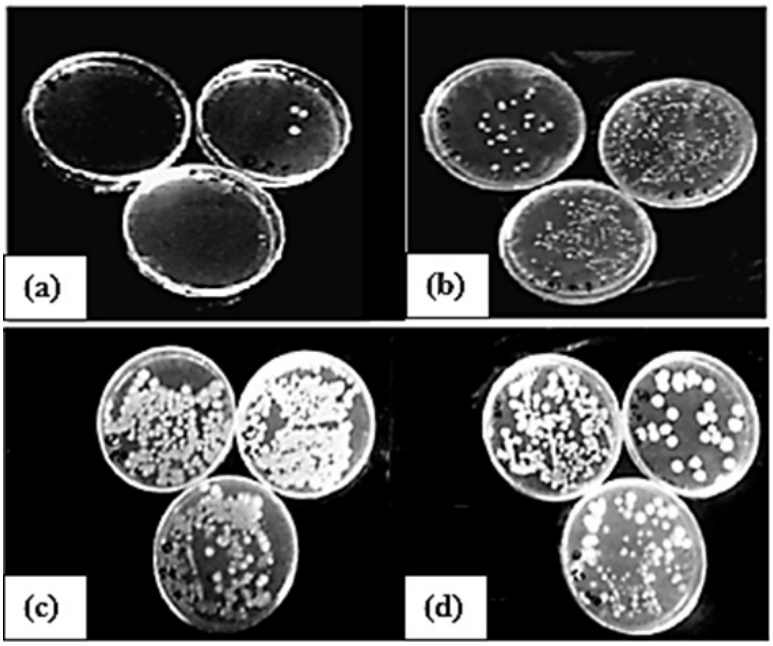

Figure 8: Restoration of soil microbial abundance and diversity in paddy agroecosystem using *in-vitro* developed microbial biofilms; (a) 100% chemical fertilizer (CF) alone practice, (b) 50% chemical fertilizer practice, (c) 50% chemical fertilizer + biofilm application, (d) forest soil.

Microscopic view of gut bacteria under the simulated gut environment with and without BFEx application

Bacteria were presented as planktonic cells in the mixed microbial cultures without BFEx application, whereas fungal-bacterial associations in biofilm mode were observed in the presence of the BFEx (Figure 9). The BFEx might have triggered the biofilm formation of the microbes. Generally, this leads to an enhanced metabolic corporation and significant upregulation of genes of biofilm-resident microbes resulting in better microbial growth (Resch et al., 2005).

Brine shrimp lethality assay for BFEx

After 48 hours, 100% survival of brine shrimp nauplii was observed only in the treatment having 10% BFEx (Figure 10). All the brine shrimp nauplii died within 72 hours in the control having only artificial seawater, possibly due to starvation. Thus, the BFEx showed no toxicity on the brine shrimp nauplii, instead, it supported their survival for a while by supplying food sources like polysaccharides, proteins, and fatty acids in the 10% BFEx concentration. As such, the optimum BFEx concentration for the brine shrimp was observed to be 10%.

In general, dietary diversity leads to food compound (biochemical) diversity which is transformed by the gut microbiota into microbial metabolites like short-chain fatty acids (SCFAs) that in turn increase microbial diversity (Gentile & Weir, 2018; Rinninella et al., 2019b). However, scientists revealed that monotonous diets such as Western-style diets are incompetent to be converted into SCFAs via trimethylamine N-oxide (TMAO), which leads to rising TMAO levels in the body causing many disorders and diseases (Gatarek & Kaluzna-Czaplinska, 2021; Ramos & Martin, 2021). As this is the case, biofilm interventions like BFEx can enable the above transformation via microbial and biochemical diversities, and their stability leads to improve human health (Herath et al., 2013; Buddhika et al., 2013; Johnson et al., 2019; Ramos & Martin, 2021).

It is reported that mushroom-forming fungi establish mutually beneficial interactions with bacteria, and these fungi also play an important role in human societies to produce mycelium materials, as a source of medicinal compounds, and as food (Braat *et al*., 2022). These mushrooms can be considered natural fungal-bacterial biofilms (Jayasinghearachchi & Seneviratne, 2006). Further, as an edible fungus, the Lion's Mane Mushroom (*Hericium erinaceus*) represents the most used fungus for all the disorders of the gastrointestinal system, and has a long history of usage in traditional Chinese medicine for the protection of gastric ulcers, mucous membranes, acute and chronic gastritis, and nervous degeneration (Spisni et al., 2021).

A single protein, called HEP3, isolated from this mushroom and administered to rats treated with trinitrobenzene sulfonic acid (TNBS) to induce experimental colitis similar to inflammatory bowel diseases (IBD), was capable of restoring the microbiota diversity in the treated rats (Diling et al., 2017). In a chronic pancreatitis mice model, the compromised microbiota profile was partially restored by Chaga (*Inonotus obliquus*) mushroom's polysaccharides administration, which was able to increase microbiota diversity and richness and also improve mouse clinical conditions (Hu et al., 2017). Likewise, naturally occurring mushrooms in complex and undefined FBB modes provide numerous benefits to human health. Thus, it is hypothesized that FBBs developed *in-vitro* and their BFEx may also render such benefits for a healthy life (Premarathna et al., 2022).

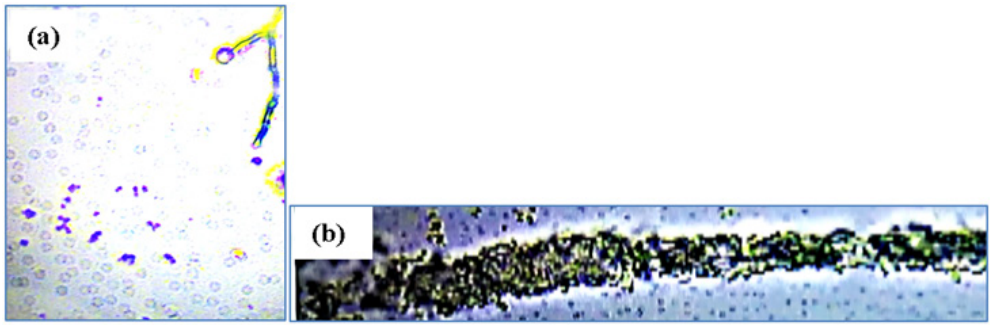

Figure 9: (a) Free-living bacteria in the mixed microbial cultures in the absence of BFEx (magnification: ×400); (b) bacteria attached to the fungal mycelia in the mixed microbial cultures with the application of BFEx (magnification: ×400).

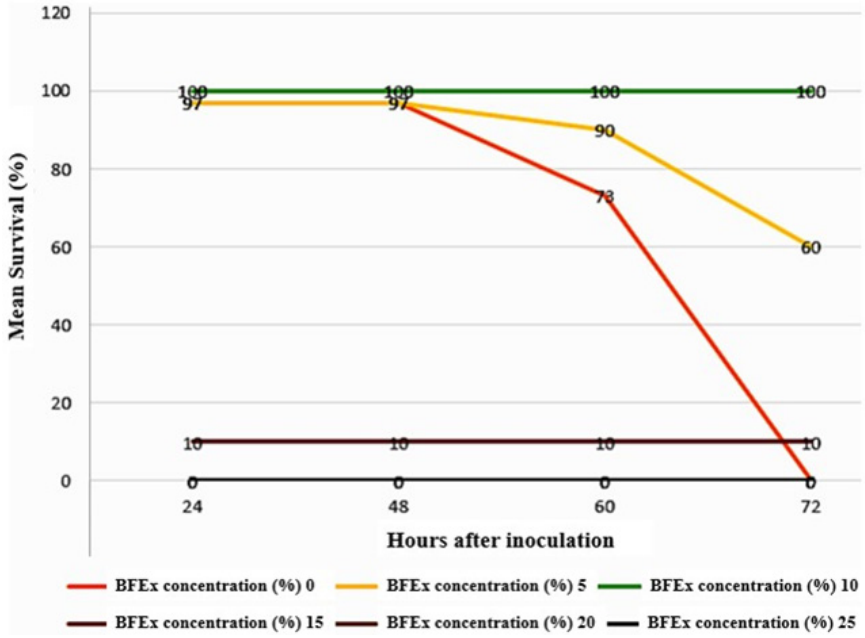

Figure 10: Survival (as a %) of brine shrimp nauplii with the application of BFEx at different concentrations (0, 5, 10, 15, 20 and 25%).

CONCLUSIONS

The BFEx encouraged the growth and possibly the dormancy-breaking of the tested gut microbes, and therefore they can be developed as biofilm-based interventions to restore gut microbiota for improved human health. So far, diet-based interventions have been developed to shape the gut microbiota. However, BFEx may be a better candidate to do the same job as they are user-friendly and also they do not restrict peoples' dietary preferences, unlike diet-based interventions. Conclusively, the biofilm-based interventions derived from BFEx can be considered as the next-generation medicines or rather nutraceuticals, which confidently will provide solutions for numerous human health issues in the future. Furthermore, this study suggests that the diseased human body ecosystem due to bad foods and habits can be treated from biofilm-EPS biochemicals by recovering the gut microbiota, thus reinstating the overall health for a happy life.

So far, postbiotics i.e. metabolites released by probiotics have been used to improve health by improving specific physiological functions in the human body. However, the effects of EPS biochemicals released by microbial biofilms on the growth and dormancy breaking of human gut microbes have still not been studied, hence this is the first study reporting a possible avenue to break the dormancy of microbiota in the diseased human gut for improved health. The next step of this study would be to evaluate this microbial intervention in animals and then in humans.

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DECLARATION OF CONFLICT OF INTEREST

Authors have no conflict of interest.

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