PREPRINT

PERTANIKA JOURNALS

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

Application of Membrane-less Microbial Fuel Cell in Reducing Human Hazards from Dewatered Sludge

Fatin Nur Izzati Mohd Fadzil[1], Chen Sep Ngee[1], Mohammed Zharif Asyrani Mohammed Alias[1], Muhammad Adib Fadhlullah Muhammad Lukman[2], Amira Suriaty Yaakop[3], Muaz Mohd Zaini Makhtar[1] and Ana Masara Ahmad Mokhtar[1,4]*

[1]Small G Protein Research Group, School of Industrial Technology, Universiti Sains Malaysia, 11800 Penang, Malaysia
[2]National Poison Centre, Universiti Sains Malaysia, 11800 Penang, Malaysia
[3]School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
[4]Green Biopolymer Coating and Packaging Centre, School of Industrial Technology, Universiti Sains Malaysia, 11800, Penang, Malaysia

ABSTRACT

Membrane-less microbial fuel cell (ML-MFC) technology has emerged as a potential for wastewater treatment and electricity generation. Despite its benefit in green energy production, studies have yet to determine its role in minimizing the human hazards stemming from dewatered sludge (DS). Hence, this research aims to investigate the effects of ML-MFC-treated DS on cell toxicity and its benefits in reducing protein-denaturation-related inflammation and antimicrobial resistance (AMR) dissemination. MTT assay was performed to determine the cytotoxic effect of ML-MFC-treated DS on 3T3-L1 and Hep G2 cells at 24 h. The anti-inflammatory property of ML-MFC-treated DS was determined using a protein denaturation assay. Next, the antibiotic susceptibility of bacteria isolated from ML-MFC-treated samples was determined using the disk-diffusion method. All the data obtained were statistically analyzed using GraphPad Prism software (Version 9.2.0) with a p-value ≤ 0.05 was considered significant. Interestingly, ML-MFC-treated DS showed 80% cell viability on 3T3-L1 and slight toxicity on Hep G2 cells. ML-MFC-treated DS exhibited anti-inflammatory properties with 62.43% protein denaturation inhibition and displayed fewer antibiotic-resistance

ARTICLE INFO

Article history:
Received: 08 May 2023
Accepted: 23 October 2023
Published: 04 April 2024

DOI: https://doi.org/10.47836/pjst.32.3.12

E-mail addresses:
fatinfadzil@student.usm.my (Fatin Nur Izzati Mohd Fadzil)
sepngee@gmail.com (Chen Sep Ngee)
zharif.asyrani.alias@gmail.com (Muhammad Zharif Asyrani Mohammed Alias)
adib.padolah@gmail.com (Muhammad Adib Fadhlullah Muhammad Lukman)
amirasuriaty@usm.my (Amira Suriaty Yaakop)
muazzaini@usm.my (Muaz Mohd Zaini Makhtar)
anamasara@usm.my (Ana Masara Ahmad Mokhtar)
*Corresponding author

ISSN: 0128-7680
e-ISSN: 2231-8526

bacteria than the untreated. Overall, the ML-MFC technology showed novel applications by decreasing DS-related health hazards.

Keywords: Antimicrobial resistance (AMR), cytotoxicity, dewatered sludge, inflammation, microbial fuel cell (MFC)

INTRODUCTION

Over the years, rapid development and growing population in Malaysia have led to increased sewage sludge production. It generates approximately 3 million cubic meters of sewage sludge annually within wastewater treatment plants and is expected to increase substantially in the future (Safuan et al., 2014). Generally, sewage sludge, or biosolids, refers to the residual, semi-solid substances produced as a by-product during the treatment of municipal or industrial wastewater (Kumar & Chopra, 2016). Sewage sludge normally contains high water content (92–99.5%), which contributes to the high costs of its further treatment (Górka et al., 2018). Hence, dewatering becomes a crucial step in sludge treatment by reducing the water content, consequently minimizing its weight and volume, resulting in a more economical disposal operating cost. The yield of this process is commonly referred to as dewatered sludge (DS). DS potentially benefits industries and communities, as demonstrated by its conversion into fertilizers and application in various land uses, including agriculture, landfills and composting (Gerba & Pepper, 2009). There is also a growing interest in deriving renewable energy from DS as it has a high potential to be converted into energy and reduce hazardous emissions compared to fossil fuel (Yacob et al., 2006). However, when managing waste from various sources, sewage workers are routinely exposed to harmful components within the sewage or sewage sludge, including biological and chemical irritants such as toxic gases, pathogens, genotoxic agents, and harmful organic chemicals (Straub et al., 1993). Despite treatment, the sewage sludge might retain resilient pathogens and bacteria capable of surviving the current treatment methods. For example, certain pathogens, such as *C. perfringens*, can remain in treated sewage sludge, displaying resistance to various disinfection methods (Al-Gheethi et al., 2018; Alonso et al., 2004).

Furthermore, the hepatitis A virus (HAV) is stable in the environment for an extended period and is resistant to current wastewater treatment practices (Ouardani et al., 2016). These pathogens, associated with food poisoning and hepatitis, can be transmitted to humans through various routes, ingesting contaminated vegetables or water, inhalation or direct skin contact. Acute and continuous exposure to these harmful substances may initiate inflammatory reactions and enhanced nitric oxide or pro-inflammatory cytokines production, leading to various inflammation-related diseases such as skin irritation, pulmonary diseases, and even cancer (Straub et al., 1993).

Moreover, the implications of wastewater treatment plants (WWTPs) are noteworthy. These plants serve as a major reservoir of antimicrobial resistance (AMR) amplification because they provide an ideal environment for the survival of AMR bacteria (ARBs) with AMR genes (ARGs) (Osińska et al., 2020). AMR, also known as drug resistance, is widely recognized as a global threat to human health that demands immediate action in countries worldwide. AMR has emerged as a significant concern in recent years, as it could lead to a post-antibiotic era in which antibiotics are no longer effective, impacting humans, animals, and the environment (Hocking et al., 2021). Although the wastewater treatment process can aid in eliminating or minimizing the ARB load, some remaining surviving ARBs with non-biodegradable ARGs will spread the genes to other bacteria via horizontal gene transfer (HZT) (Sun et al., 2019). Ultimately, it will facilitate the transmission of pathogenic AMR or ARBs to animals and humans via several routes, endangering their health (Osińska et al., 2020). Consequently, the dissemination of AMR in the environment could result in the inability to effectively treat infections, exacerbating inflammation and leading to prolonged illnesses such as cancer (Michael et al., 2014; Mokhtar et al., 2022).

Therefore, an alternative is needed to reduce the human hazards stemming from the land application of DS, and one of the potential technologies is Microbial Fuel Cell (MFC). MFC is a bioelectrical device that operates electrochemically by incorporating electrogenic bacteria (EB), an organism that can transfer electrons to extracellular electron acceptors to generate electricity (Mahmoud et al., 2022; Obileke et al., 2021). Previous studies show the potential use of MFC treatment in providing electricity and reducing chemical oxygen demand (COD) in DS (Makhtar & Tajarudin, 2020; Makhtar & Vadivelu, 2019; Muaz et al., 2019). Additionally, MFC technology can also effectively replace conventional non-renewable resources such as natural gas and coal that are continuously depleted through time (Muaz et al., 2019) and could reduce the emission of greenhouse gases that can cause global warming and climate change (Anderson et al., 2016). In line with this, the utilization of EB, such as *B. subtilis*, demonstrated its capacity to secrete anti-inflammatory metabolites like exopolysaccharide (EPS), that can stimulate the development of anti-inflammatory M2 macrophages to inhibit T cell activation *in vivo* (Paynich et al., 2017; Rhayat et al., 2019).

Nevertheless, in the current research landscape, the emphasis within MFC studies has predominantly revolved around its electricity generation capabilities, leaving a notable gap in understanding its potential to mitigate DS-related human hazards. Hence, exploring the potential of MFC technology in DS treatment gained significant attention, particularly for its role in mitigating inflammation and countering the spread of antimicrobial resistance (AMR) in forthcoming research endeavors. Therefore, this study investigated the promise of ML-MFC as a functional approach to producing electricity and concurrently reducing the long-term risk of human hazards stemming from DS.

MATERIALS AND METHODS

Collection and Preparation of ML-MFC-treated DS Samples

DS was obtained from the Juru municipal wastewater treatment plant. The ML-MFC setup was constructed following Muaz et al. (2019)'s approach. *B. subtilis* served as the EB. This study considered two sets of samples: ML-MFC-untreated DS (T0) and ML-MFC-treated DS obtained after 120 hours (T120). 1 g of sample was mixed with 10 mL sterile distilled water. Subsequently, centrifugation at 3500 rpm for 15 min was conducted to obtain the supernatant. The supernatant was then filtered with a 0.22 µm membrane filter and stored in a -20°C freezer for further analysis.

Cell Maintenance

Mouse fibroblast, 3T3-L1 cell and hepatocellular carcinoma, Hep G2 cancer cell was maintained in Dulbecco's Modified Eagle Medium (DMEM) and Minimum Essential Medium (MEM), respectively, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotic, in the incubator with 5% CO_2 at 37°C. Cells were subcultured after reaching 80-90% confluency.

MTT Assay

The toxic effect of ML-MFC-treated DS on 3T3-L1 and Hep G2 cells was identified using an MTT assay adapted from Horiuchi et al. (1988) with slight adjustments. Briefly, cells were seeded in a 96-well plate at a cell density of 50,000 cells/mL overnight. The cells were treated with 25 mg/mL, diluted, filtered samples before being further incubated for 24 h. 5 mg/mL MTT solution was added to each well and incubated for another 4 h at 37°C. After 4 h, the media was removed, and DMSO solution was added to dissolve the MTT-formazan crystals. Finally, the absorbance value for each sample was measured at 540 nm with 620 nm as a reference. The percentage of cell viability of each sample was calculated as Equation 1:

$$\text{Percentage of cell viability} = \frac{(\text{A treatment} - \text{A blank})}{(\text{A control} - \text{A blank})} \times 100\% \quad [1]$$

where A = absorbance value for each sample

Protein Denaturation Assay

The ability of MFC technology to reduce DS-induced protein denaturation-related inflammation was determined via bovine serum albumin denaturation assay as described by Padmanabhan and Jangle (2012), with slight modifications. Firstly, the mixtures were prepared by mixing DS samples, 7% BSA solution and PBS solution (pH 6.9) before being heated at 75°C for 20 min. A mixture of PBS and BSA was used to form the control tube. Then, the mixtures were left to cool down at room temperature for 5 minutes. After

the mixtures were cooled down, each sample's absorbance values were measured at 660 nm in triplicate, and the readings were recorded. The percentage of inhibition of protein denaturation was calculated based on Equation 2 to identify the anti-inflammatory activity of the samples involved.

$$\% \text{ Inhibition of protein denaturation} = \left(1 - \left[\frac{\text{Abs (sample)}}{\text{Abs (control)}}\right]\right) \text{x } 100 \qquad [2]$$

Antibiotic Susceptibility Testing

The Kirby-Bauer disk diffusion susceptibility test was performed to determine the sensitivity or resistance of bacteria to various antibiotics in ML-MFC-treated DS samples. Firstly, the ML-MFC-treated DS samples were diluted up to 10^{-3} dilutions. The bacterial colonies were then isolated using the spread plate method and incubated at 37°C for a week. The well-isolated bacterial colonies obtained from the isolation step were further purified on the streak plate and then further incubated overnight. After incubation, a loopful of pure culture from different dewatered samples was diluted and spread onto the nutrient agar plate. Then, the antibiotic discs (streptomycin sulfate, kanamycin monosulphate, tetracycline free base, chloramphenicol, amoxicillin, penicillin G, and ampicillin) were applied to the nutrient agar plate, followed by incubating the plate in the incubator at 37°C for 24 h. After 24 h, the diameter of the zone of inhibition (ZOI), in mm, for each antibiotic was measured and determined according to the guidelines published by the Clinical and Laboratory Standards Institute (CLSI, 2020). Zone diameters of susceptibility testing results were categorized as sensitive, intermediate, or resistant based on the CLSI breakpoint.

Bacterial Identification of Selected Isolates

The colony PCR method was used to identify the selected isolates genetically. The 16S rRNA gene of selected bacteria was amplified by using a forward primer (27F: 5'-AGAGTTTGATCCTGGCTCAG-3') and a reverse primer (1492R: 5'-GGTTACCTTGTTACGACTT-3'), as described by Lane (1991). The quality of the PCR product was quantified using NanoDrop (Thermo Fisher Scientific). Centre for Chemical Biology (CCB) USM performed gene sequencing. Sequences obtained were run in the Basic Local Alignment Search Tool (BLAST) feature from the National Center for Biotechnology Information (NCBI) to find regions of local similarity between sequences for identification.

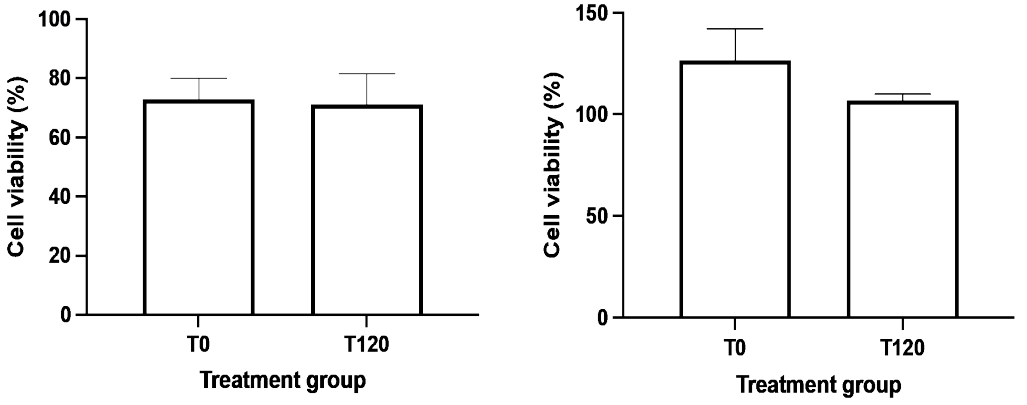
Statistical Analysis

The statistical analysis was performed using GraphPad Prism version 9.2.0. All results were presented as mean ± standard deviation (SD) of three independent experiments. An unpaired t-test was used to compare the means between groups with a *p*-value less than or equal to 0.05 ($p \leq 0.05$) considered significant.

RESULTS AND DISCUSSION

ML-MFC-treated DS Showed Slight Toxicity to Cancer Cells

ML-MFC-treated DS at 120 h was chosen in this research because it was when the power generation and biomass were in the log phase (Makhtar et al., 2021). Besides, according to Sabri et al. (2021), MFC-treated DS at 120 h (day 5) shows an increment of voltage at peak (88 mV). It indicates that *B. subtilis* reacted positively in ML-MFC on day 5, resulting in the fast growth of the bacteria and high voltage output due to the acceleration of the metabolic rate of *B. subtilis*. The toxic effect of ML-MFC-treated DS on normal cell, 3T3-L1 cell was identified using MTT assay for 24 h. As shown in Figure 1, both samples show more than 60% cell viability, with 67.74% for T0 and 65.99% for T120, respectively. There was no statistical difference between both samples, indicating that with or without ML-MFC treatment, there was no toxic effect on normal cells.



(a) (b)

Figure 1. The determination of cell viability percentage using MTT assay for (a) 3T3-L1 and (b) Hep G2 cells. Values were expressed as the mean ± standard deviation (n = 3). T0: untreated DS at 0 h; T120: MFC-treated DS at 120 h.

Next, the effect of ML-MFC-treated DS on hepatocellular carcinoma cell toxicity, Hep G2, was determined using an MTT assay for 24 h. According to Figure 1, although it is not significant, there is a slight decrease in cancer cell viability after being treated with T120. Hence, the data suggests that ML-MFC treatment can potentially reduce the number of cancer cells. The inhibitory effect observed in ML-MFC-treated DS may be due to the activity of *B. subtilis*-secreted bacteriocins such as lipopeptides, surfactin, and iturin (Zhao et al., 2018). This bacteriocin was found to kill cancer cells such as K562 (myelogenous leukemia cells) (Zhao et al., 2018), LoVo (human colon carcinoma cell) (Kim et al., 2007), MCF-7 (breast cancer cells) (Cao et al., 2010), and 95D (human lung cancer cell line) (Yin et al., 2013). Bacteriocin exerts its activity by inducing paraptosis, a planned cell death characterized by cytoplasmic vacuoles and swelling of the mitochondria and endoplasmic

reticulum (ER). It is comparable to cell necrosis, but necrosis is generally followed by blebbing of the cell membrane (Zhao et al., 2018). Kim et al. (2007) also reported that surfactin from *B. subtilis* has an anti-proliferation effect on LoVo cells by promoting pro-apoptotic activity and interrupting the cell cycle. Nevertheless, the effect was expected to increase if we prolonged the incubation time, such as up to 72 h. However, caution must be taken as normal cells might also be affected.

Potential Anti-Inflammatory Properties of ML-MFC-Treated DS

Protein denaturation has been linked to inflammation and the development of various inflammatory-associated-disorders (Osman et al., 2016). During protein denaturation, the biological characteristics of a protein are impaired, contributing to a disruption in its functional activity. Therefore, a BSA protein denaturation assay was performed to determine the potential of ML-MFC-treated DS in reducing protein denaturation and, hence, its ability to reduce inflammation. Interestingly, as *B. subtilis* has been supplemented as a catalyst in the ML-MFC treatment process, the macromolecules or metabolites secreted by *B. subtilis* may have some anti-inflammatory properties. As postulated, Figure 2 shows an increasing percentage of protein denaturation inhibition with increased treatment times, wherein 32.77% and 62.43% were recorded for T0 and T120, respectively. The high percentage of protein denaturation inhibition observed on day five is probably due to the increased metabolic rate of *B. subtilis*, which leads to the high secretion of macromolecules or metabolites with anti-inflammatory properties (Sabri et al., 2021).

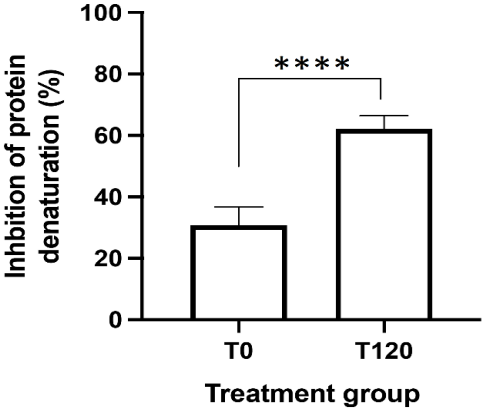


Figure 2. Protein Denaturation inhibition of ML-MFC-treated DS. An unpaired t-test was used to compare the means between groups with **** $p \leq 0.0001$. Values were expressed as the mean ± standard deviation (n = 3). T0: untreated DS at 0 h; T120: MFC-treated DS at 120 h.

Based on Figure 2, the T0 sample recorded a lower percentage of protein denaturation inhibition than the ML-MFC-treated DS. Nevertheless, treatments with ML-MFC significantly decreased protein denaturation compared to the untreated samples. The release of anti-inflammatory metabolites, EPS by *B. subtilis* that act as a catalyst during the ML-MFC treatment process might contribute to it. EPS are high-molecular-weight polymers produced by microorganisms outside their cell wall. EPS may stabilize the BSA protein by modifying its functional properties, leading to lower protein denaturation levels (Deep et al., 2012). It is supported by the high percentage of protein denaturation inhibition compared to the untreated.

ML-MFC Technology Promotes Bacteria Sensitivity Against Antibiotics

Eight types of antibiotics were involved in the testing, including streptomycin sulfate, kanamycin monosulphate, tetracycline free base, chloramphenicol, amoxicillin, penicillin G, and ampicillin. The distilled water and ethanol extract acted as the negative.

The diameter of the zone of inhibition (ZOI), in mm, for each antibiotic was measured and compared to the CLSI breakpoint criteria published by CLSI to determine the bacteria's susceptibility to specific antibiotics. Table 1 shows the size of the ZOI for each sample and control.

Table 1

Size of the zone of inhibition (ZOI) for negative control and different antibiotics

Group	Size of Zone of Inhibition (ZOI) (mm)		Antibiotic Resistance (Sensitive/Intermediate/ Resistant)	
	Sample			
	T0	T120	T0	T120
	Mean ± S.D	Mean ± S.D		
Negative Control				
Distilled Water (DW)	-	-	-	-
Ethanol (EtOH)	-	-	-	-
Antibiotics				
Streptomycin sulfate (S)	20 ± 0	25 ±1.41	**Sensitive**	**Sensitive**
Kanamycin mono sulfate (K)	28 ± 2.83	30 ± 2.83	**Sensitive**	**Sensitive**
Amoxicillin (AX)	20 ± 0	20 ± 0	**Sensitive**	**Sensitive**
Penicillin (P)	27 ± 1.41	33 ± 1.41	**Resistant**	**Sensitive**
Ampicillin (Amp)	-	24 ± 0	**Resistant**	**Sensitive**
Chloramphenicol (C)	33 ± 2.83	18 ± 0	**Sensitive**	**Sensitive**

Based on the disk diffusion antibiotic susceptibility test, the bacteria growth in the T0 and T120 samples were generally sensitive to Streptomycin sulfate (S), Kanamycin monosulphate (K), Amoxicillin (AX) and Chloramphenicol (C). It can be seen with a larger zone of inhibition around an antibiotic-containing disk than the standard zone of inhibition, indicating the presence of ARBs with ARGs (supplementary data). Interestingly, the bacterial colonies isolated from the T0 sample were shown resistance to both Penicillin (P) and Ampicillin (Amp). However, after treatment with ML-MFC, the bacteria found

in the T120 sample were susceptible to them, indicating that the ML-MFC technology is beneficial in reducing ARBs with ARGs, which could help reduce AMR distribution to the environment.

It is probably due to MFC's ability to break down antibiotics and ARGs, as proven by Ondon et al. (2020) and Xue et al. (2019), who found MFC technology to be able to remove 85.1% and 65.5% of sulfamethoxazole (SMX) and norfloxacin (NFLX), respectively. Additionally, the number of ARGs and integrons after MFC treatment was significantly less than that discovered in WWTPs. For example, the relative abundance of the *intI1* is between 63.11 and 652.00 copies/mL(g) in the MFC product compared to 109 to 1011 copies/mL in WWTPs (Chen et al., 2021; Mandal & Das, 2018).

MFC's ability to promote the susceptibility of certain bacteria in DS might also be due to the release of certain bacteriocin from *B. subtilis* that will help kill those ARBs (Joseph et al., 2013). Bacteriocins are a group of ribosomally synthesized antimicrobial peptides produced by bacteria, capable of controlling bacterial pathogens and clinically relevant susceptible and drug-resistant bacteria (Benítez-Chao et al., 2021). Alternatively, *B. subtilis* produces subtilin and subtilosin, which are active against many strains of gram-positive bacteria and act as antimicrobial or killing peptides, directly inhibiting competing strains or pathogens (Joseph et al., 2013).

Microbes Isolated from MFC-Treated DS and Its Potential as Anti-Inflammatory Agents

Four isolates were obtained from the T120 sample, and upon analysis, they were found to share more than 90% similarities with *Bacillus licheniformis* (*B. licheniformis*), *Bhargavaea beijingensis* (*B. beijingensis*), *Oceanobacillus caeni* (*O. caeni*) and *Oceanobacillus caeni* (*O. caeni*) (Table 2).

Interestingly, among these bacteria, *B. licheniformis* is known as one of the most important bacteria to produce bacitracin to combat pathogens, promote balance in the intestinal flora, and also enhance the nutritional quality of animal feeds (Lan & Kim, 2019; Wang et al., 2020). In addition, *B. licheniformis* has also been shown to exhibit anti-inflammatory properties by reducing the production of pro-inflammatory cytokines, Interleukin (IL)-8, upon induction with *Salmonella enterica* serovar Typhimurium (Aperce et al., 2010). Other microbes isolated from ML-MFC-treated DS were *B. beijingensis* and *O. caeni*. There were no reports on *O. caeni's* benefits in regulating inflammation However, interestingly, *B. beijingensis* DSM19038, isolated from the internal tissue of ginseng roots, was shown to affect nitrite production (Verma et al., 2012), suggesting its potential as an anti-inflammatory agent.

Intriguingly, the absence of isolated *B. subtilis* from the DS sample, particularly at T120, can be attributed to the complex and competitive nature of the microbial community present

within the DS. This environment fosters intense competition among various microorganisms for limited resources, potentially leading to the dominance of certain species (Bauer et al., 2018). Notably, it is conceivable that *B. licheniformis* could outcompete *B. subtilis* and other bacterial counterparts, further influencing the composition of the microbial community observed. This phenomenon underscores the dynamic interplay between microbial species within the DS ecosystem, where ecological factors and resource availability shape the prevalence of specific bacteria over others. In addition to the observed outcomes, there is potential for improvement by employing bacterial identification of the chosen isolates through 16S rRNA gene sequencing at multiple time points rather than solely relying on a single time point. This expanded approach would provide a more comprehensive understanding of the dynamic changes in the microbial composition within the DS ecosystem over time.

Table 2

16S rRNA identification of bacteria isolated from ML-MFC-treated DS at 120 h

Test Strain	Representative species	Percentage similarity (%) (BLAST)	Query Coverage (%)	Proven Anti-inflammatory properties	References
A	*Bacillus licheniformis*	90.80	13	Decrease Salmonella-induced 1L-8 secretion Improve subhealth state by reshaping gut microbiota, lowering inflammation, suppressing hypothalamic-pituitary-adrenal axis hyperactivity, regulating neurotransmitter levels, and relieving a negative mood. Enhance isovaleric acid in the colon, which alleviates abdominal pain and diarrhea.	Aperce et al. (2010); Feng et al. (2022); Lan and Kim (2019); Li et al. (2022); Roselli et al. (2017)

Table 2 *(Continue)*

Test Strain	Representative species	Percentage similarity (%) (BLAST)	Query Coverage (%)	Proven Anti-inflammatory properties	References
B	*Bhargavaea beijingensis*	98.05	97	Positive for nitrite reduction	Verma et al. (2012)
C	*Oceanobacillus caeni*	97.69	94	N/A	
E	*Oceanobacillus caeni*	96.67	96	N/A	

Note. Not applicable denoted by N/A

CONCLUSION

To conclude, ML-MFC technology demonstrates remarkable anti-inflammatory properties in a time-dependent manner, exhibiting its highest inhibitory effect on protein denaturation (62.43%) at T120. Furthermore, this treatment successfully restored the sensitivity of previously resistant bacteria to penicillin and ampicillin. Additionally, ML-MFC-treated DS exhibited minimal cytotoxicity towards normal cells while demonstrating moderate effectiveness against cancer cells, thus indicating its potential as an anti-cancer agent. These noteworthy benefits may be attributed to the secretion of numerous metabolites, including but not limited to bacteriocins, subtilin, EPS, and subtilosin. However, further in-depth research, encompassing both *in vitro* and *in vivo* studies, is necessary to fully elucidate the underlying mechanisms responsible for these properties. Undoubtedly, this research holds significant promise for applications in healthcare and pharmaceuticals.

ACKNOWLEDGEMENT

This work was supported by Universiti Sains Malaysia (Apex ERA) (Grant numbers No. 1001.PTEKIND.881006) granted to Ana Masara Ahmad Mokhtar (AMAM), Universiti Sains Malaysia, Penang, Malaysia.

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