JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

ISOLATION AND CHARACTERIZATION OF ACTIVE METABOLITES PRODUCED FROM PROBIOTIC ISOLATES AGAINST DANDRUFF CAUSING *MALASSEZIA FURFUR* (MTCC: 1374^{T})

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https://doi.org/10.15414/jmbfs.3522

ARTICLE INFO

Received 29. 7. 2020
Revised 19. 12. 2020
Accepted 30. 12. 2020
Published 1. 6. 2021

Regular article

OPEN ACCESS

ABSTRACT

Dandruff is commonly known as seborrheic dermatitis, a common scaling condition, causing itching and discomfort. Anti-dandruff shampoos contain compounds harmful to the environment and humans; therefore, a solution with potential anti-dandruff activity with minimal or no harm is needed for an hour. This study aims to screen and identify the likely anti-dandruff probiotic organisms and characterize their active metabolites. Three morphologically different probiotic microorganisms were isolated from local dairy products and identified by gram staining. 16S rDNA of the isolates were amplified and sequenced for molecular identification. 16S rDNA sequences confirmed the isolates as *Lactobacillus rhamnosus* (98%), *Enterococcus faecalis* (98%), and *Enterococcus faecium* (97.65%). Phylogenetic analysis reveals that *L. rhamnosus* (MK951691) indicated high sequence similarity with *L. paracasei.* Similarly, *E. faecium* (MK951690) represented close similarity with *E. faecalis* (MK951689). A maximum zone of inhibition of 7 mm was recorded by the extracellular fractions of bacterial isolate *L. rhamnosus*, followed by the other two isolates of *Enterococcus* sp. The MIC value of the extracellular fraction of probiotic bacterial strains was optimized to be 100 mg/mL. GCMS analysis revealed that all three extracellular bacterial isolates had propionic acid, lactic acid, phenol, 2,4-bis(1,1-dimethyl ethyl), hexadecanoic acid, octadecanoic acid, and 3-isobutyl hexahydro pyrrolo [1,2-a]pyrazine-1,4-dione. In conclusion, these isolates are enriched with antifungal components, and hence, they could be considered an antifungal target against *Malassezia furfur.*

Keywords: Antifungal activity; Dandruff*;* Extracellular compounds; *Malassezia furfur;* Probiotics

INTRODUCTION

Dandruff (seborrheic dermatitis) is a common relapsing inflammatory scalp scaling condition that causes discomfort and an unpleasant image. Dandruff is also a sign of several immune system disorders. Dandruff leads to an itchy scalp, hair fall, acne formation, recurrent ear eczema, and dead skin build-up, making it essential to get rid of it. The etiological factors of dandruff include Malassezia species, sebaceous secretions, and individual susceptibility **(Kim *et al.*, 2016)**. *M. furfur (Pityrosporum ovale)*, a lipophilic yeast-like fungus that causes seborrheic dermatitis pityriasis Versicolor and Tinea circinata **(Kindo *et al.*, 2004)**. *M. furfur*, the primary dandruff-causing agent, can be isolated from scalps in significantly high amounts. *M. furfur* secretes lipases that contribute to dandruff formation. Most anti-dandruff shampoos and lotions consist of chemicals such as ketoconazole, zinc pyrithione, and selenium sulfide, which are harmful to the environment as well as humans.

As per the approved definition by the United Nations Food and Agriculture Organization and the World Health Organization, probiotic organisms are live microorganisms, thought to be safe and beneficial to the host organism beyond essential nutrition **(WHO, 2001)**. Probiotic bacteria play an essential role in maintaining the human intestinal tract, promote proper digestion, boost immune function, increase resistance to infection, inhibit the growth of harmful bacteria, and maintain a balance in the intestinal microflora **(Helland *et al.*, 2004; Sieladie *et al.*, 2011)**. The common probiotic bacteria are *Lactobacillus, Propionibacterium, Peptostreptococcus, Bacillus, Lactococcus, Enterococcus, Streptococcus, Bifidobacterium,* and *Bacteroides* **(George Kerry *et al.*, 2018)**. Various commercial probiotic preparations are available in the market in the form of capsules, liquid/gel, and powdered that claim to prevent infectious diseases **(Simova *et al.*, 2009)**. Probiotics have been shown to cure skin conditions such as acne, eczema, rosacea and restore hair growth.

The effects of the probiotic species on atopic dermis were observed by **Kim and Ji, (2012)**. The result of probiotics on *Salmonella enterica* and *Clostridium difficile* in an *in vitro* model showed that probiotics inhibit the pathogen by producing short-chain fatty acids (lactic acid, acetic acid, propionic and butyric acid). It helps to maintain the pH in the colonic lumen **(Tejero-Sarinena *et al.*, 2013)**. Probiotics compounds like peptides and bacteriocins were mainly involved in membrane permeability **(George Kerry *et al.*, 2018)**. The bacterial STDs like Gonorrhea and Chlamydia are treated using *Lactobacillus* species by inhibiting the vaginal microbial flora via the supplements of probiotics **(Waigankar and Patel, 2011)**. Probiotic bacteria produced metabolites that are useful in treating skin disorders. Probiotic bacteria are promising alternatives to harmful chemical compounds, as the antimicrobial compounds have the potential to suppress food-borne yeasts and molds **(Gajbhiye and Kapadnis, 2016)**. Therefore in the present study, isolation and identification of anti-dandruff probiotics strains were carried out, and their active metabolites for antifungal activity were characterized.

MATERIALS AND METHODS

Strains and Medium used

The fungal culture *M. furfur* 1374^{T} used in this study was obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India. Emmons modified Sabouraud's agar (Dextrose - 20g/L, Peptone - 10g/L, Agar - 17g/L, Corn oil - 1 mL/L) at pH 5.5 was used for culturing *M. furfur* 1374^{T}. De Man Rogosa and Sharpe (MRS) agar were used for culturing probiotic strains. All the chemicals used in the study were obtained from HiMedia Pvt.Ltd, India.

Screening and Characterization of Probiotic Strains

Commercial probiotic samples of curd, yogurt, and probiotic tablets were collected from the local market, Guduvancherry, Chennai, India. The samples were serially diluted and plated on MRS agar. The plates were incubated at 37°C for 48 h. Morphologically distinct colonies were selected and subjected to Scanning Electron Microscope analysis (SEM). Whole cells were used for SEM analysis by pelleting liquid cultures of all isolates. The cell pellet dissolved in PBS was placed on round glass coverslips and left to air dry. The fixed samples

were coated with Au (Sputter-coater Quorum SC 7620) and viewed under SEM (TESCAN VEGA3, SBH, Tescan, Brno, Czech Republic).

Molecular Characterization of Bacterial isolates

The isolated strains active against *M. furfur* were selected and subjected to 16s rDNA amplification and sequencing. The PCR amplification of the 16srDNA region was performed in Eppendorf Master cycler nexus GX2 thermocycler (Germany) using the universal forward primer (5'AGAGTTTGATCCTGGCTCAG 3') and the reverse primer (5′ ACGGCTACCTTGTTACGACTT 3′) as reported by **Weisburg *et al.*, (1991)**. Colonies were subjected to the heat lysis method described by **Alam *et al.*, (1999)** and used as a template for the PCR amplification reaction. The reaction conditions were initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min 30 sec, extension at 72 °C for 1 min 30 sec, and a final extension at 72 °C for 2 minutes. PCR products were visualized on 1 % agarose gel stained with ethidium bromide (0.5 µg/mL).

Sequencing and Phylogenetic analysis

The purified PCR products were sequenced by AgriGenome Labs Pvt. Ltd, Kochi, Kerala, India. The sequences obtained were then processed using BioEdit, a biological sequence alignment editor tool (http://www.mbio.ncsu.edu/bioedit/bioedit.html), which generated contig sequences for each primer pair **(Hall, 1999)**. *Lactococcus lactis* (NR103878.1) and *Myroides odoratimimus* (KU382740.1) are considered as outgroups for this study. This contig sequence was then compared with the 16S ribosomal RNA sequences (Bacteria and Archaea) database of NCBI using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the most similar sequences were found out and sorted by the E score. The first fifteen similar sequences were aligned using ClustalW, and a phylogenetic tree was generated using MEGA7 software **(Kumar *et al.*, 2016)**.

Agar well diffusion assay

Intracellular and extracellular fractions of isolated probiotic strains were checked against *M. furfur* by agar well diffusion assay **(Bulgasem *et al.*, 2016)**. Emmons modified Sabouraud's agar plates, and the mid-log phase culture of *M. furfur* was swabbed on the plate. The agar plates were allowed to dry, and 9 mm diameter wells were made with sterile borer on inoculated plates. 100 µL of extracellular and intracellular fractions were loaded in the respective wells on the plates. The plates were incubated for seven days at 30 °C. The zone of inhibition (mm) was measured. This assay was performed in triplicates.

Minimum Inhibitory Concentration (MIC) assay

The microdilution method was performed with 96 microtiter plates to determine the minimum inhibitory concentration of cell-free Supernatants (CFS) of bacterial isolates **(Leong *et al.*, 2017)**. Different concentrations of lyophilized CFS as 10, 30, 50, 70, 90,100 µg/mL were dissolved in DMSO and used as test samples. Positive control contained 160 µL of Emmons modified Sabouraud's broth, 10 µL of climbazole drug (2 mg/mL), 10 µL of *M. furfur*, and 20 µL of resazurin dye (1 mg/mL). The negative control contained 170 µL of Emmons modified Sabouraud's broth, 10µL of *M.furfur* culture, and 20 µL of resazurin dye (6.75 mg/mL^{-1}). The microtiter plate was incubated at 30 °C for 48 h. The minimum inhibitory concentration values for the extracellular fractions of isolates dissolved in DMSO were determined by measuring OD_{540} and OD_{590} using a multimode plate reader (BioTek, USA).

Gas Chromatography-Mass Spectrometry (GCMS) analysis

Culture supernatants of the isolates were extracted with ethyl acetate and subjected to GCMS analysis. These extracts were analyzed on an Agilent Technologies 7890B GC with FID. The software used for the study of extracted metabolites was Mass hunter software. The GC was mounted with an HP-5MS column with a length of 30 m, width - 0.25 mm, and film thickness of 0.1µm. Helium was used as carrier gas flowing at 0.8 mL/min. 1µL of samples were injected with a split ratio of 1:100 in an injector kept at 250 °C. The temperature of the flame ionization detector was held at 240 °C. The temperature gradient was used for low boiling metabolites; the oven was held at 40 C for 2 min initially, then increased to 240 °C at a rate of 20 °C/min. For metabolites with higher boiling temperature, the oven initially held at 150 °C for 2 min, then increased to 240 °C at the rate of 20 °C/min. It was held at 240 °C for 10 min **(Honore *et al.*, 2016)**.

RESULTS AND DISCUSSION

Morphological and Molecular Identification of the Isolates

A total of three probiotic organisms were isolated from the local dairy products. The organisms' morphological characteristics belong to the genus *Lactobacillus* (1) and *Enterococcus* (2). Further morphological characterization of these bacterial isolates was analyzed by SEM. SEM analysis showed that *Enterococcus* species (Fig.1A and B) were found to be cocci, and *Lactobacillus* species (Fig.1C) was a rod in shape. The DNA from the isolates were collected and subjected to molecular identification. The 16S rDNA gene of 1500 bp was amplified from all three bacterial isolates and confirmed on 1% agarose gel stained with ethidium bromide (0.5 µg/mL) (Fig.2). Following BLASTN analysis, similar sequences were retrieved from Genbank, and a phylogenetic tree was generated using the neighbor-joining method. These isolates showed high similarities with *L. rhamnosus* (MK951691) (98 %), *E. faecium* (MK951689) (97.65 %), and *E. faecalis* (MK951690) (98 %). All the sequences were deposited to Genbank with the accession numbers mentioned above. The phylogenetic analysis of *L. rhamnosus* indicated high sequence similarity with *L. paracasei* strain NBRC 15883 (Fig.3a). **Smokvina *et al.*, (2013)** stated that *L. paracasei* and *L. zeae* and *L. rhamnosus* were closely related species *E. faecium* indicated high sequence similarity with *E. faecalis* (98 %) (Fig.3b). Castillo-Rojas et al. (2013) reported that *E. faecalis* and *E. faecium* have the highest sequence similarity, up to 84.3 %. *M. odoratimimus* and *L. lactis* were considered as outgroups.

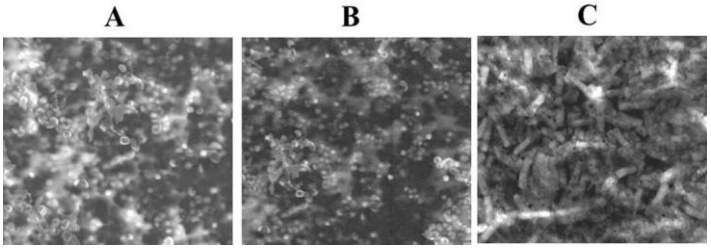


Figure 1 Scanning electron microscopy micrographs of probiotic isolates: *Enterococcus* species (A and B), (C) *Lactobacillus* species. Scale bar: 30 µm; Magnification: 5000x.

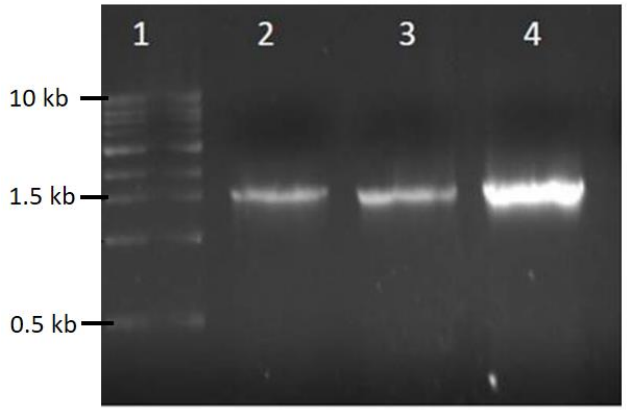


Figure 2 Agarose gel electrophoresis of colony PCR amplified 16s rDNA region of bacterial isolates, 1 - 1 Kb ladder, 2 - *E. faecium*, 3- *E. faecalis*, 4 -*L. rhamnosus.*

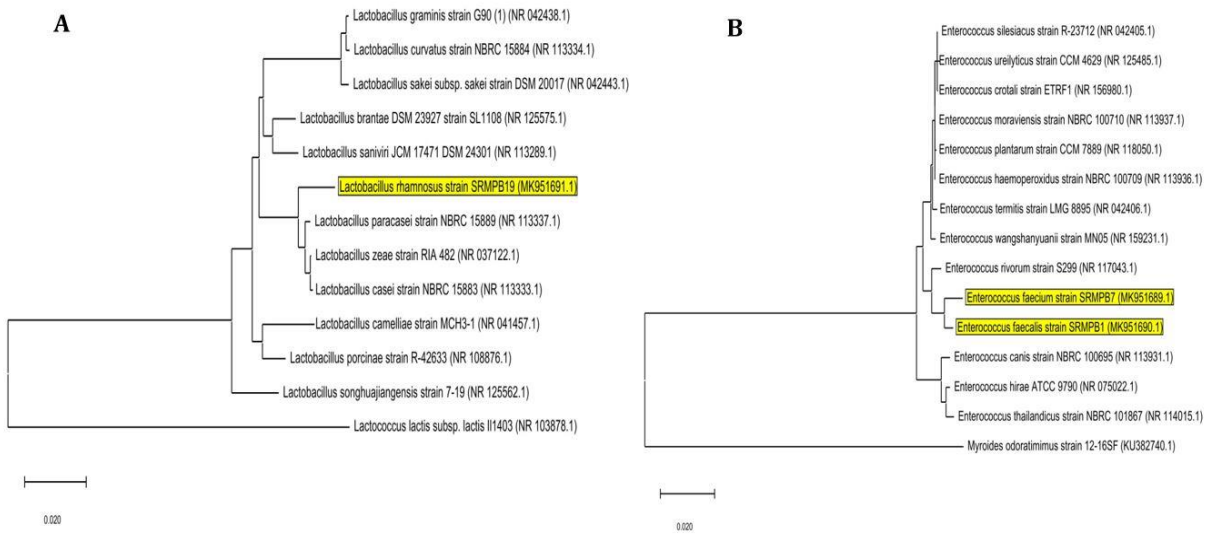


Figure 3 Phylogenetic analysis of 16S rDNA sequences of the isolates (A) *L. rhamnosus* (B) *E. faecium and E. faecalis*

Agar well diffusion assay

Probiotic organisms were isolated from local dairy products, including curd, yogurt, and probiotic tablets, and screened for antifungal activity against *M. furfur*. Our results showed that extracellular fractions of the probiotic isolates had an inhibitory effect against *M. furfur*. The zone of inhibition was recorded in diameters (mm), and the maximum inhibition of 7 ± 0.5 mm was observed by the extracellular fractions of bacterial isolate *L. rhamnosus*, followed by *E. faecium* (5 ± 0.3 mm) and *E. faecalis* (3.5 ± 0.2 mm) respectively (Fig.4). Climbazole (50 mg/mL) used as a positive control, showing an inhibitory zone of 8 ± 0.2 mm. No zone of inhibition was confirmed by the intracellular fractions of the bacterial isolates, indicating that intracellular components do not affect the growth of *M. furfur*. **Karami *et al.*, (2017)** reported that *Lactobacillus* sp. from dairy products were able to inhibit the growth of *S. typhimurium* (2.8 mm) *and S. aureus* (8 mm). *L. rhamnosus* showed the highest inhibitory activity on *S. equinus*, as reported by **Masalam *et al.*, (2018)**. A similar study was done by **Nami *et al.*, (2019)** showed that the *Enterococcus sp*. had a strong inhibitory effect on *E. coli* (33.3 mm), *S. flexneri* (22 mm), *B. subtilis* (7.9 mm) and *S. aureus* (7.4 mm). The inhibition property of *Enterococcus* sp. could be due to the production of *bacteriocin* **(Hassanzadazar *et al.*, 2014)**.

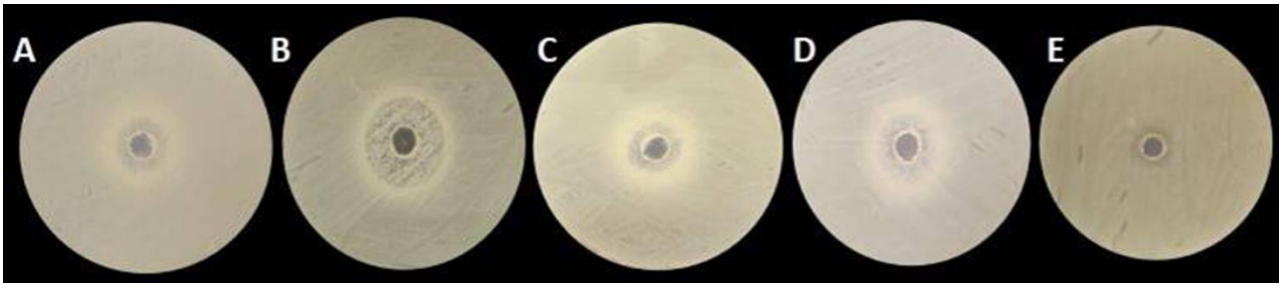


Figure 4 Agar well diffusion assay for the extracellular fractions of bacterial isolates, A) positive control Climbazole (50 mg/mL), B) *L. rhamnosus*, C) *E. faecium*, D) *E. faecalis*, E) Negative control (MRS broth)

Minimum Inhibitory Concentration (MIC) assay

The isolates were subjected to an antifungal susceptibility test using the microdilution method. The MIC_{50} value for the strains was measured by subtracting OD_{540} from OD_{590}. The MIC for all three isolates were found to be 100 mg/mL (Fig.5). The standard drug, Climbazole, used in the study, had a MIC value of 2 mg/mL. Antimycotic activity of fluconazole was found to be 2 μg/mL. In a similar study by **Arena *et al.*, (2016),** CFS of *L. plantarum* strains could inhibit the growth of food-borne pathogens at ≥ 25 % (25:75, CFS: growth medium). The antifungal activity of the cell-free supernatant of *L*actobacillus species against different fungi (*A. niger*, *A. fumigatus*, *C. albicans*, *F. verticillioides*, *F. solani*, *P. chrysogenum*, *Botrytis elliptica*) and maximum growth inhibition was recorded by *B. elliptica* (79.58 %) followed by *A. fumigatus* (67.3 %) **(Deepthi *et al.*, 2016)**. In the study by **Valan Arasu *et al.*, (2013)** 20 % of CFS obtained from *L. brevis* FF2 inhibited the growth of moulds of *A. flavus* and *A. carbonarius. L. brevis KR3, KR4 and KR51* isolated from "katak", completely suppress the growth of *P. claviforme*, *A. awamori*, and *A. niger* but exhibited a lower inhibitory activity of *A. flavus and T. viride* **(Tropcheva *et al.*, 2014)**.

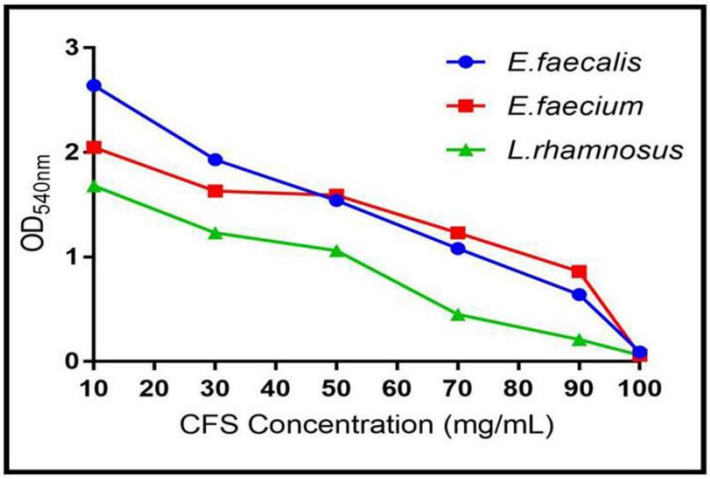


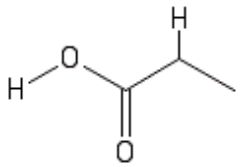
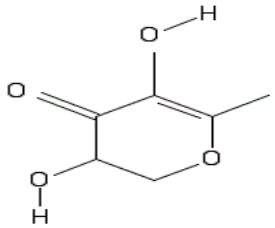
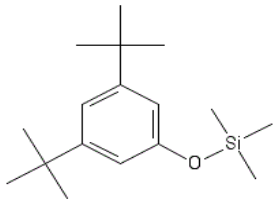
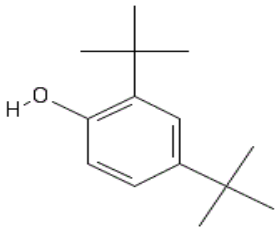
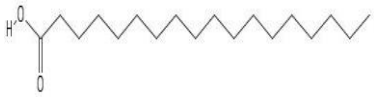
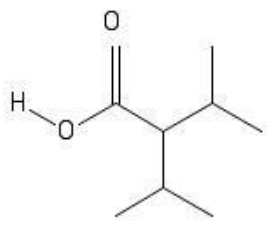
Figure 5 MIC analysis for extracellular fractions of probiotic bacterial isolates against *M. furfur*

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GCMS analysis of the antifungal probiotic extracts of three bacterial isolates consisted of propionic acid, lactic acid, hexadecanoic acid, Octadecanoic acid, phenol, 2,4-bis(1,1-dimethyl ethyl), and 3-Isobutyl hexahydro pyrrolo [1,2-a]pyrazine-1,4-dione (Table 1). Our study showed that *L. rhamnosus* and *E. faecium* produce all five extracellular volatile compounds. 3-Isobutyl Hexahydro

Pyrrolo[1,2-a]pyrazine-1,4-dione was found to be a major antifungal active metabolite in *E. faecalis*. Similar to our study, propionic acid possesses antimicrobial activity against pathogenic bacteria *S. typhimurium* via inhibition and expression of genes in *Salmonella* **(Saad *et al*., 2010)**. A recent study reported that lactic acid produced by CFS of *L. plantarum* and *L. buchneri* could inhibit *P. nordicum* by the production of Ochratoxin A **(Guimaraes *et al*., 2018)**. Similarly, decanoic acid production by *L. plantarum* was already proven to inhibit food spoilage fungi *A. fumigatus* and a film-forming yeast *Pichia kudriavzevii* GY1. The inhibitory mechanism of decanoic acid could be due to the detergent-like properties that damage organisms' cellular membranes **(Ryu *et al*., 2014)**. Moreover, the volatile compounds such as acetic acid, lactic acid, propionic acid, octadecanoic acid, and eicosanoic acid produced by *L. pentosus* showed activity against filamentous fungi **(Lipińska *et al*., 2018)**. The phenol-2,4-bis (1,1-dimethyl ethyl) reported to inhibit sporulation in *Alternaria solani* **(Gao *et al*., 2017).** 3-Isobutyl Hexahydro Pyrrolo [1,2-a]pyrazine-1,4-dione is a major compound found in all three bacterial isolates. Moreover, this compound was already reported against rice pathogen *Pyricularia oryzae* **(Awla *et al*., 2016)**. *L. plantarum* has been studied to show inhibitory activity against many fungal species through the production of phenolic compounds in a study by **Valan Arasu *et al*., (2013)**.

Table 1 - Extracellular antifungal volatile compounds produced by bacteria *E. faecium, E. faecalis,* and *L. rhamnosus.*

COMPOUND IDENTIFIED	ORGANISMS	MOL. FORMULA	MOL. WEIGHT	STRUCTURE
Propionic acid	*E. faecium* and *L. rhamnosus*	$C_3H_6O_2$	74.08	
Lactic acid	*E. faecium* and *L. rhamnosus*	$C_3H_6O_3$	90.08	
Phenol, 2,4-bis(1,1-dimethylethyl)	*E. faecium* and *L. rhamnosus*	$C_{22}H_{30}O$	206.32	
Hexadecanoic acid	*E. faecium* and *L. rhamnosus*	$C_{16}H_{32}O_2$	256.4	
Octadecanoic acid	*E. faecium* and *L. rhamnosus*	$C_{18}H_{36}O_2$	284.48	
3-Isobutyl Hexahydro Pyrrolo[1,2-a]pyrazine-1,4-dione	*E. faecalis*, *E. faecium* and *L .rhamnosus*	$C_{11}H_{18}N_2O_2$	210.27	

CONCLUSION

The three different bacterial species like *L. rhamnosus*, *E. faecalis*, and *E. faecium* were isolated *from* dairy products and identified based on 16S rDNA sequences. The extracellular fractions of the isolates were shown potential probiotic activity against dandruff, causing *M. furfur*. All three strains produced more amounts of acidic metabolites that could be responsible for the production of antifungal activity**.** The overall result suggested that the identified components can be incorporated in anti-dandruff formulations as they have less harmful effects than the conventional anti-dandruff drugs. Further studies have to be carried out in the purification and stability analysis of the metabolites.

Conflict of interest: The authors declare that they have no conflict of interest.

Acknowledgments: The authors acknowledge the SRM Institute of Science and Technology, Kancheepuram district, Tamil Nadu, for providing the facilities to utilize the research.

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