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Article

Metabolites from *Lactococcus lactis* subsp. *lactis*: Isolation, Structure Elucidation, and Antimicrobial Activity

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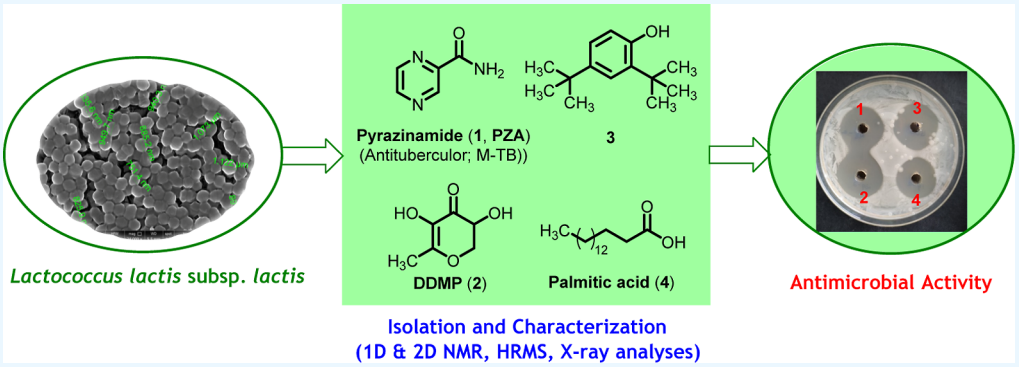

ABSTRACT: Herein, we disclose the identification of novel metabolites from a potential probiotic strain, *Lactococcus lactis* subsp. *lactis*, obtained from traditional dairy milk samples collected in Maharashtra, India (in January 2021). Isolated metabolites include pyrazin-2-carboxamide [**1**, pyrazinamide, a potential antitubercular drug], 3,5-dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one (**2**, DDMP), 2,4-di-*tert*-butylphenol (**3**), and hexadecanoic acid (**4**, palmitic acid). The chemical structures of these metabolites were elucidated through extensive 1D NMR (^{1}H and ^{13}C) and 2D NMR (HSQC, HMBC, and NOESY) analyses, high-resolution mass spectrometry, high-performance liquid chromatography, and single-crystal X-ray crystallography. Furthermore, these novel metabolites exhibited potent inhibitory activities against various bacteria, fungi, and yeast strains with minimum inhibitory concentrations ranging between 1.56 and 25 μg/mL, and compounds **1** and **3** were found to be most active against a wide range of microbial strains tested.

INTRODUCTION

In 1929, the discovery of penicillin from *Penicillium notatum* marked a crucial turning point in antibiotic research. This finding increased the importance of microbial natural products in drug discovery because of their availability, diversity, structural uniqueness, and excellent antimicrobial potential.[1] Overuse and misuse of various antibiotics and personal care products for long term lead to antimicrobial resistance (AMR). AMR is a significant concern and threatens our ability to treat common infections. The increase in AMR results in severe threats to human health, resulting in 700,000 deaths per annum, which is predicted to increase to 10 million per annum by the year 2050.[2] This catastrophe is manifested by fungi and bacteria.[3,4] The spread of infectious diseases poses severe risks to public health and the global economy.[5] In addition, over the past 20 years, livestock farming has experienced the highest expansion in the agricultural sector, and more livestock farms are being established, which increases the need for antibiotic growth promoters in animals raised for food applications.

The genera of lactic acid bacteria (LAB) such as *Lactobacillus*, *Lactococcus*, *Enterococcus*, and *Pediococcus* have gained much attention due to the balance of the gut microbiome, biological and bio-therapeutic applications, antimicrobial potential, and immunomodulatory effects on humans and livestock.[6] These LAB exhibit probiotic potential and are eco-friendly. Hence, they are generally considered safe when administered in controlled amounts.[2] Various *Lactococcus* species, including *Lactococcus lactis*, have gained more importance due to their applications in the production of food and healthcare products.[7,8] Furthermore, these bacteria can produce potent bioactive molecules, peptides, proteins, and live cells that are accountable for improving digestion, modulating the immune system, and protecting against

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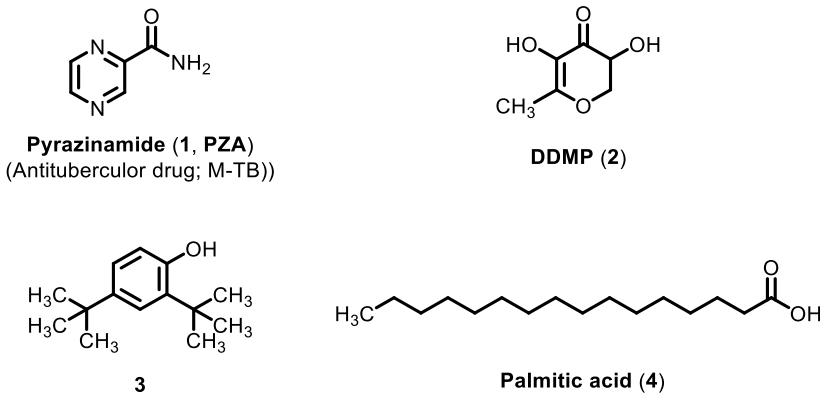

Figure 1. Chemical structures of secondary metabolites **1**, **2**, **3**, and **4** isolated from *L. lactis*.

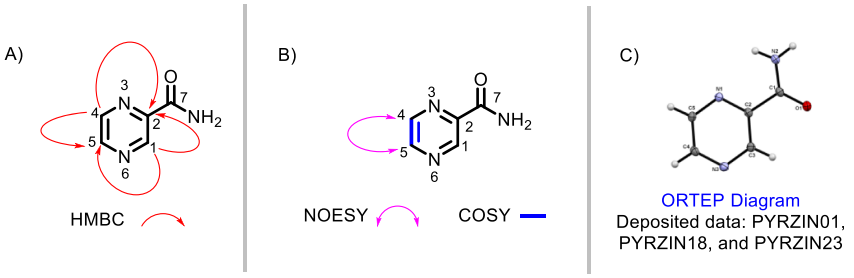

Figure 2. (A) Important HMBC correlations, (B) important NOESY and COSY correlations, and (C) ORTEP of compound **1**.

diabetes, cardiovascular diseases, advanced glycation end products, and mycotoxin neutralization from food and feed.[9,10]

L. lactis isolated from various sources produces a variety of bioactive molecules, including bacteriocins (nisins A, Z, Q, F, and U), enterolysin A, subtilin, zoocin A,[11] pediocin,[12] lantibiotics,[13] and lactococcin G.[14] It has also been reported to produce 2,4-di-*tert*-butyl-phenol,[15] cyclo-(Leu−Pro), tetradecanoic acid,[16] and several volatile molecules such as aldehydes, ketones, and hydrocarbons.[17] Inspired by the exciting profile of *L. lactis* and as part of our investigations in finding bioactive secondary metabolites from diverse bacteria and their applications in medicinal and food chemistry and natural product chemistry,[18] herein we report the isolation, structural elucidation, and antimicrobial profile of pyrazin-2-carboxamide [**1**, pyrazinamide (PZA)], 3,5-dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one (**2**), and 2,4-di-*tert*-butyl-phenol (**3**) along with a fatty acid hexadecanoic acid (**4**, palmitic acid) from *L. lactis* for the first time.[19] It is noteworthy that we have disclosed the isolation of pyrazinamide (**1**, pyrazine-2-carboxamide, PZA) for the first time from a bacterial strain (*L. lactis*), which is the first-line approved drug for tuberculosis treatment. It functions by preventing the growth of *Mycobacterium tuberculosis*.[20] An extensive literature survey revealed that the *Lactobacillus pentosus* S-PT84 strain is known to produce another metabolite isolated in this work, 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (**2**, DDMP), that stimulates nerve activity and has anticancer activity (colon cancer) by inhibiting NF-kappa B, which causes cell death;[21] it contributes to the antioxidant properties of intermediates of the Millard reaction (heat-induced reaction between reducing sugars and proteins).[21] Besides, hexadecanoic acid (palmitic acid) is produced by plants, animals, and microbial sources and is known to play essential roles at the cellular and tissue levels. It has anti-inflammatory properties and is essential during infancy.[21−24]

RESULTS AND DISCUSSION

In the present report, we have collected *L. lactis* from dairy milk samples from the Western Ghats region (Kolhapur), India. The fermented broth's (30 L) cell-free supernatant was used for the isolation, purification, and structural elucidation of metabolites. The EtOAc extract of the cell-free supernatant was fractionated by passing over silica gel and screened for antimicrobial activity. This bioactivity-guided fractionation resulted in the identification of four metabolites, namely pyrazinamide (**1**, PZA), 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP, **2**), 2,4-di-*tert*-butyl phenol (**3**), and hexadecanoic acid (**4**, palmitic acid). In addition, purified metabolites **1**−**4** were evaluated for their antimicrobial activity (Figure 1).

Compound **1** gave the molecular formula $C_5H_5N_3O$, as deduced from the ESI-HRMS at m/z 123.12 $[M + H]^+$, which suggested the presence of five degrees of unsaturation. The ^1H and ^{13}C NMR data of **1** (Table S1) revealed the presence of three aromatic protons [δ_H 9.43 (d); δ_C 144.8 (C1); δ_H 8.78 (d); δ_C 147.7 (C4) and δ_H 8.58−8.53 (m); δ_C 142.9 (C5)], one quaternary carbon at δ_C 144.3 (C2), and one carbonyl carbon at δ_C 165.5 (C6). As summarized in Figure 2, ^1H−^1H COSY, HSQC, and HMBC NMR experiments were carried out to verify the complete skeletal connections of this metabolite. The HMBC correlation of H-1 to C-2 and C-5, H-4 to C-5, and H-5 to C-1 and C-4, NOESY, and COSY correlation of H-4 with H-5, the presence of three nitrogens, and five degrees of freedom revealed the presence of a pyrazine ring. Ultimately, signal-crystal X-ray diffraction analyses of **1** rigorously established it as pyrazine-2-carboxamide (**1**) (crystallized from a 1:9 mixture of dichloromethane and hexanes and correlated with known deposited data with PYRZIN01, PYRZIN18, and PYRZIN23, Figure 2, entries A−C).[25]

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ESI-HRMS analyses of compound **2** (m/z 144.12 [M + H]$^+$) gave the molecular formula $C_6H_8O_4$, suggesting three degrees of unsaturation. NMR analyses of **2** (^{1}H and ^{13}C NMR, Table S2) showed the presence of one methyl group [δ_H 2.04 (s); δc 15.7 (C-7), one oxygen-bearing methine proton δ_H 4.01 (m); δc 71.07 (C-3), and one methylene δ_H 4.44 (m); δc 67.27 (C-2). It was subsequently analyzed employing ^{1}H−^{1}H COSY, HSQC, and HMBC NMR experiments to get insight into the complete structural connections. The HMBC correlation of H-2 to C-3, C-4, and C-6 and H-3 to C-2, C-4, and C-6 revealed the presence of a dihydropyran ring, and the correlations of the methyl proton H-7 to C-5 and C-6 indicated that C-7 is linked to the C-6 quaternary carbon. In addition, NOESY correlations of H-2 with H-3 and COSY correlations of H-2 with C-3 established the complete structure of **2** as 3,5-dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one (**2**, DDMP) (Figure 3, entries A and B).[25]

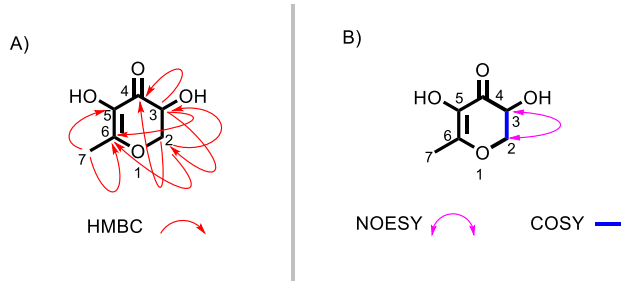

Figure 3. (A) Important HMBC correlations, and (B) important NOESY and COSY correlations of **2**.

Compound **3** gave the molecular formula $C_{14}H_{22}O$, deduced from the ESI-HRMS at (m/z 206.17 [M + H]$^+$), which indicated the four degrees of unsaturation. The structure of **3** was established based on single-crystal X-ray diffraction analyses and extensive NMR spectroscopy (including ^{1}H and ^{13}C NMR, HMBC, NOESY, and COSY). The ^{1}H and ^{13}C NMR data of **3** (Table S3) revealed the existence of six methyl groups [δ_H 1.39−1.28 (m), 1.52−1.41(m); δ_C 29.7 (C-10), 31.7 (C-10)] and three aromatic protons [δ_H 6.62 (dd, J = 1.1, 8.1 Hz); δ_C (C-6), δ_H 7.16−7.04 (m); δ_C (C-5) and δ_H 7.36−7.33 (m); δ_C (C-3)]. The HMBC data analyses established connectivities between quaternary carbons. The HMBC correlation of H-3 to C-1, C-5, and C-8 indicated the presence of quaternary phenolic carbon, and the correlation of H-5 to C-1 and C-3; H-6 to C-4; H-9 to C-2 and C-7; and H-10 to C-4 and C-8 established the position of di-*tert*-butyl groups at C-2 and C-4 positions (concerning the phenyl ring). Moreover, single-crystal X-ray diffraction analyses led to the rigorous structural assignment of compound **3** as 2,4-di-*tert*-butylphenol (crystallized from hexanes) (Figure 4, entries A−C).[25]

Compound **4** gave the molecular formula $C_{16}H_{32}O_2$ (deduced from the ESI-HRMS at m/z 256.43 [M − H]$^+$) and suggested one degree of unsaturation. The ^{1}H and ^{13}C NMR spectra (Table S4) showed one methyl group at δ_H 0.89 (t, J = 6.9 Hz), δ_C 14.3 (C-16), and 14 methylene protons at δ_H 1.63 (m), 1.33 (m), 1.30 (m), and 1.28 (m), which indicated the presence of a linear alkyl chain. The ^{13}C NMR spectrum and DEPT data revealed the presence of 16 carbons and found one sp^3 methyl resonating at δ_c 14.3 as well as 14 sp^3 methylene carbons and one carbonyl carbon at δ_C 179.7 (C-16). The HMBC correlation shows the connection between H-16 to C-15 and C-14 and H-2 to C-3 and C-4. Furthermore, key ^{1}H−^{1}H− COSY and HSQC correlations (see the Supporting Information) established compound **4** as a linear long-chain containing a saturated fatty acid, hexadecanoic acid (palmitic acid) (Figure 5, entries A and B).[25]

Antibacterial Activity. The isolated compounds were evaluated for microbial efficacies against bacteria, and streptomycin sulfate (STRS) was used as a positive control. Compounds **1**−**4** (**C-1** to **C-4**) showed potent microbial activity against both Gram positive and Gram negative bacterial strains, with minimum inhibitory concentrations (MICs) varying from 1.56 to 25 μg/mL. All the investigated strains were significantly inhibited by compound C-1, which is also more active against *Mycobacterium smegmatis* with MIC 12.5 μg/mL, and other strains were less susceptible with an MIC of 25 μg/mL. Compound C-2 is most active against *Serratia marcescens* and *Staphylococcus aureus* with an MIC value of 3.12 μg/mL, moderately active against *Pseudomonas desmolyticum*, *Escherichia coli*, and *Bacillus subtilis* with an MIC of 6.25 μg/mL each, and least active against *M. smegmatis* with an MIC of 12.5 μg/mL. Furthermore, for compound C-**3**, the highest antibacterial activity was found against *S. marcescens* (MIC, 1.56 μg/mL), followed by *M. smegmatis*, *E. coli*, and *B. subtilis* (MIC, 3.12 μg/mL), and the least activity was reported against *S. aureus* (MIC, 6.25 μg/mL). Similarly, C-4 also showed good antibacterial activity against the tested strains (MIC, 12.5 μg/mL), except for *M. smegmatis* (MIC, 25 μg/mL) (Table 1).

Antifungal Activity. Next, compounds **1**−**4** (**C1** to **C4**) were screened for their antifungal activity employing amphotericin B (AMB) as a positive control. Compound C-1 was less effective against *Fusarium graminearum* (MIC, 50 μg/mL), and for *Candida albicans* and *Fusarium verticillioides* an MIC of 12.5 μg/mL was observed (Table 1). Compound C-2 showed an inhibitory effect toward all the tested fungal strains (MIC, 3.12 to 6.26 μg/mL). Compound C-3 was more active against *F. verticillioides*, and *F. graminearum* exhibited the MIC

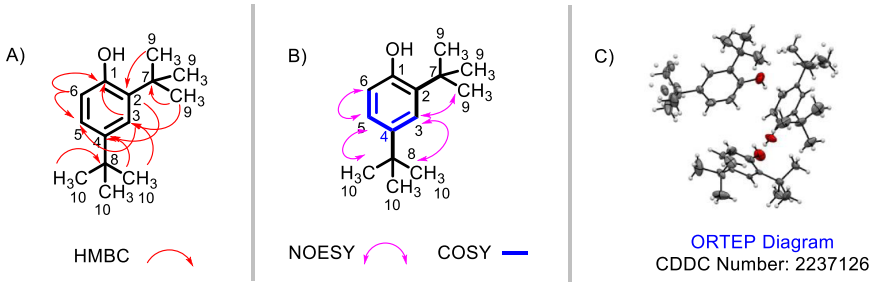

Figure 4. (A) Key HMBC correlations, (B) key NOESY and COSY correlations, and (C) ORTEP diagram of compound **3**.

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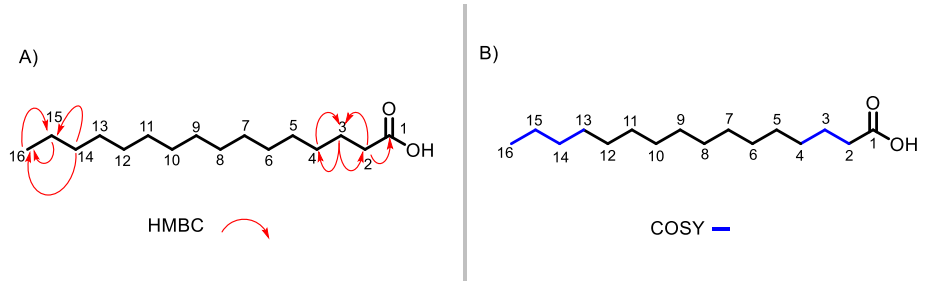

Figure 5. (A) Key HMBC correlations, and (B) COSY correlations of compound **4**.

Table 1. Antimicrobial Efficacy of Compounds 1−4[a]

	MIC (μg/mL)				
bacterial strain	C-1	C-2	C-3	C-4	STR
S. aureus NCIM 2079	25.0	3.12	6.25	12.5	0.39
B. subtilis NCIM 2010	25.0	6.25	3.12	25.0	0.39
E. coli NCIM 2065	25.0	6.25	3.12	12.5	0.78
S. marcescens NCIM 2919	25.0	3.12	1.56	6.25	1.56
P. desmolyticum NCIM 2112	25.0	6.25	6.25	12.5	0.39
M. smegmatis NCIM 5138	12.5	12.5	3.12	12.5	6.25
	MFC (μg/mL)				
fungal strain	C-1	C-2	C-3	C-4	AMB
Candida albicans NCIM 3557	12.5	3.12	12.25	12.5	0.78
Fusarium verticillioides BIONCL4	12.5	3.12	6.25	25.0	1.56
Fusarium graminearum MTCC1893	50.0	6.25	6.25	12.5	1.56
Aspergillus niger BIONCL 12	25.0	3.12	12.50	25.0	3.12

[a]C refers to compounds STRS—streptomycin sulfate and AMB—amphotericin B, MIC—minimum inhibitory concentration, MFC—minimum fungicidal concentration, NCIM—National Collection of Industrial Microorganisms, and MTCC—Microbial Type Culture Collection and Gene Bank.

of 6.25 μg/mL; *Aspergillus niger* and *C. albicans* showed the least inhibition with an MIC of 12.50 and 12.25 μg/mL, respectively. However, compound C-4 exhibited good activity, and an MIC of 12.50 μg/mL was observed against *C. albicans* and *F. graminearum*; other fungal stains tested were susceptible at 25.00 μg/mL (Table 1).

CONCLUSIONS

The fermentation of probiotic strain *L. lactis* subsp. *lactis* recovered from traditional milk led to the discovery of novel antimicrobial compounds: pyrazin-2-carboxamide (**1**, pyrazinamide (PZA), a potential antitubercular drug), 3,5-dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one (**2**, DDMP), 2,4-di-*tert*-butylphenol (**3**), and hexadecanoic acid (**4**, palmitic acid). Structural elucidation was carried out using 1D (^{1}H and ^{13}C NMR), 2D NMR (HMBC, HSQC, and NOESY), and ESI-HRMS analyses. Compounds **1** and **3** were meticulously established through single-crystal X-ray crystallography analyses. All of these isolated metabolites exhibited promising antimicrobial activity against various bacterial and fungal strains, with MICs varying between 1.56 and 50 μg/mL. Compound **2** had shown strong antimicrobial potential against *Mucor marcescens* (MIC, 3.12 μg/mL), *C. albicans*, *Fusarium*, and *Aspergillus* species (MIC, 3.12 μg/mL). Similarly, compound **3** showed potent antimicrobial activity against *M. marcescens* (MIC, 1.56 μg/mL).

EXPERIMENTAL SECTION

General information: thin layer chromatography (TLC) was performed using silica gel 60 F254. Subsequent visualization of TLC was accomplished employing short-wave UV light, PMA, or anisaldehyde staining solutions, followed by heating. Column chromatography was performed using silica gel (100−200 mesh), standard techniques, and eluting with solvents, as indicated in the experimental procedure. On Bruker 500 spectrometers, ^{1}H and ^{13}C NMR spectra were recorded using indicated solvents, and chemical shifts (δ) are presented in ppm. The chemical shifts were translated to the TMS scale, and residual solvent signals are used as references ($CDCl_3$: H = 7.27 ppm, C = 77.16 ppm). The following abbreviations were used to show the multiplicity of signals: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; AB q, AB quartet; dd, doublet of doublet; td, triplet of doublet; and br, broad. A Thermo Scientific Q-Exactive, Accela 1250 pump was used for the HRMS analyses. Experimental procedures for all known and new compounds without published procedures are furnished in below sections. High-performance liquid chromatography (HPLC) analyses were carried out using a HPLC Waters, a 2545 Quaternary Gradient Module (Waters, USA), and an X-bridge C18 column (5 μm, 4.6 × 250 mm). The Bruker D8 Advance was used for the single-crystal XRD analyses, and the data were collected using high-resolution (0.78 Å) Cu Kα radiation at a low temperature (100 K).

Isolation and Identification of Bacteria (*L. lactis* subsp. *lactis*). *L. lactis* subsp. *lactis* was recovered from milk samples collected in the Western Ghats region (Kolhapur), India. Bacterial species associated with milk samples were isolated as suggested by Sellamani et al., (2016).[26] Briefly, 100 μL dilutions were spread and cultured on the de Man, Rogosa, and Sharpe (MRS) agar medium (Hi Media, India). Thus, prepared plates were incubated anaerobically at 37 °C for 48 h. After the completion of incubation, the pure colonies were further sub-cultured on MRS medium (Figure S1) and morphologically identified using Gram staining and field-emission scanning electron microscopy observations (Figure S2). Furthermore, molecular identification was conformed using 16S rRNA gene sequencing and identified as *L. lactis* subsp. *lactis*, showing 100% similarity with the strain *L. lactis* subsp. *lactis* (Genbank no. MH669384). The top BLAST hits were used to draw a phylogenetic tree using MEGA-X software with 1000 bootstrap values and formed a clade with *L. lactis* subsp. *lactis* (Figure S3). The sequence of the identified strain *L. lactis* subsp. *lactis* BIONCL 17752 was deposited at the National Center for Biotechnology Information (NCBI) under the gene bank accession number MG917752.

Probiotic Characterization. The probiotic characterization of the BIONCL 17752 strain was performed as per

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the standard guidelines suggested by the Indian Council of Medical Research (ICMR) and the Department of Biotechnology, India.[27]

Antimicrobial Sensitivity Assay. The *L. lactis* subsp. *lactis* strain BIONCL 17752 was subcultured on solidified MRS agar and grown for 48 h at 37 °C anaerobically. A loop full of grown colonies was transferred into 100 mL of MRS broth with a pH of 6.5 and adjusted before sterilization. Equal volumes of ethyl acetate (EA) were used to extract the fermented cell-free broth. The organic fraction was collected and concentrated using a rotary evaporator. The crude extract was tested for biological activity.

The bacterial strains were tested for antimicrobial potential by the agar overlay method.[28] The BIONCL 17752 strain was found to be active on an agar plate against the *F. verticillioides* strain, with a zone of inhibition covering more than 50% of the overall plate area (Figure S4A). The crude extract of the BIONCL 17752 strain was solubilized in HPLC-grade EA (2 mg/mL). To test the antimicrobial activity, 100 μL of crude extract was tested against *E. coli*, *B. subtilis*, and *F. verticillioides* using the agar well diffusion assay. The crude EA extract (CE) of the BIONCL 17752 strain showed 14, 18, and 16 mm zones of inhibition against *F. verticillioides*, *B. subtilis*, and *E. coli*, respectively (Figure S4B).

L. lactis inhibits the vegetative growth of several pathogenic and toxigenic bacterial and fungal pathogens. It was reported to have antibacterial activity against *Serratia typhimurium*, *S. aureus*, *E. coli*, and *Listeria monocytogenes*.[29,30] Besides, antifungal activity against *Aspergillus niger*, *Penicillium chrysogenum*, *Fusarium oxysporum*,[15] *F. verticillioides*,[31] *Colletotrichum capsici*,[32] *Penicillium expansum*, *Cladosporium cladosporioides*, *Mucor hiemalis*, and *Ulocladium chartarum* has been reported.[33]

Culture and Fermentation Conditions. The strain BIONCL 17752 was subcultured on MRS agar plates and grown at 37 °C for 48 h. A pure single colony was transferred into 50 mL of MRS broth. The culture was incubated on a rotary shaker incubator, as discussed above. After the completion of the incubation period, 1 mL of grown culture was subcultured into 200 mL of seed culture and incubated till the optical density (OD) reached 1.8. The seed inoculum (2.0%) was inoculated into 1 L of fermentation medium/production medium. The microbial fermentation conditions were optimized at a small-scale level using a 2 L fermenter (Biostat B, Bangalore, India) to obtain maximum biomass and metabolite production. The fermentation conditions, such as growth media (MRS, 1% yeast extract), pH 6.5, temperature 37 °C, and dissolved oxygen (DO) at 10% saturation with constant agitation at 75 rpm, were optimized as ideal conditions. The scale-up was carried out using a 10 L fermenter (New Brunswick BioFlo/CelliGen115, Germany) with the same conditions. During the fermentation process, the OD of the culture was measured by taking absorbance at 600 nm after 2 h of time intervals. As the OD reached a maximum of around 7.0, the fermentation was stopped, and the broth was harvested. The cells from the fermented broth were separated by centrifugation at 4000 Xg for 20 min. The cell-free broth was further processed for metabolite extraction and structural characterization.

Extraction and Purification of Metabolites. The fermented cell-free filtrate (CFS) was extracted with EA (EtOAc, 1:1, v/v) three times (n = 3). Next, the resulting aqueous layer was reextracted with dichloromethane (DCM). All organic phases were collected and concentrated under reduced pressure (rotary evaporator). This led to the procurement of 16 g of a semi-solid extract from 30 L of fermented broth. Based on TLC profiling, EtOAc and DCM extracts were pooled and processed for silica-gel column chromatography, employing petroleum ether (PE) with a gradually increasing EtOAc percentage (gradient elution). 12 fractions (F-1 to F-12) were collected and processed for bioactivity. Among these 12 fractions, 4 fractions (F-1 to F-4) showed a bioactive profile (Figure S5) and were subjected to second-stage silica-gel column chromatography to obtain pure compounds. Fraction-1 was eluted with 2.5% EtOAc/PE to give major compound **3** (230 mg), and following elution with 5% EtOAc/PE furnished compound **4** (70 mg). Purification of F-4 by 40% EtOAc/PE gave compound **2** (145 mg), and elution of F-5 with 50% EtOAc/PE delivered compound **1** (35 mg).

*Pyrazine-2-carboxamide (**1**).* Obtained as a white crystalline solid (0.035 g from 30 L, in 0.21% yield) TLC: R_f = 0.5 (SiO_2, 80% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.43 (d, J = 1.3 Hz, 1H), 8.78 (d, J = 2.5 Hz, 1H), 8.58−8.53 (m, 1H), 7.65 (br s, 1H), 5.91 (br s, 1H);^{13}C NMR ($CDCl_3$, 126 MHz): δ 165.5, 147.7, 144.8, 144.3, 142.9. HR-ESI m/z: $[M + H]^+$ (calcd for $C_5H_6N_3O$, 124.0505; found, 124.0505).

*3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (**2**).* Obtained as a yellowish semi-solid (0.145 g from 30 L, in 0.90% yield) TLC: R_f = 0.5 (SiO_2, 50% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.45−4.32 (m, 2H), 4.10−3.98 (m, 1H), 2.04 (s, 3H); ^{13}C NMR ($CDCl_3$, 126 MHz): δ 188.2, 160.8, 131.4, 71.0, 67.3, 15.7. HR-ESI m/z: $[M + H]^+$ (calcd for $C_6H_9O_4$, 145.0493; found, 145.0495).

*2,4-Di-tert-butylphenol (**3**).* Obtained as a white solid (0.230 g from 30 L, in 1.43% yield) TLC: R_f = 0.5 (SiO_2, 5% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.36−7.33 (m, 1H), 7.14−7.09 (m, 1H), 6.62 (dd, J = 1.1, 8.1 Hz, 1H), 4.67−4.63 (m, 1H), 1.48−1.44 (m, 9H), 1.35−1.29 (m, 9H); ^{13}C NMR ($CDCl_3$, 126 MHz): δ 151.9, 143.1, 135.3, 124.2, 123.7, 116.1, 34.9, 34.4, 31.8, 29.8. HR-ESI m/z: $[M + H]^+$ (calcd for $C_{14}H_{23}O$, 207.1739; found, 207.1743).

*Hexadecanoic Acid (**4**).* Obtained as a white crystalline solid (0.070 g from 30 L, in 0.43% yield) TLC: R_f = 0.5 (SiO_2, 20% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.36 (t, J = 7.6 Hz, 2H), 1.68−1.58 (m, 2H), 1.35−1.28 (m, 6H), 1.26 (s, 18H), 0.89 (t, J = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 126 MHz): δ 179.7, 34.1, 32.1, 31.8, 29.8, 29.7, 29.6, 29.5, 29.4, 29.2, 24.8, 22.8, 14.3. HR-ESI m/z: $[M - H]^-$ (calcd for $C_{16}H_{31}O_2$, 255.2319; found, 255.2328).

Single-Crystal XRD Analysis. Single-crystal X-ray diffraction measurements were performed to determine the crystal structures of compounds **1** and **3** at 100 K using the APEX3 (Bruker, 2016; Bruker D8 VENTURE Kappa Duo PHOTON II CPAD) diffractometer having monochromatized graphite (Mo Kα = 0.71073 Å). The X-ray generator was operated at 50 kV and 30 mA. 36 frames were used to create a preliminary set of unit cell parameters and an orientation matrix., and the cell refinement was performed by SAINT-Plus (Bruker, 2016). An optimized strategy used for data collection consisted of different sets of φ and ω scans with 0.5° steps φ/ω. The data were collected with a time frame of 10 s for both the components by setting the sample to detector distance at 40 cm. All the data points were corrected for Lorentzian, polarization, and absorption effects using SAINT-Plus and SADABS programs (Bruker, 2016). SHELXS-97 (Sheldrick,

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2008) was used for structure solution and full-matrix least-squares refinement on F2.1, 2 The molecular graphics of ORTEP diagrams were performed by Mercury software. The crystal symmetry of the components was cross-checked by running the cif files through PLATON (Spek, 2020) software, which indicated that no additional symmetry was observed. Encifer software was used to correct the cif files.

The SC-XRD experiments confirm the structures of the newly isolated metabolites. This includes pyrazine-2-carboxamide (**1**), 3,5-dihydroxy-2-methyl-4*H*-pyran-4-one (**2**), and 4-di-*tert*-butylphenol (**3**). Detailed crystallographic information on the parameters of all the components and the main driving force for forming molecular crystals is provided in Tables S5 and S6, respectively. Compound **1** (pyrazine-2-carboxamide) crystallizes in a monoclinic $P2_1/c$ space group with one molecule in the asymmetric unit. However, the crystal structure of compound **1** has been deposited in the Cambridge Structural Database Software; the crystallographic parameters of compound **1** are matched with the β form of pyrazine-2-carboxamide (Tables S5 and S6); the refcodes are PYRZIN01, PYRZIN18, and PYRZIN23. The crystallization of compound **3** takes place in the triclinic $P\bar{1}$ space group, with two molecules in the asymmetric unit. The residual factor for this molecule is relatively high, i.e., 16.3; this is due to the availability of poor-quality single crystals.

HPLC Analysis. HPLC was performed for confirmation and purity of the isolated compounds. The Waters HPLC system, 2545 Quaternary Gradient Module (Waters, USA), was used with an X-Bridge C18 column (5 μm, 4.6 × 250 mm). HPLC analysis of compounds **1** and **2** was carried out with the mobile phase acetonitrile and water (10:90). Compound **1** was eluted with an RT of 4.68, which correlates with a reference standard RT of 4.54 (Figure S15), and compound **2** was eluted with an RT of 4.38 (Figure S24). Furthermore, compound **3** and reference standard analysis were carried out using acetonitrile (ACN) and water (90:10) in the mobile phase with an injection volume of 10 μL, and absorbance was recorded at 254 nm with a flow rate of 0.7 mL m^{-1}. Compound **3** was eluted at a retention time (RT) of 6.71 min, which correlates with a reference standard RT of 7.07 (Figure S33).

Antimicrobial Activity. The purified compounds (**1**−**4**) were initially evaluated for antimicrobial activity by a well diffusion assay as mentioned above and showed a 10 to 18 mm zone of inhibition against *F. verticillioides* and *S. marcescens* (Figure S6). The MICs of purified compounds were studied by following the Clinical and Laboratory Standards Institute (CLSI) guidelines.[34] The antimicrobial activity was carried out against *S. aureus*, *B. subtilis*, *M. smegmatis*, *E. coli*, *P. desmolyticum*, and *Serratia marcescens*. Furthermore, antifungal activity was tested against mycotoxin-producing fungi such as *F. verticillioides*, *F. graminearum*, *A. niger*, and the opportunistic pathogenic yeast *C. albicans*. The stock solution was prepared by dissolving individual compounds in dimethyl sulfoxide (DMSO). The different concentrations of compound (0 to 100 μg mL^{-1}) and 50 μl bacterial culture (5×10^5 CFU mL^{-1}) were transferred to an individual well of a 96-well microtiter plate except for the control well. The broth, without treatment culture, DMSO, and streptomycin were referred to as negative and positive controls, respectively. The final volume was adjusted to 200 μL with a growth medium and cultured for 24 h at 37 °C under shaking conditions. After the completion of 24 h, OD was taken at 600 nm using a multimode plate reader (Bio-Rad, USA). The antimicrobial activity of *M. smegmatis* was carried out using the same protocol with Middlebrook 7H9 medium (Hi-media, Mumbai, India) and incubated for 48 h. For antifungal activity, RPMI-1640 (Sigma-Aldrich, India) was used as a growth medium. The compounds with different concentrations as described above and a 50 μL suspension of fungal spores (1×10^6 mL^{-1}) were added to an individual well of a 96-well microtiter plate except for media control and cultured for 48 to 72 h at 28 °C under shaking conditions. The media broth, untreated culture, DMSO as a control, and amphotericin B were used as the standard drugs. The minimum concentration that inhibited the visible growth of microorganisms, defined as the MIC value, was calculated for the individual compound. Furthermore, % growth inhibition was found out using the formula, growth inhibition (%) = Ac−At/Ac×100, where Ac and At represent the absorbance of the control and test sample (compound or standard, respectively).

MIC Determination by the Resazurin Assay. Furthermore, following the above experimental plates, resazurin dye (resazurin sodium salt, 2.5 mg mL^{-1}) was added and incubated under dark conditions for 4 h. All the plates were protected by aluminum foil since resazurin is a light-sensitive dye. The color transition from pink to blue is considered MIC. All turbidimetric and resazurin assays were compared to ensure the MICs of all the isolated molecules produced by probiotic potentials of *L. lactis* (data not shown).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01662.

Copies of ^{1}H, ^{13}C, and 2D NMR spectra for all new compounds (PDF)

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Author Contributions

K.R.V. and R.K designed the project, directed the research, and examined experimental results. V.D.N carried out isolation, fermentation, purification, and bioactivity studies; B.R.B contributed to the structural elucidation investigations. G.R.K. carried out X-ray crystallography studies. All authors revised the manuscript and the Supporting Information.

Notes

The authors declare no competing financial interest.

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