

Characterization of an Antibacterial Compound, 2-Hydroxyl Indole-3-Propanamide, Produced by Lactic Acid Bacteria Isolated from Fermented Batter

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Abstract Lactic acid bacteria are known to produce numerous antimicrobial compounds that are active against various pathogens. Here, we have purified and characterized a novel low-molecular-weight (LMW) antimicrobial compound produced by *Lactobacillus* and *Pediococcus* isolated from fermented *idly* and *uttapam* batter. The LMW compound was extracted from cell-free supernatant using ice-cold acetone, purified by gel permeation and hydrophobic interaction chromatography. It exhibited antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria sparing the probiotic strains like *Lactobacillus rhamnosus*. The molecular weight of the LMW compound was identified as 204 Da using LC-MS-ESI. In addition, the structure of the compound was predicted using spectroscopic methods like FTIR and NMR and identified as 2-hydroxyl indole-3-propanamide. The LMW compound was differentiated from its related compound, tryptophan, by Salkowski reaction and thin-layer chromatography. This novel LMW compound, 2-hydroxyl indole-3-propanamide, may have an effective application as an antibiotic which can spare prevailing probiotic organisms but target only the pathogenic strains.

Keywords *Lactobacillus* · *Pediococcus* · Antibacterial · NMR · FTIR · 2-Hydroxyl indole-3-propanamide

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Introduction

Lactic acid bacteria (LAB) are a group of generally recognized as safe (GRAS) organisms which produce several low-molecular-weight (LMW) compounds acting as antimicrobials and/or antifungals and flavoring agents that enhance the stability of food products. They produce several metabolites like diacetyl, acetoin, butanediol, flavone, organic acids, and various volatile components depending on the sources of fermentation [1]. During fermentation process, they metabolize certain flavonol glycoside in the plant materials into 4-hydroxybenzoic acid [2] and gallic acid [3] that exhibited antimicrobial activity [2] and antioxidant activity [3]. Some *Lactobacillus* spp. and *Pediococcus* spp. were known to produce 2-pyrrolidone-5-carboxylic acid that exerts antimicrobial activity against spoilage bacteria [4]. Also, other LMW compounds like benzoic acid, glycyl-L-leucine, methyl hydantoin, mevalonolactone [5], 5-hydroxyl ferulic acid [6], 3-phenyllactic acid [7], 3-hydroxydecanoic acid [2], hydroxyl fatty acid [8], and more exhibited antimicrobial activity.

Reuterin, chemically known as β -hydroxypropionaldehyde produced by *Lactobacillus reuteri*, was a non-peptide broad-spectrum antimicrobial compound synthesized from glycerol exhibiting broad-spectrum antimicrobial activity against prokaryotes and eukaryotes [9]. Reuterin as an analogue of D-ribose also inhibits B1 subunit of ribonucleotide reductase and thioredoxin, suggesting its ability to inhibit sulfhydryl enzymes [10]. Although it was first identified from the *Lactobacillus reuteri*, it was also produced by other LAB like *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus collinoides*, and *Lactobacillus coryniformis* [11]. In addition, bacteriocins are well-known proteinaceous antimicrobial compounds widely studied for their use as food preservatives [12]. However, other antimicrobial compounds produced by LAB have not been evaluated extensively, as they are not produced in high concentrations in the medium. In this study, a successful attempt was made in isolation and purification of low-molecular-weight (LMW) antimicrobial compound produced by *Pediococcus pentosaceus* VJ13 [13], *Lactobacillus plantarum* JJ18, and *Lactobacillus plantarum* subsp. *plantarum* JJ60 [14] isolated from fermented *idly* batter, and *Lactobacillus pentosus* SJ65 [15] and *P. pentosaceus* PJ7 isolated from fermented *uttapam* batter. All these isolates produced the same compound which was identified by spectroscopic methods, and its antimicrobial activity was evaluated against various pathogens and probiotic organisms.

Materials and Methods

Chemicals and Media

All the chemicals and media were procured from HiMedia, Mumbai, while indole-3-lactic acid was purchased from Sigma, Bangalore, and DL-tryptophan from SRL, Mumbai. The solvents procured for HPLC, NMR, and FTIR are from Merck India, Mumbai.

Microbial Strains

The indicator strains used in this study were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. All LAB were maintained and propagated in de Man, Rogosa, and Sharpe (MRS)/Brain Heart Infusion (BHI) whereas

pathogenic organisms in Tryptone Soy Broth (TSB)/Luria Bertani (LB). The cultures and isolates were stored at $-20\text{ }^{\circ}\text{C}$ with 20 % glycerol.

Accession Numbers

The isolates *P. pentosaceus* VJ13 [13], *Lactobacillus plantarum* JJ18, and *Lactobacillus plantarum* subsp. *plantarum* JJ60 [14] isolated from idly batter and *Lactobacillus pentosus* SJ65 [15] and *P. pentosaceus* PJ7 isolated from uttapam batter. The 16S rRNA gene sequences of these isolates from batter were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) whose accession numbers were JN573609, JN573601, JN573602, JN573623, and KC439681.

Extraction and Purification of LMW Antimicrobial Compound

The isolates were grown in 1 L of de Man, Rogosa, and Sharpe (MRS) broth with 0.5 % of inoculum of overnight culture for 36 h at $37\text{ }^{\circ}\text{C}$ under static condition, and the cell-free supernatant (CFS) was collected by centrifugation at $8000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$. The CFS was concentrated to 10-fold (100 mL) in rotavapor (Buchi, Flawil, Switzerland), and ice-cold acetone was added to attain 70 % (v/v) saturation initially. The supernatant was collected by centrifugation at $8000\times g$ for 20 min at $-4\text{ }^{\circ}\text{C}$ and was further subjected to 85 % (v/v) saturation with ice-cold acetone and incubated at $-4\text{ }^{\circ}\text{C}$. After 2 h of incubation, the supernatant was collected by centrifugation at $8000\times g$ for 10 min at $-4\text{ }^{\circ}\text{C}$. The acetone in the supernatant was removed completely using rotavapor (Buchi, Flawil), and the extract was dissolved in 9 mL of 5 mmol L^{-1} of acetate buffer, pH 5.2 (i.e., approximately 111-fold concentration of original CFS), which was then passed through Sephadex G25 (100-cm \times 2-cm column) pre-equilibrated with 5 mmol L^{-1} acetate buffer, and the fractions were collected at the flow rate of 0.5 mL min^{-1} with same buffer. The fractions were monitored at 280 and 260 nm and evaluated for antimicrobial activity against *Staphylococcus aureus* and *Listeria monocytogenes* by agar well diffusion assay [16]. The active fractions were pooled and passed through Sephadex G15 (50-cm \times 1.8-cm column) pre-equilibrated with 5 mmol L^{-1} of acetate buffer, pH 5.2, to obtain a single homogenous peak and eluted at the flow rate of 0.5 mL min^{-1} . The fractions from Sephadex G15 were further purified by solid-phase extraction technique for spectroscopic studies. The sample was passed through Sep-Pak C18 (Waters, Bangalore) cartridge pre-equilibrated with water having 0.1 % trifluoroacetic acid (TFA) and was washed with 10 % acetonitrile (ACN) having 0.1 % TFA to remove the contaminants while the fraction eluted with 60 % ACN containing 0.1 % TFA after removal of solvent using SpeedVac (Christ, Osterode) concentrator showed antimicrobial activity against *S. aureus* and *Listeria monocytogenes* by agar well diffusion assay. This active fraction was lyophilized and used for further studies.

Antimicrobial Spectrum of LMW

The LMW fraction obtained from Sephadex G15 was evaluated for further antibacterial activity against various organisms as indicated in Table 3 using agar well diffusion method [16].

Determination of Molecular Mass of LMW

The lyophilized LMW was dissolved in 0.5 mL of water, and 50 μL was applied to HPLC (Waters 600, Millipore Corp., Milford, MA) connected with reverse-phase column C18 (Luna

5 μm , 100 \AA , 30 \times 2 mm), pre-equilibrated with 3 % of ACN having 0.1 % TFA. Fractions were eluted using water having 0.1 % TFA (A) and ACN having 0.1 % TFA (B) in gradient of 0 to 15 min with 3 % B and 16 to 40 min with 60 % B. The flow rate was maintained at 1.5 mL min^{-1} , and fractions were monitored at 215 nm. The mass of the compound was identified on a liquid chromatography equipped with diode array (Waters 600, Millipore Corp., Milford, MA) connected to time-of-flight mass spectrometer using QStar pulsar (Applied Biosystems, Foster City, CA). The compound was applied (10 μL) to the C18 column and eluted using the above-mentioned mobile phase and gradient at the flow rate of 1.5 mL min^{-1} . The liquid chromatography (LC)-MS/time-of-flight (TOF) parameters used were as follows: end plate offset voltage, 500 V; capillary voltage, 4500 V; nebulizer pressure, 1.2 bar; dry gas flow, 6 L min^{-1} ; dry temperature, 200 $^{\circ}\text{C}$; collision energy, 18 eV/z; transfer time, 100 μs ; and collision RF, 400 Vpp.

UV Absorption Spectrum of the LMW

The lyophilized LMW was dissolved in water and scanned for absorption spectrum at UV region using UV/vis spectroscopy (Shimadzu, Japan) from 200 to 300 nm.

FTIR Spectroscopy

The Sep-Pak purified fraction was lyophilized, and 1 mg of sample was grounded with 100 mg of KBr to get a fine powder and made as pellet. The FTIR spectrum was recorded in the frequency range of 4000 to 400 cm^{-1} using Nicolet 6700 FTIR spectrometer (Thermo, Madison, WI).

NMR Spectroscopy

The NMR spectrum was recorded with Bruker 400-MHz spectrophotometer at 295 K. The lyophilized Sep-Pak purified sample was dissolved in D_2O for deuterium exchange and analyzed for ^1H and ^{13}C NMR. The CH , CH_2 , and CH_3 were identified by distortionless enhancement by polarization transfer (DEPT) analysis. For the identification of hydroxyl group, the lyophilized LMW was dissolved in DMSO and OH exchange was done with a drop of D_2O . The spectrum was recorded before and after exchange with D_2O . The chemical shift was recorded using tetramethylsilane (TMS) as internal reference.

Salkowski Reaction

The LMW, tryptophan, and indole-3-lactic acid (5 mg mL^{-1}) were treated with Salkowski reagent and incubated for 30 min to differentiate the indole derivatives [17].

Thin-Layer Chromatography

The TLC plate was prepared with the uniform slurry of silica gel GF in water (2:5) and poured in glass plates with the thickness of 2 mm. After activation at 100 $^{\circ}\text{C}$ for 2 h, the LMW sample (having molecular mass similar to tryptophan) and tryptophan (5 mg mL^{-1}) was applied on the TLC plate with applicator. After air-drying, the plate was developed in chamber saturated with acetone/ammonia/isopropanol (0.45:1.25:5.6) and the spots were identified using iodine vapors.

Results and Discussion

The demand for antimicrobial compounds is constantly growing with regard to the threat caused by pathogenic strains; hence, purification and characterization of novel antimicrobial compounds produced by LAB are currently being focused. In recent years, many organic compounds produced by LAB that were able to inhibit other microbes were identified [18]. Idly and uttappam batter are indigenous south Indian fermented food sources from which various organisms were isolated and characterized [13–15] that exhibited effective antimicrobial and probiotic properties [19–21].

In this study, a novel antimicrobial compound produced by the LAB isolates was extracted from cell-free supernatant (CFS) with 85 % acetone, purified, and characterized. The antimicrobial compounds from crude 85 % supernatant were purified by passing through gel filtration chromatography Sephadex G25 followed by Sephadex G15, which gave a Gaussian peak with the elution volume (V_e) of 34 mL with no contaminants of higher molecular weight (data not shown). Further purification by hydrophobic interaction chromatography yielded a pure compound as depicted by LC profile (Fig. 1a). In this study, the 85 % supernatant after acetone precipitation was considered as crude LMW fraction, as CFS from LAB culture in MRS broth has bacteriocin as the major antimicrobial compound [22–24]. This LMW was present in very low concentration in the CFS as reflected by absence of antimicrobial activity after protease treatment of CFS. The supernatant obtained after precipitation of bacteriocin by acetone (85 %) and concentrated to 9 mL (111-fold concentrate of original CFS) showed

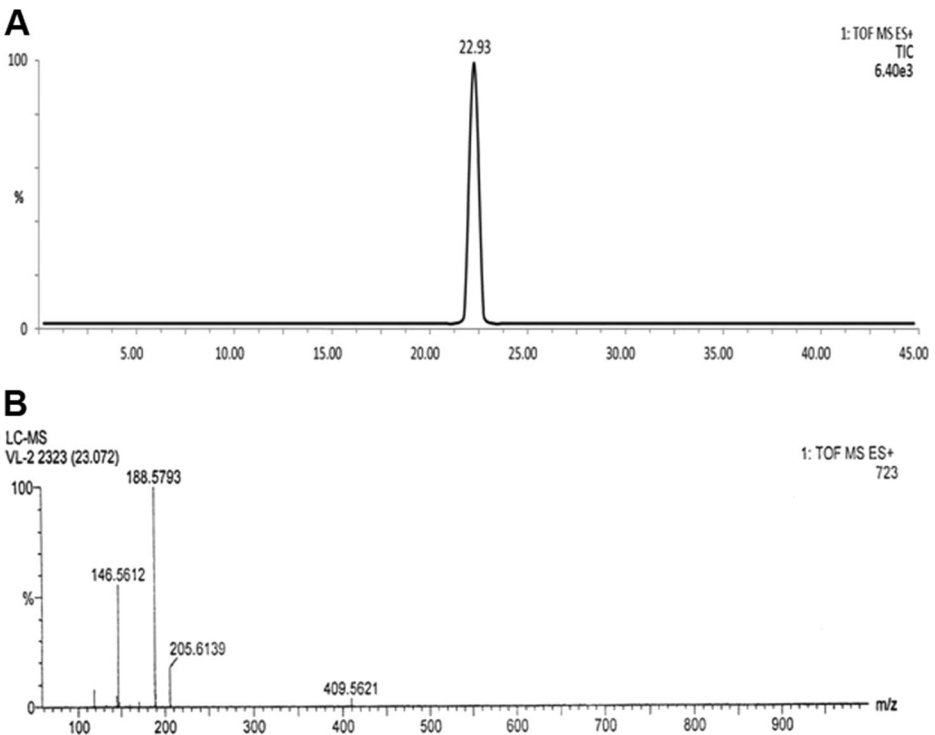


Fig. 1 Liquid chromatography showing retention time of LMW compound (a) and mass spectra of LMW compound (b)

Table 1 Physiochemical properties of LMW

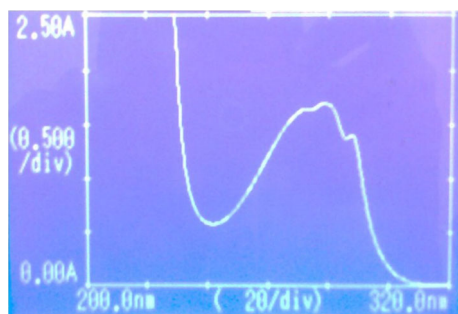
	2-Hydroxyl indole-3-propanamide
Molecular formula	C ₁₁ H ₁₂ N ₂ O ₂
Appearance	Pale yellow
(+) ESI-MS	205.614 (M+H)
Calculated	205.226 (M+H)
Solubility	Soluble in water and acetone Insoluble in benzene and chloroform

antimicrobial activity (taken as crude). This upon further purification resulted in a yield of 11 % (Supplementary file S1). The purified LMW compound was active at a limited range of pH, up to pH 5.5 while the activity was lost at pH 6.5. A similar observation was made in an earlier study where a fatty acid derivative lost its antimicrobial activity at the pH of 6.0 [25]; however, certain fatty acid derivative compounds exhibited antifungal activity [8] while LMW in the present study did not exhibit any antifungal activity.

The physiochemical properties of the purified compound are given in Table 1. The compound was readily soluble in polar solvents like water, methanol, and acetone but insoluble in chloroform and benzene.

The Sep-Pak purified sample had retention time of 23 min. The ESI-MS showed a base peak at 205.61 Da [M+H] with fragmentation pattern of *m/z* 188 (M+H) and 146 (M+H) (Fig. 1b). The fragmentation pattern corresponds to loss of NH₃ (17 Da), ethanone (42 Da), and CO (28 Da,) suggesting that the compound exhibited fragmentation except indole ring (117 Da) and correlate with the structure of the compound. The determined molecular weight of the LMW was 205.614 Da (M+H) using TOF-MS-ES⁺, and the calculated molecular mass was 205.225 Da (M+H). Although the reason for the difference in the molecular mass is not known exactly, it might be of the limitations of instrument and its resolution. Since further spectroscopic studies have confirmed the structure of the LMW compound, high-resolution MS for the compound was not performed.

The LMW isolated from all isolates exhibited absorption maximum (λ_{\max}) in H₂O at 287.6 and 279.2 nm (Fig. 2) which was also similar to tryptophan, suggesting that the compound might be a derivative of tryptophan. The FTIR spectra of LMW compounds from all isolates have shown similar pattern as depicted in Fig. 3, suggesting the presence of similar functional groups, and the data are correlated in Table 2. Thus, LMW compounds produced by all isolates were same. The ¹³C NMR of LMW showed presence of 11 carbons of which 8 are in aromatic

**Fig. 2** UV absorption spectrum of LMW compound that shows absorption maximum at 287 and 279 nm

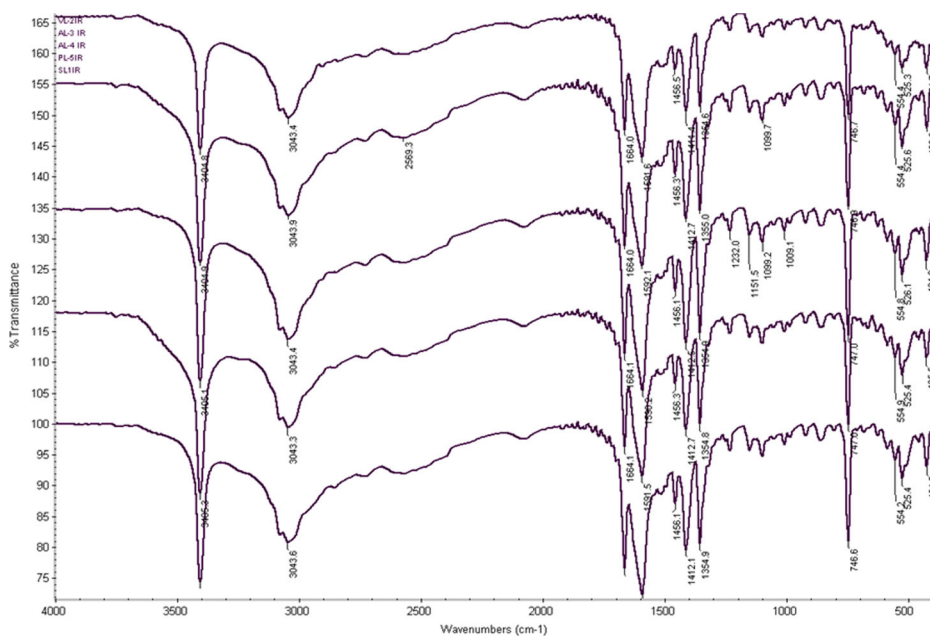


Fig. 3 Overlapped IR spectrum of five LMW compounds in KBr cells indicating similar spectra

region. The ^{13}C NMR report was (100 MHz, D_2O) δ (174.44, 136.27, 126.58, 124.96, 122.07, 119.40, 118.38, 111.88, 107.42, 55.01, 26.33) (Supplementary file S2). The proton NMR analysis report was ^1H NMR (400 MHz, deuterium oxide) δ 7.73 (dd, $J=8.0, 1.2$ Hz, 1H), 7.57–7.50 (m, 1H), 7.33–7.15 (m, 3H), 4.05 (dd, $J=8.1, 4.8$ Hz, 1H), 3.53–3.43 (m, 1H), and 3.30 (dd, $J=15.3, 8.1$ Hz, 1H) (Supplementary file S3). The DEPT 135 analysis showed inverted peak at $\delta -26.3$, and the peak at $\delta -55$ confirmed the presence of CH_2 and CH in the alkyl carbon (Supplementary file S4). The FTIR analysis showed the presence of amide and hydroxyl group coupled with CH. The ^{13}C NMR confirmed the presence of 11 carbons in the LMW, out of which 8 are in heterocyclic ring indicating an indole derivative. The peaks in the range of 7 to 8 and 100 to 140 in ^1H NMR and ^{13}C NMR, respectively, correspond to the heterocyclic ring. The presence of aliphatic CH stretch is confirmed in ^1H NMR by the peaks in the range of 3.2 to 3.5 and the peaks in the range of 4.0 corresponding to $-\text{CH}(\text{OH})-$ stretch. In ^{13}C NMR, the presence of carbonyl and its derivatives was determined by the presence of peak in 174, carbon linked with hydroxyl group by the peak at 55 [26], and the presence of CH_2 in aliphatic chain is determined by the peak at 26. To further confirm the

Table 2 Correlation table of IR spectroscopy

Band type	Wave number (cm^{-1})	Possible bonds
Sharp	3405	Secondary amine N–H
Broad	3043	C–H or aromatic or alkyl
Sharp	1664	Amide C=O
Sharp	1590	N–H amide bend

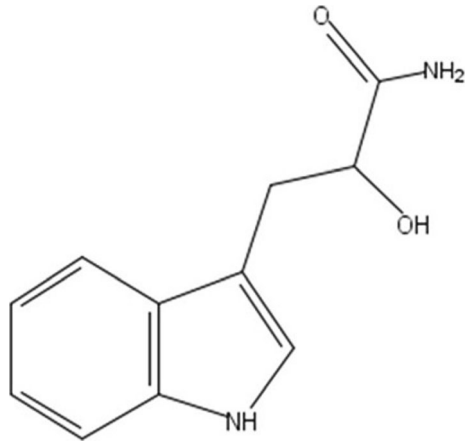


Fig. 4 Predicted structure of LMW compound 2-hydroxyl indole-3-propanamide

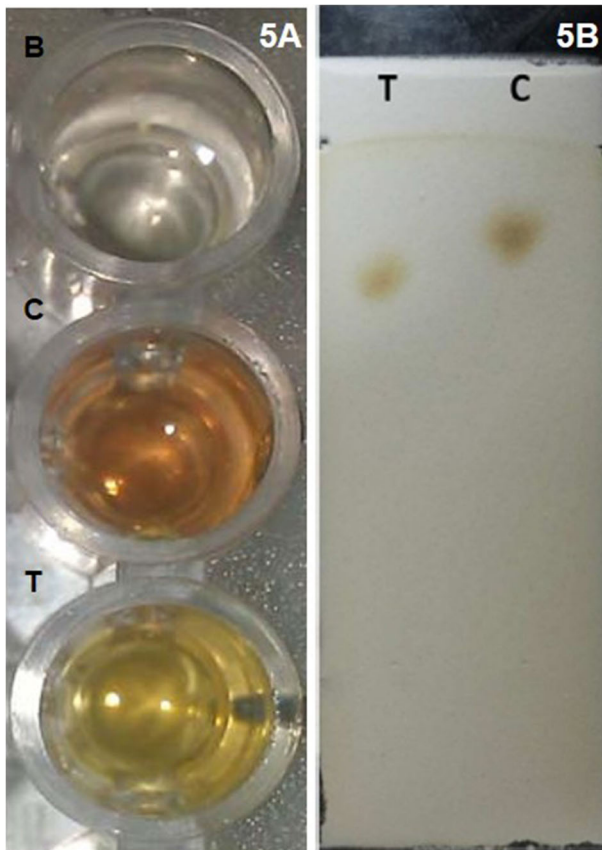


Fig. 5 Differentiation of tryptophan (*T*) and LMW compound (*C*) against blank (*B*) using Salkowski reagent (a) and thin-layer chromatography (b)

Table 3 Inhibitory spectrum of LMW against various indicator organisms

Indicator	Activity (mm)
<i>Lactobacillus brevis</i> MTCC 1750	–
<i>Lactobacillus rhamnosus</i> MTCC 1408	–
<i>Lactobacillus plantarum</i> MTCC 6161	–
<i>Brevibacterium casei</i> MTCC 1530	–
<i>Leuconostoc mesenteroides</i> MTCC 107	12±2
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MTCC 440	13±2
<i>Lactobacillus fermentum</i> MTCC 1745	14±3
<i>Aeromonas hydrophila</i> MTCC 1739	16±2
<i>Escherichia coli</i> MTCC 728	16±4
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MTCC 3038	20±4
<i>Micrococcus luteus</i> MTCC 106	20±3
<i>Bacillus subtilis</i> MTCC 619	20±3
<i>Bacillus cereus</i> MTCC 1272	21±3
<i>Vibrio parahaemolyticus</i> MTCC 451	22±2
<i>Pseudomonas aeruginosa</i> MTCC 2295	23±2
<i>Listeria monocytogenes</i> MTCC 657	24±3
<i>Staphylococcus aureus</i> MTCC 737	24±4
<i>Candida albicans</i> MTCC 3017	–

Inhibition zone in millimeter inclusive of well diameter 6 mm; values are mean±SD of three independent experiments performed in duplicate; (–) indicates no activity

hydroxyl group in LMW, after recording in DMSO, the hydroxyl exchange was done with addition of a few drops of D₂O into DMSO and the spectrum recorded again. The peak at δ 8.19 in DMSO disappeared after addition of D₂O, suggesting the presence of hydroxyl group, while that at δ 11.04 suggesting the presence of –NH– group, thus confirming the compound (Supplementary file S5). Thus, the ¹H, ¹³C, and HMBC spectra (Supplementary files S2 to S6) have established the structure of LMW as 2-hydroxyl indole-3-propanamide and are depicted in Fig. 4. The tryptophan, indole-3-lactic acid (ILA), and the LMW were differentiated with Salkowski reagent (Fig. 5a). The LMW gave orange color, while tryptophan gave yellow color. Further differentiation of tryptophan and the LMW was achieved by thin-layer chromatography (TLC) using acetone/ammonia/isopropanol (0.45:1.25:5.6) (Fig. 5b).

For LMW and tryptophan having similar molecular mass, NMR and FTIR spectra were differentiated by TLC and Salkowski color reaction. Many indole compounds and their derivatives were well known to exhibit antimicrobial activity [27, 28]. Indole derivatives have been identified as secondary metabolites acting as potent antimicrobials from several sources especially from plants, microbes, and marine sources. The salient role of indole primarily involves signaling molecules in acid resistance, biofilm formation, and more [29]. Earlier studies in *Bifidobacterium* have demonstrated ILA, a metabolite from tryptophan exhibiting antifungal activity [30].

Indole propanamide derivative was known to exhibit antimicrobial and antiviral activity [27], and chemically derived indole-3-propanamide also acts as an immunosuppressive agent [31]. The LMW exhibited a wide spectrum of activity against various LAB and pathogens as summarized in Table 3. The LMW was not active against probiotic strains like *Lactobacillus rhamnosus*, *Lactobacillus Plantarum*, and *Lactobacillus brevis*. On the other hand, it exhibited effective inhibition against various food-borne pathogens like *Listeria monocytogenes* and *S. aureus*, while less effective against *Escherichia coli* and *Aeromonas hydrophila*. Although

various compounds produced during fermentation by LAB were studied [2], this is the first to report production of 2-hydroxyl indole-3-propanamide exhibiting antibacterial activity. In recent years, some LAB were reported to produce certain other antimicrobial compounds like benzene acetic acid and 2-propenyl ester exhibiting antifungal activity [32]. This LMW isolated has effective antibacterial activity against Gram-positive and Gram-negative bacteria but spares the probiotic strains like *Lactobacillus rhamnosus*, suggesting that the compound can be used against clinical pathogens without harming the resident probiotics. The probable active moiety that exhibits antimicrobial activity of the LMW may be the amide group which is a strong nucleophile-attacking molecule. Even though, in the present study, the LMW compound exhibited its activity at the concentration of 50 μM , further substitutions in the side chains of indole might enhance the antimicrobial activity and stability of the compound in alkaline conditions.

Conclusion

Thus, 2-hydroxyl indole-3-propanamide from LAB strains isolated in this study showed broad-spectrum antibacterial activity against various Gram-positive and Gram-negative pathogenic organisms sparing the probiotic organisms like *Lactobacillus rhamnosus*, may have the potential as antibiotics, and play a significant role in maintenance of human health. As the molecular weight of LMW being 204 Da, it may be of use as antibiotic for several clinical applications. Further, using this compound as a lead molecule, additional quantitative structure–activity relationship (QSAR) studies, which quantitate structure and activity of a molecule, may yield a better understanding of molecule and its activity against other pathogenic organisms. Hence, the identified compound could have significant application in medicine.

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