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ORIGINAL RESEARCH ARTICLE

Lactococcus lactis subsp. *cremoris* of Plant Origin Produces Antifungal Cyclo-(Leu-Pro) and Tetradecanoic Acid

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Abstract The antifungal cyclo-depeptide and the fatty acid were isolated and purified from an indigenous strain of *Lactococcus lactis* subsp. *cremoris.* Maximal activity was observed at pH 5.5 and 6.5, and at 30 °C under stationary conditions, which was detected in the culture supernatant 8 h post-inoculation in MRS broth until 22 h. The activity of antifungal compounds in the culture supernatant was sensitive to pH and temperature; and was protease-resistant. The antifungal compounds were concentrated by freeze-drying and ultrafiltration with activity retained in 1 kDa filtrates indicating low molecular weight metabolites. The compounds were further extracted by using different solvents amongst which, ethyl acetate provided the highest recovery. Antifungal compounds were separated on a silica gel column into two active fractions that were revealed to be tetradecanoic acid and cyclo-(Leu-Pro), a cyclic dipeptide, by GC–MS. Herein, we describe and attribute the biocontrol potential of *L. lactis* subsp. *cremoris* to the low molecular weight antifungal compounds isolated, which is the first report of their isolation from this strain. The broad antifungal spectrum of this candidate advocates further exploration of its biocontrol potential in managing fungal infections in different food and feed systems.

Keywords Lactic acid bacteria · Tetradecanoic acid · Cyclo-(Leu-Pro) · Pomegranate · Biocontrol

Introduction

Lactic acid bacteria (LAB) are food-grade microbes with a 'Generally Recognized As Safe' (GRAS) status. LAB produce a myriad variety of metabolites that are antibacterial and antifungal compounds (AFC) [1]. The antifungal LAB can be isolated from aerial plant surfaces, vegetables, fermented products, grass silage, malted barley, sourdough, pickled cabbage, wax gourd, maize leaf, malted cereals, sheep milk, intestinal samples of human, mouse, pig and bovine etc. Antifungal LAB have a wide range of applications related to the prevention of fungal spoilage of several feeds and foods [2]. The ability of LAB to prevent fungal rot in commercially valuable fruits is well documented [3, 4], highlighting its biocontrol potential in plants. The antifungal potential of the LAB is primarily due to the decrease in pH by the production of organic acids [5]. The major antimicrobials identified from LAB are lactic acid, acetic acid, caproic acid, diacetyl, reuterin, hydrogen peroxide, 3-hydroxy fatty acids, phenyllactic acid, proteinaceous compounds and cyclic dipeptides [6]. Most of the published literature focuses on the isolation of AFC from the genus *Lactobacillus*, while others remain under-studied. There is a real possibility of discovering new types of AFC from other genera of LAB and new antimicrobial compounds from them, as demonstrated in this study.

LAB produce a variety of AFC, however, the concentration of such compounds may not be sufficient to sustain inhibitory activity. Thus, the evaluation of the time-course of production of AFC and its correlation to the growth

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phase in different cultural conditions is advantageous for maximizing the production and recovery of AFC [7]. Biosynthesis of similar metabolites by LAB is growth-associated and extracellular and/or released after cell lysis [8]. The AFC produced under controlled conditions can be further used to prepare antifungal product formulations for applications in the management of fungal infections in crops. Under natural conditions, concentrations of AFC produced by LAB are low. Many times, the inhibitory activity of AFC results from the synergistic action of such metabolites. Thus, novel approaches, techniques and methodologies are required for the isolation and identification of AFC. Purification of the AFC produced by LAB may be performed by using various techniques like solvent extraction, ultrafiltration and column chromatography. The high-resolution analytical techniques used for the identification of AFC are GC–MS, HPLC–MS, MS/MS and nuclear magnetic resonance (NMR) spectroscopy [9].

Given this background, the major objective of this work was the optimization of different parameters for maximizing the production of AFC and purification and identification of AFC from antagonistic *L. lactis* subsp. *cremoris* (*Llc*). This study's results can undoubtedly guide further studies to develop formulations to manage phytopathogenic fungi in sustainable, organic and green bioeconomy.

Materials and Methods

Lactic Acid Bacterial Strain

A potent antifungal LAB, *Lactococcus lactis* subsp. *cremoris* (*Llc*) (GenBank accession number: JN792509), an indigenous isolate from pomegranate bud was used in this study [10].

Antifungal Activity Assay

The antifungal activity assay was performed using the modified agar overlay technique [4]. The microtiter plate well method [7] was used to determine the concentration of AFC in the culture supernatant (CS). The relative amount of antifungal compound in the broth was determined by the highest dilution inhibiting 100% spore germination and expressed as dilution units (DU). The inhibition (%) of the test fungus by CS was determined by measuring optical density at 550 nm in a microplate reader. The inhibition (%) was calculated as follows: (OD of control − OD of test) ÷ OD of control × 100. The antifungal assays were performed against *Penicillium* GF29, one of the etiologies of pomegranate fruit rot.

Time-Course and Optimization Studies

Optimization of the cultural conditions used to produce AFC was done according to Gajbhiye and Kapadnis [4]. The effect of pH, temperature, media and agitation on the production of AFC by *Llc* was evaluated. CS was prepared from the aliquots withdrawn after every 2 h from *Llc* inoculated MRS broth and tested for the antifungal activity, pH and cell density for the determination of time-course of production of AFC.

Characterization of Culture Supernatant

The pH of aliquots of CS was adjusted from 3 to 9 and their antifungal activity was determined. Another set of aliquots was exposed to 50, 70 and 100 °C for 1 h and at 121 °C for 15 min followed by determination of residual activity. Similarly, 10 ml aliquots were treated with proteinase K, trypsin and pepsin, at pH 7.6, 7.6 and 2.0, respectively [7]. Following incubation at 37 °C for 1 h, the pH was readjusted and residual activity was determined.

Purification of Antifungal Compounds

Concentration by Lyophilization

CS from *Llc* was pre-frozen in a cooling bath (Christ CB 18–40, Germany) containing glycerol at − 60 °C. Freeze-drying was carried out at 0.08 mbar vacuum pressure and ice condenser temperature of − 55 °C for 12 h in a lyophilizer (Christ Alpha 1–2 LD plus, Germany). After this, the total volume was adjusted with sterile phosphate buffer (pH 6.5, 50 mM) to get 15 fold concentrated CS (CCS).

Fractionation by Ultrafiltration

The fractionation of CCS was done using an ultrafiltration centrifugal device (Pall Life Sciences, USA). The CCS was passed through 10, 3 and 1 kDa membrane gradually [11]. For this, 15 ml of CCS was added to the centrifugal device and spun at 4500 g for 30 min at 25 °C. Following every step, the retentate and filtrate were checked for antifungal activity by the microtiter plate well method.

Extraction by Solvents

Ethyl acetate, butanol, cyclohexane, petroleum ether and chloroform were used for the extraction of AFC. Each of the solvents was mixed with ultrafiltrate in 1:1 ratio separately in a separating funnel and agitated overnight. The two liquid phases, viz., extraction phase (solvent phase) and retentive phase (aqueous phase) were separated and tested for activity. Ultrafiltrates from MRS broth were

likewise treated. The ethyl acetate extracts were further concentrated in a rotary vacuum evaporator at 50 °C. The extracts were analyzed by GC–MS.

Silica Gel Column Chromatography

The extract was further purified by silica gel column chromatography. A stepwise elution was carried out using a mixture of ethyl acetate and hexane (7:3 v/v). The fractions were collected at a rate of 3 ml/min. The fractions were evaporated until dry and antifungal activity was checked by the microtiter plate well method by dissolving it in ethyl acetate. The purity of each active fraction was checked by the thin layer chromatography and then analyzed by GC–MS.

Gas Chromatography–Mass Spectroscopy Analysis

The analysis of active fractions was performed on a GCD-HP1800A GC/MS instrument (Hewlett-Packard, USA). The GC oven was maintained at 120 °C initially and then gradually increased to 280 °C at 4 °C per 3 min. Helium was used as carrier gas at a flow rate of 30 ml/min. The identification of compounds was based on fingerprinting analysis by comparison with reference compounds present in the MS spectra library.

Statistical Analysis

The data was analyzed for the significant difference between the groups by Post Hoc Test (Tukey's HSD procedure) using SPSS 18 at a 95% confidence level where $P \leq 0.05$ was considered to be statistically significant.

Results and Discussion

Optimization Studies and Characterization of Antifungal Activity of Culture Supernatant

The maximum antifungal activity of *Llc* was found at pH 5.5 and 6.5 and at temperature 30 °C on MRS agar which is in agreement with reports of other researchers [1, 12]. *Llc* showed very low activity on Elliker's, M-17 and APT, whereas, maximum activity was recorded on MRS followed by TSA. The relationship between cell density, pH and antifungal activity was studied over for *Llc* in MRS broth at 30 °C over 40 h (Fig. 1). Antifungal activity was detected exclusively in CS between 8 and 22 h, suggesting compounds from the metabolism of live cells secreted extracellularly rather than a release from dead cells. The maximum observed activity using CS was 8 DU with an inhibition zone of 13 mm at the end of the log phase which

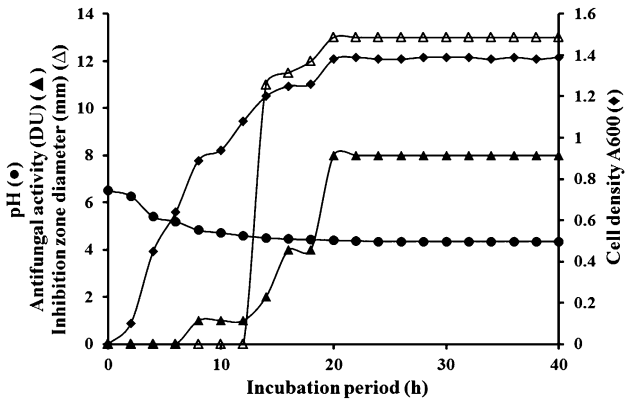


Fig. 1 Time course of antifungal activity of *Lactoccocus lactis* subsp. *cremoris*. pH of culture supernatant (filled circle), cell density in terms of A_{600} (filled diamond), antifungal activity in terms of dilution units (DU) (filled triangle) and inhibition zone diameter (mm) (open triangle)

remained unaffected thereafter. Many reports demonstrate that the production of AFC is growth associated. According to Coloretti et al. [13], the AFC are released from lactobacilli during the early growth phase and late growth phase. Sjogren et al. [14] have reported the synthesis of antifungal hydroxy fatty acids from *Lact. plantarum* during the log phase and not during the stationary phase. On similar lines, Dalie et al. [8] described the production of AFC to secondary metabolites by *Pediococcus pentosaceus* beginning at the end of its logarithmic phase and plateauing at the end of the stationary phase. According to a recent optimization study, *Lact. rhamnosus* produces AFC at 34 °C under shaking conditions [15].

The effect of pH on the antifungal activity of CS from *Llc* was studied from pH 3 to 9. Herein, the maximal activity of CS was observed at pH 3 and 4, which rapidly decreased with the increase in pH until pH 7 (Table 1). Exposure of CS to high temperatures from 50 to 121 °C brought a significant reduction in the activity ($P < 0.05$) suggesting the thermolabile nature of AFC. However, when treated with proteases, the activity of CS retained. Altogether, the antifungal activity of CS was pH-dependent and protease-resistant. The antifungal activity of LAB is due to the production of a variety of metabolites, and organic acids are the major inhibitory substances present in CS [1, 5]. The AFC produced by many lactobacilli are thermostable and resistant to protease degradation [4, 8]. Li et al. [11] have described the pH-dependent and protease-resistant antifungal activity of CS from *Lact. casei.* However, Abouloifa et al. [16] have reported protease and lipase sensitive antifungal activity of culture supernatant indicating their proteinaceous and lipid nature.

Table 1 Effect of different parameters on antifungal activity of culture supernatant

Parameter	Inhibition* (%)	
	CS from *Llc*	MRS**
pH		
3	96.77 ± 1.98a	96.05 ± 1.33a
4	96.06 ± 0.93a	26.84 ± 1.67b
5	91.56 ± 0.93b	12.60 ± 1.45c
6	55.45 ± 0.34c	1.20 ± 0.93d
7	2.38 ± 1.21d	0.86 ± 0.99d
8	1.36 ± 0.54e	0.82 ± 0.92d
9	1.33 ± 0.88e	0.74 ± 0.44d
Untreated	97.77 ± 0.33a	0.86 ± 1.82d
Temperature (°C)		
50	96.78 ± 2.33a	1.44 ± 0.36a
70	94.54 ± 2.1b	1.55 ± 1.22a
100	66.89 ± 1.22c	3.77 ± 0.56b
121	52.98 ± 0.56d	3.78 ± 0.46b
Untreated	97.87 ± 1.45a	1.08 ± 1.44a
Proteolytic enzymes		
Proteinase K	93.73 ± 0.89a	0.83 ± 1.29a
Trypsin	93.82 ± 0.25a	0.93 ± 0.61a
Pepsin	94.46 ± 1.33a	0.45 ± 0.69a
Untreated	94.77 ± 1.06a	0.55 ± 0.12a

*Determined by microtiter plate well assay using *Penicillium* sp. GF29; each data point represents mean of triplicate ± SD; values with different letters in a column for each parameter are significantly different ($P < 0.05$) according to Tukey's HSD

**Served as control

Table 2 Antifungal activity of fractions of culture supernatant subjected to ultrafiltration

Sample type	Inhibition* (%)	
	Llc	MRS**
CCS	99.51 ± 0.23a	7.33 ± 0.33a
10 kDa filtrate	99.33 ± 0.18a	6.22 ± 0.56a
10 kDa retentate	4.23 ± 1.23b	6.12 ± 0.12a
3 kDa filtrate	98.33 ± 0.94a	6.27 ± 0.18a
3 kDa retentate	2.34 ± 0.64c	5.12 ± 0.99b
1 kDa filtrate	98.78 ± 0.31a	4.77 ± 0.91b
1 kDa retentate	2.11 ± 0.65c	4.61 ± 0.36b

*Determined by microtiter plate well assay using *Penicillium* sp. GF29; each data point represents mean of triplicate ± SD; values with different letters in a column are significantly different ($P < 0.05$) according to Tukey's HSD

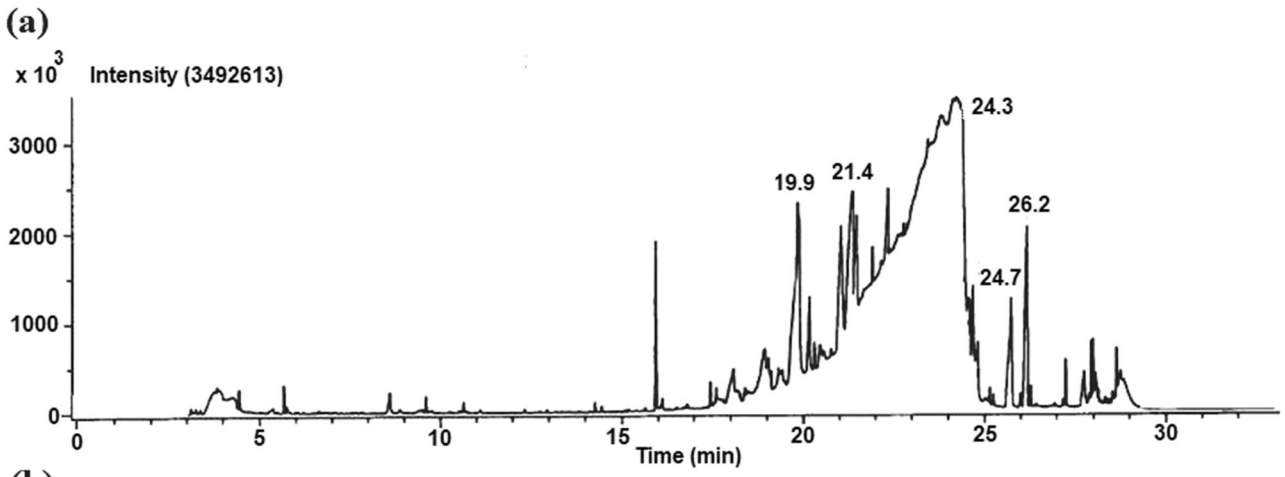
**Served as control

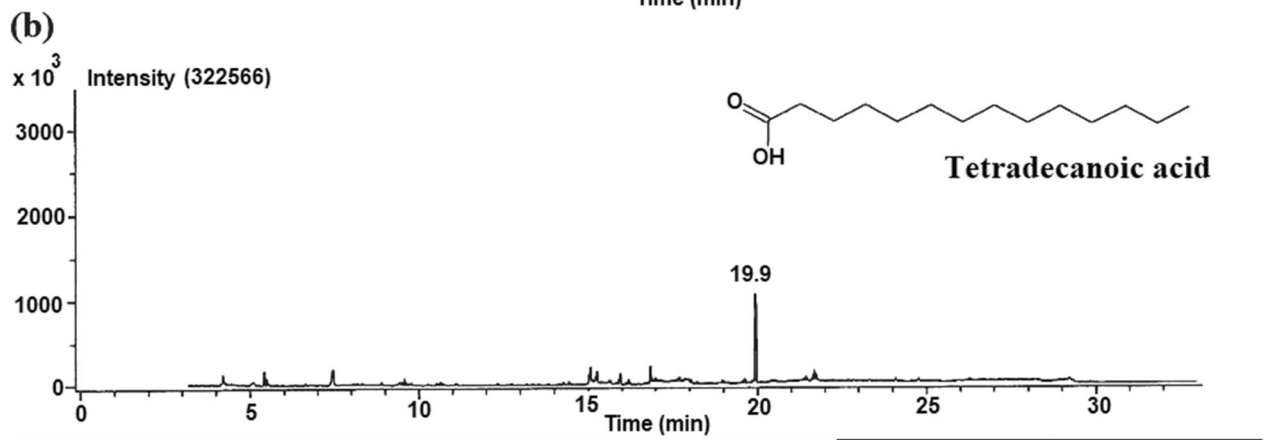
Purification of Antifungal Compounds

Following the fractionation of CCS, the ultrafiltrates showed significant activity as compared with retentates (Table 2) indicating metabolites of molecular weight lower than 1 kDa. The extraction of AFC from 1 kDa ultrafiltrate was attempted with different solvents. Ethyl acetate gave the highest recovery of the AFC as its extract showed strong inhibition and has a broad-spectrum of activity (data not shown). The extraction of AFC from CS of LAB using ethyl acetate has been reported recently [17]. Thus, all the AFC extracted in the ethyl acetate were low molecular weight compounds and similar findings are reported by Dalie et al. [8] and Li et al. [11].

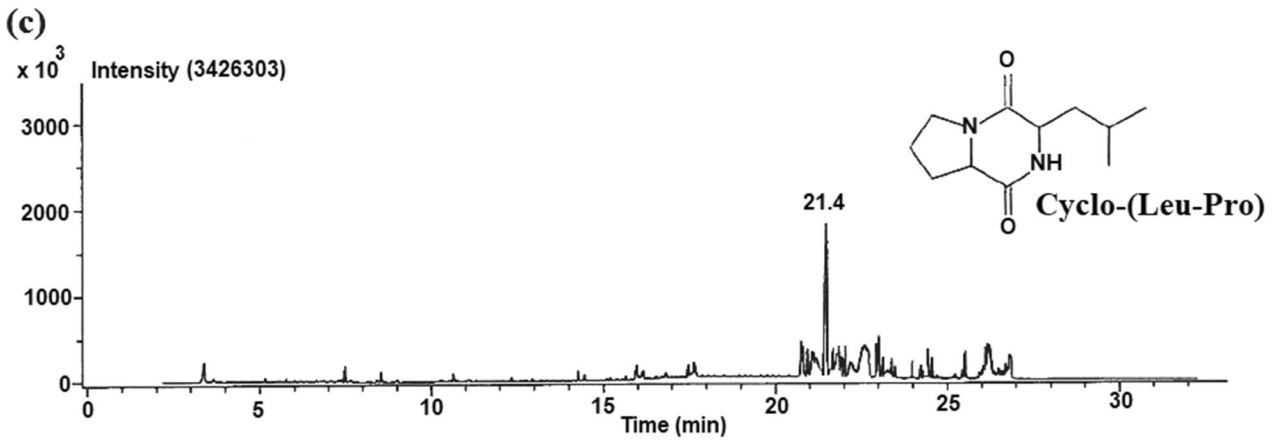
GC–MS analysis of ethyl acetate extract revealed the presence of tetradecanoic acid; pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) i.e., cyclo-(Leu-Pro); pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(phenylmethyl) i.e., cyclo-(Phe-Pro) and Octadecanoic acid (Fig. 2a). On further chromatographic purification, around 200 fractions were collected and the antifungal activity of these was determined intermittently. The antifungal fractions from 20 to 60 were pooled and reloaded into a silica gel column. Three fractions (41, 42 and 49) displayed antifungal activity. Thin layer chromatography showed the presence of the same compound in 41st and 42nd fraction. GC–MS analysis of the active fractions led to the identification of tetradecanoic acid (Fig. 2b) and cyclo-(Leu-Pro), a cyclic dipeptide (Fig. 2c). There are several reports on the biosynthesis of antifungal peptides and cyclic dipeptides by LAB. AFC of proteinic nature with a molecular mass of 3 kDa has been isolated from *Lact. coryniformis* subsp. *coryniformis* [7]. The production of antifungal cyclic dipeptides, cyclo-(Phe-Pro) and cyclo-(Phe-4-OH-Pro) by *Lact. plantarum* MiLAB 393 was first reported by Strom et al. [12]. *Lact. plantarum* produces several cyclic dipeptides (cyclo-(Leu-Pro), cyclo-(Phe-Pro) and cyclo-(Val-Pro)) [18], and similar compounds (cyclo (Phe-Pro), cyclo (Ile-Pro)) were detected in CS from several dairy propionibacteria [19]. Li et al. [11] have described the presence of a novel antifungal cyclic dipeptide, cyclo-(Leu-Pro), in CS from *Lact. casei* along with 5,10-Diethoxy-2,3,7,8-tetrahydro-1*H*,6*H*-dipyrrolo [1,2-a;1′,2′-d] pyrazine and 2,6-diphenyl-piperidine. Antifungal peptide or proteinaceous compound is another subset of AFC that has been discovered from several LAB such as *Lact. plantarum* [20–22] and *Leuconostoc mesenteroides* [23]. Bazukyan et al. [24] have proposed that the antifungal activity of *Lact. rhamnosus* is attributed to the proteinaceous AFC associated with the bacterial cell wall.

A fatty acid, tetradecanoic acid (myristic acid) was detected in the purified fraction of ultrafiltrates from *Llc*. Several publications report the antifungal properties of





Peak Number	Time [min]	Type	Peak Width (FWH) [min]	Area [Intens.*sec]	Height	Description	Start Point Time [min]	Start Point Height	End Point Time [min]	End Point Height
1	19.9	BB	0.0399	3320166.16	1213629.28		19.8	264593	20.0	366148



Peak Number	Time [min]	Type	Peak Width (FWH) [min]	Area [Intens.*sec]	Height	Description	Start Point Time [min]	Start Point Height	End Point Time [min]	End Point Height
1	21.4	BB	0.0838	15030949.13	1219131.98		21.3	901352	21.5	1380632

Fig. 2 GC spectrum of compounds present in ethyl acetate extracts of ultrafiltrates from *Lactococcus lactis* subsp. *cremoris* (**a**); GC spectrum of antifungal compound present in purified fraction I (**b**) and fraction II (**c**). Right hand corner of **b** and **c** shows structural formulae of tetradecanoic acid and cyclic dipeptide [cyclo-(Leu-Pro)], respectively

fatty acids from LAB [25]. *Lact. plantarum* MiLAB14 produces antifungal 3-hydroxy fatty acids namely, (3-(*R*)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecanoic acid, 3-(*R*)-hydroxydodecanoic acid and 3-(*R*)-hydroxytetradecanoic acid) [14]. Fatty acids such as butyric, caproic, caprylic, capric, lauric and palmitic acid have been proven to be effective in the management of fungal infections in tomato and cucumber [26]. Recently, Bukhari et al. [17] revealed the presence of fatty acids in the antifungal fractions obtained from *Lact. plantarum* and *Lact. coryniformis*. Thus, with reference to the literature survey, it can be noticed that research on the isolation of AFC is mainly focused on *Lactobacillus* species [27] however, the present investigation signifies the possibility of obtaining AFC from other genera of LAB. Some of the AFC may remain unidentified due to a lack of effective methodology or tools for their detection at low concentrations. Such investigations could lead to the isolation of novel antifungal bioprotective agents for the management of fungal infections in crops.

Conclusion

The production of AFC by *Llc* was growth associated and, the antifungal activity of CS was pH-dependent, sensitive to elevated temperature and unaffected by the action of proteases. Following the use of a cascade of isolation and purification techniques, AFC produced by novel *L. lactis* subsp. *cremoris* were identified as cyclo-(Leu-Pro) and tetradecanoic acid. These studies are the first report internationally on the production of these AFC from *Llc* of indigenous plant origin. Studies and findings of this work are vital to LAB research's knowledge base and to understand their varied and vast untapped potential. It also demonstrates new possibilities in managing fungal diseases in organic, sustainable and eco-friendly green chemistry way. Demonstrated antifungal activity and range towards phytopathogenic fungi; clearly show very high potential for developing it as a biocontrol agent in controlling fungal phytopathogens in agriculture and post-harvest applications.

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Compliance with Ethical Standards

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

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