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ESSENTIAL OILS AFFECT THE GROWTH AND FATTY ACID COMPOSITION OF *LACTOBACILLUS ACIDOPHILUS*

RAM KULKARNI* AND ASHWIN WADASKAR

Symbiosis School of Biomedical Sciences,
Symbiosis International University,
Lavale, Pune 412115, India

ABSTRACT

Lactobacillus acidophilus is an important lactic acid bacterium found in healthy human gut and it also has many food-related applications. Essential oils or their components are used as additives to the dairy products fermented by lactic acid bacteria for imparting the desired flavor. The objective of this study was to assess whether some essential oils affect the growth and fatty acid composition of *L. acidophilus*. Out of the nine oils tested, cinnamon and clove oils were the inhibitoriest to the growth of *L. acidophilus* as suggested by broth dilution as well as disc diffusion method. Neem and cinnamon oil caused increase in the proportion of unsaturated and cyclopropane fatty acids in *L. acidophilus*, respectively. This study signifies the potent application of essential oils for altering the growth and fatty acid composition of lactic acid bacteria which are significant parameters for industrial and health-related usage of these bacteria.

KEYWORDS: **Antimicrobial, lactic acid bacteria, lipids, fragrance, flavor, FAMEs**

RAM KULKARNI
Symbiosis School of Biomedical Science, Symbiosis International University,
Lavale, Pune 412115, India
ramskulkarni@gmail.com

INTRODUCTION

Bacteria belonging to genus *Lactobacillus* represent an important group with many significant properties for various applications. They are naturally found in various conventional fermented foods, commercially used for the production of lactic acid and fermentation of dairy products, and more importantly, are a part of microbiota of healthy human gut conferring numerous health benefits to the host [1,2]. One of the major ways by which some *Lactobacillus* species act as probiotics is by generating conjugated and short-chain fatty acids in the gut pointing towards the unique fatty acid metabolizing capabilities of the lactic acid bacteria [3]. It has also been shown that such metabolism of fatty acids by lactic acid bacteria in lumen profoundly affect the lipid profile and consequently host health [4,5]. Furthermore, it is also known that during their use in fermentation, lactic acid bacteria can alter the fatty acid content of the food which is undoubtedly linked to human health [6].Essential oils represent an important class of plant natural products which are in use by humans since ancient age. They have numerous applications in the flavor and fragrance, food, cosmetic and pharmaceutical industries. Essential oils have various biological activities such as antimicrobial, antifungal, antioxidant, anti-inflammatory, anti-cancer, anti-parasitic, insect-repelling, hypoglycemic, anti-ulcer, carminative, sedative, antidepressive, which are attributed to their main chemical constituents such as terpenes, phenylpropanoids, aldehydes, ketones, alcohols, etc [7,8,9,10].Plant related flavors mainly coming from the essential oils are gaining popularity for their usage in the fermented dairy products for imparting aesthetic value and biological activity [11,12,13]. Considering this and *Lactobacillus* being one of the most dominant genera of the dairy microorganisms, we analyzed inhibitory activity of some essential oils against *Lactobacillus acidophilus*. Further, we also assessed whether essential oils alter the fatty acid composition of *L. acidophilus.*

MATERIALS AND METHODS

Bacterium, growth condition and essential oils

L. acidophilus NDRI-BD-4 was obtained from National Centre for Industrial Microorganisms (NCIM) at CSIR-National Chemical laboratory, Pune. The bacterium was obtained as a stab culture and was grown in deMan rogosa and Sharpe (MRS) medium (broth and agar) statically at 37°C for 16-17 hours. Nine essential oils (basil, cedar wood, cinnamon, clove, eucalyptus, lemon, orange, neem and tea tree) were selected and procured based on their reported antibacterial activity and availability in the local market.

Determination of minimum inhibitory concentration by broth dilution method

Stocks of essential oils were made in DMSO to aid the solubilization. Specific volumes of stocks were directly added to MRS broth to get final essential oil concentrations of 0.0015, 0.0031, 0.0063, 0.013, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 % v/v. Ten microliter of the starter culture (OD_{600} 0.6) was inoculated in 1ml MRS broth containing essential oil of above dilutions. After incubation for 16-17 hours at 37°C, OD_{600} was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific). The lowest concentration of essential oil which showed the OD_{600} close to the control was taken as a measure of MIC.

Assessment of anti-microbial activity of essential oils using disc-diffusion method

Overnight grown culture of *L. acidophilus* was spread onto MRS-agar plate (1 x 10^5 cfu). Ten microliters of essential oil at its respective minimum inhibitory concentration in DMSO was impregnated on sterile disc loaded on a MRS agar cultured plate. The agar plates were incubated anaerobically at 37°C for 16-17 hours and the zone of inhibition was measured.

Preparation of fatty acid methyl esters (FAMEs)

1% of started culture (OD_{600} 0.6) was inoculated in 50 ml of MRS medium containing essential oil at the concentration equivalent to MIC. The cultures were grown anaerobically at 37°C for 16-17 hours and the cells were harvested by centrifugation 4500 rpm for 15 min. The pellets were washed with phosphate buffered saline to remove traces of the essential oil. Fatty acid methyl esters were prepared as described earlier [14] and extracted in hexane. The samples were dehydrated using sodium sulfate and analyzed by gas chromatography.

Analysis of fatty acid methyl esters (FAMEs) by gas chromatography

For gas chromatography, 7890B GC system (Agilent Technologies) coupled with Agilent 5977A MSD with SP™ 2560 capillary column (60 m x 180 µm x 0.18 µm) (Sigma-Aldrich) was used. Oven temperature was maintained at 130°C for 5 min, increased to 230°C at 5°C min^{-1} and held there for 20 min. Injector and detector (FID) temperatures was 250°C. Helium was used as a carrier gas with flow rate of 1 ml min^{-1} and split ration of 10:1. Mass spectra were obtained using 7890B GC couple with 5977A MSD gas chromatograph–mass spectrometer instrument (Agilent Technologies) at 70 eV with a scan time of 0.2 s for m/z 20–400 under the GC conditions same as described above. Fatty acids were identified by comparing acquired mass spectra with those of authentic standards and with the help of NIST 2011 Mass Spectral Library and Wiley registry of mass spectral data (10th edition). Some of the fatty acids were also identified by comparing their retention times with the authentic standards. The amounts of fatty acids were expressed as percent of total fatty acid and the unsaturation index was determined using a formula [% of monoenes + (2 × % of dienes) + (3 × % of trienes) / 100].

RESULTS AND DISCUSSION

Antimicrobial activity of essential oils against L. acidophilus

L. acidophilus culture was grown in the presence of various concentrations (0.0015%-1.8% v/v) of the essential oils (basil, cedar wood, cinnamon, clove, eucalyptus, lemon, orange, neem and tea tree) to analyze their inhibitory effect on the growth of *L. acidophilus*. DMSO was selected to dissolve essential oils considering the hydrophobic nature of most of the

essential oil and the initial results which showed that DMSO itself does not affect the bacterial growth (data not shown). In almost all the cases, bacterial growth, as indicated by OD_{600}, decreased with the increasing concentrations of essential oils (Fig. 1, Table 1). Cedarwood, cinnamon, eucalyptus and tea tree oils almost completely inhibited the bacterial growth exhibiting OD_{600} of 0.065, 0.005, 0.004 and 0.006, respectively, at MIC. On the other hand, basil, clove, lemon, neem and orange oils caused only partial growth inhibition with OD_{600} of 0.398, 0.137, 0.261, 0.129 and 0.164, respectively, at the highest concentration used. Further increase in the essential oil concentration in these cases resulted in increase in OD_{600}. This could be because of immiscibility of the oils in MRS medium at higher concentrations, although DMSO was used to aid the solubilization. Cinnamon oil was found to be the strongest growth inhibitor with the lowest MIC (0.025% v/v); whereas cedar wood, lemon, neem and orange oils were among weaker inhibitor oils with the highest MIC (1.6% v/v). Basil oil displayed the mildest antibacterial activity with only partial reduction in OD_{600} (till 0.398) at the highest concentration of 1.6% v/v.The essential oils were further compared to each other for their inhibitory potential against *L. acidophilus* by disc diffusion method. Consistent with the earlier results, cinnamon oil showed the strongest activity with the largest zone of inhibition followed by cedar wood, eucalyptus, clove and basil oils with zone of inhibition in the decreasing order (Fig. 2, Table 1). On the other hand, tea tree, lemon, neem and orange oils did not show any zone of inhibition even at much higher concentrations.Previous studies have already reported antimicrobial activity of some plant essential oils against lactic acid bacteria [15–19]. However, the essential oils tested in these studies were different from those analyzed in the present study. Only very few of the essential oils used in the present study have been studied in the earlier scarce reports for their activity against lactic acid bacteria. Some of the results obtained in the present study, such as low MICs of cinnamon and clove oil against *L. acidophilus*, are consistent with these earlier studies on various lactic acid bacteria [7,20,21]. Cecchini et al[15] tested activities of the pure phytochemicals dominantly present in some of the essential oils which we studied. For example, terpinen-4-ol (a major component of tea tree oil) and 1,8- cineole (a major component of eucalyptus oil), were inhibitory to the growth of *L. acidophilus*. This is in congruence with the antibacterial activities of tea tree and eucalyptus oils observed in the present study and suggested the possible role of these major bioactive chemicals found in them.

Effects of essential oils on the fatty acid composition

To test the effect of essential oils on the fatty acid composition in *L. acidophilus*, profiling of methyl esters of six major fatty acids in *L. acidophilus* grown separately with five essential oils was carried out using gas chromatography. These five essential oils were selected to encompass broad range to MIC values (highest to lowest) and zone of inhibition (no zone to the largest zone). The fatty acids which were identified and quantified included palmitic acid, stearic acid, oleic acid, vaccenic acid, linoleic acid and dihydrosterculic acid. Untreated control and *L. acidophilus* treated with all the essential oils except neem oil showed palmitic acid as the most abundant fatty acid in the range of 36.9%-45.7% (Fig. 3). Linoleic acid was the least abundant fatty acid in the range of 0.33%-2.47% in all the *L. acidophilus* samples except those treated with neem oil. Such an abundance of palmitic acid in *L. acidophilus* is consistent to the earlier report [22]. The overall fatty acid content was also similar not just to the available reports on *L. acidophilus*, but other Lactobacilli [22–24].Treatment with neem oil caused the most dramatic change in the fatty acid profile making oleic acid the most and vaccenic acid the least dominant fatty acid with the proportion of 53.1% (13 fold increase in comparison to control) and 0.77% (38 fold decrease in comparison to control), respectively (Fig. 3). Neem oil also caused about two fold decrease and 46 fold increase in the relative proportion of stearic and linoleic acids, respectively. The effect of neem oil on the fatty acid profile of *L. acidophilus* was also clearly evident in the unsaturation index (Fig. 4). Whereas other essential oils did not affect the unsaturation index, neem oil caused about 2.4 fold increase in this value ($p < 0.005$). All these changes imposed by neem oil were statistically significant. Previous studies have shown that the proportion of unsaturated fatty acids in lactic acid bacteria increase in response to various stresses such as low pH high temperature, high salt concentration, and oxidative environment [25,26]. It is possible that the rise in the unsaturation index imposed by neem oil might be a similar kind of stress response.Cinnamon oil caused significant increase (about two fold) in the relative levels of oleic acid and dihydrosterculic acids and about 1.7 fold decrease in the proportion of linoleic acid (Fig. 3). About 7.5 fold increase in the relative amount of linoleic acid was induced by orange oil. Although other tested essential oils caused some changes in the fatty acid composition of *L. acidophilus*, the difference was not statistically significant. It has been reported that stress-inducing conditions cause increase in the levels of cyclopropane fatty acids (CFA) in many bacteria including *E. coli* [27]. Since cinnamon oil was highly inhibitory to the growth of *L. acidophilus*, the rise in the proportion of dihydrosterculic acids (a CFA) might also be a stress-related response. Nonetheless, as some of the recent studies have questioned the role of cyclopropane fatty acids in lactic acid bacteria [28], how the increase in the level of dihydrosterculic acids in *L. acidophilus* caused by cinnamon oil is correlated with the growth inhibitory properties of cinnamon oil remains an enigma.To understand the effect of essential oil on the overall fatty acid pattern of *L. acidohpilus*, the data was further subject to Principal Component Analysis. PC1 and PC2 of the score plot explained 75.1% and 18.5% of the variation in the data (Fig. 5). Essential oils scattered from each other mainly along PC2, whereas the unique placement of neem oil in second quadrant was mainly because of its different coordinates along PC1. Cinnamon and eucalyptus oils were present in the fourth quadrant; whereas, orange and cedar wood oils clustered together with control in the first quadrant. In the loading plot, oleic acid and linoleic acid formed a distinct cluster in the second quadrant; palmitic, stearic and vaccenic acid were present close together in the first quadrant; whereas, dihydrosterculic acid was isolated in the fourth quadrant. The spread of essential oils and fatty acids over the score and loading plots,

respectively, was very much correlated to each other. Wherein, the presence of neem oil in the second quadrant of loading plot can be easily correlated to the position of oleic acid and linoleic acid (high in neem oil treated *L. acidophilus*) in the score plot. Similarly, the fourth quadrant of score and loading plots were correlated to each other in having cinnamon oil and dihydrosterculic acid, respectively. The PCA analysis confirmed that among all the tested essential oils, neem and cinnamon oils caused the highest impact on the fatty acid content of *L. acidophilus* and this change was with respect to increase in the proportion of unsaturated and cyclopropane fatty acids, respectively.Many plant essential oils contain bioactive components such as mono- and sesquiterpenes, phenolics, alcohols, aldehydes and ketones. Di Pasqua et al [29] studied the effect of various such chemicals which are the major components of essential oils, on the fatty acid composition of various bacteria, and observed numerous changes. The alteration in the fatty acid composition of *L. acidophilus* induced by these essential oils might be because of the interference of such active chemicals with the bacterial fatty acid metabolism. Since some of the plant essential oils also contain fatty acids[30], the changes induced by them in the fatty acid composition of *L. acidophilus* might be because of direct incorporation of the essential oil fatty acids into the bacterial cell membrane.

Table 1
Minimum inhibitory concentrations and zone of inhibition shown by essential oils against L. acidophilus. ND: not detected

No.	Essential oil	MIC (%)	Zone of inhibition (mm)
1	Basil	0.8	0.45
2	Cedarwood	1.6	1.30
3	Cinnamon	0.025	1.93
4	Clove	0.4	0.55
5	Eucalyptus	0.8	0.6
6	Lemon	1.6	ND
7	Neem	1.6	ND
8	Tea tree	0.2	ND
9	Orange	1.6	ND

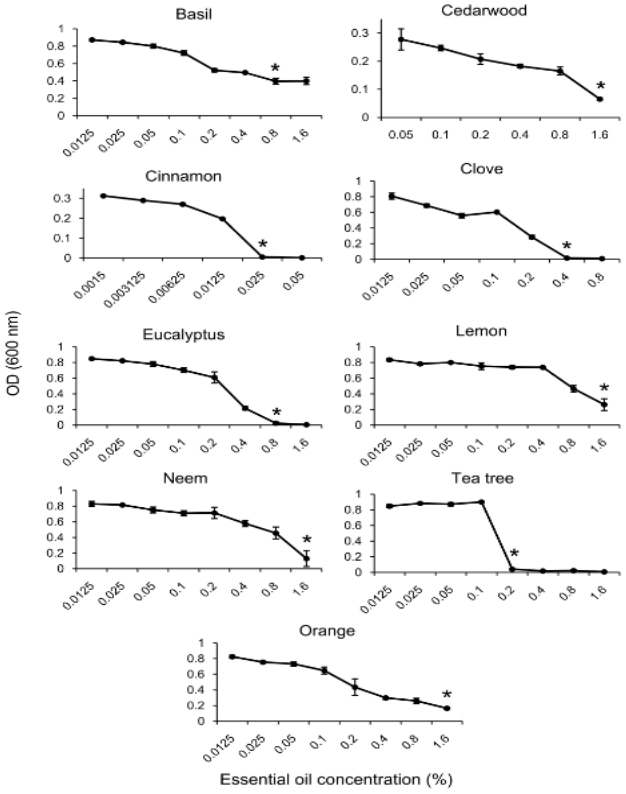

Figure 1
Growth of L. acidophilus as affected by overnight incubation with various essential oils. Error bars represent standard error of measurement with at least two biological replicates. Asterisk symbols indicate the minimum inhibitory concentrations.

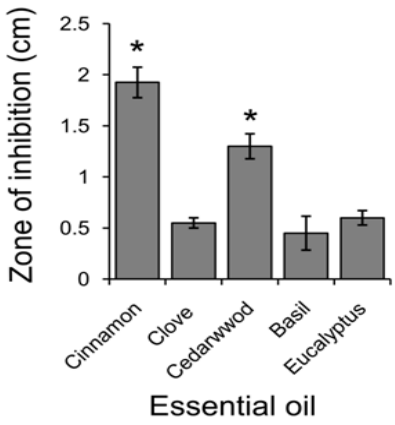

Figure 2
Zone of inhibition determined for the effect of essential oils on the growth of L. acidophilus. Error bars represent standard error of measurement with at least two biological replicates. Asterisk symbols indicate the values which were statistically ($p \leq 0.05$) different from the other values.

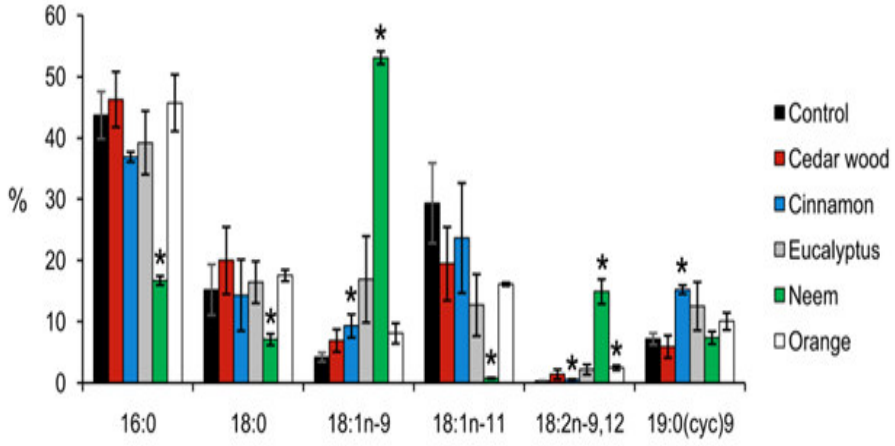

Figure 3
Relative levels of major fatty acids (16:0, palmitic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:1n-11, vaccenic acid; 18:2n-9,12, linoleic acid and 19:0(cyc)9, dihydrosterculic acid) in L. acidophilus as determined by gas chromatography upon growing it overnight in the presence of various essential oils. Error bars represent standard error of measurement with at least two biological replicates. Asterisk symbol indicates the value in that group which was significantly different from the untreated control ($p<0.05$) according to the Student's t-test.

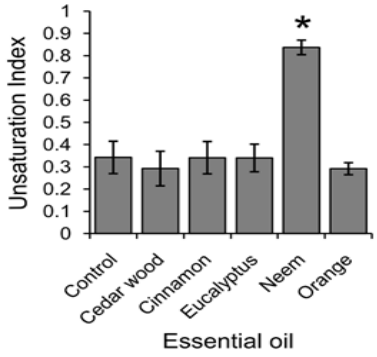

Figure 4
Unsaturation index for the fatty acid composition of L. acidophilus as determined by formula: Unsaturation index = % of monoenes + (2 × % of dienes) + (3 × % of trienes) / 100. Error bars represent standard error of measurement with at least two biological replicates. Asterisk symbol indicates the value which was significantly different from the untreated control ($p<0.05$) according to the Student's t-test. Being a cycloporpane fatty acid, dihydrosterculic acid, was not considered for determining unsaturation index.

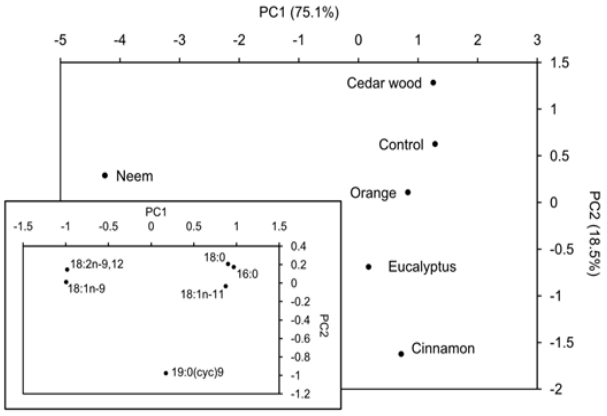

Figure 5
Score plot and loading plot (inset) of the Principal Component Analysis of fatty acid profiles of L. acidophilus upon treatment with various essential oils. The names of fatty acids mentioned in the loading plot are same as detailed in legend of Fig. 3.

CONCLUSION

This work depicts the antimicrobial effects of some plant essential oils. We also showed that essential oils profoundly affect the fatty acid composition in *L. acidophilus*. Symbiotic lactic acid bacteria play a very important role in human health. Their fatty acid composition is a significant factor not only for their industrial usage [31,32,33] but also for their probiotic properties in the human gut [34]. Considering this and the results of the present study, ancient natural products such as essential oils can have huge potential in modulating the probiotic and industrial properties of these important bacteria through the alterations in the fatty acid composition.

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COINFLICT OF INTEREST

Conflict of interest declared none.

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