Anticandidal activity of cell extracts from 13 probiotic *Lactobacillus* strains and characterization of lactic acid and a novel fatty acid derivative from one strain

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Abstract

This study investigated the anti-*Candida* activity of methanol extracts from freeze-dried probiotic cells and the isolation of some constituents in the extracts. The MIC values of the probiotic methanol cell extracts against *Candida albicans* ranged between 1.25-5 mg/ml after 48 h of incubation. However, *Lactococcus latics* subsp. *lactis* strain X and *Lactobacillus casei* strain B extracts had an MIC of 10 mg/ml after 48 h of incubation. The extracts had fungistatic rather than fungicidal activity. These extracts had a much higher antifungal activity than antifungal compounds isolated from the growth medium by many other authors.

This indicates that probiotics may also release antifungal compounds in their cells that could contribute to a therapeutic effect. Lactic acid (**1**) and 6-*O*-(*α*-D-glucopyranosyl)-1,6-di-*O*-pentadecanoyl-*α*-D-glucopyranose a novel fatty acid derivative (**2**) were isolated from methanol probiotic extracts and the structure of these compounds were elucidated by using NMR (1 and 2D) and mass spectrometry (MS).

Keywords: Probiotic; *Lactobacillus*, Constituents; Anti-*Candida*

1. Introduction

Probiotics are defined as 'live microorganisms of human intestinal origin, which when ingested in adequate amounts, impart health benefits to the consumer beyond basic nutrition' (Coeuret, Gueguen, & Vernoux, 2004; Nyanzi, & Jooste, 2012). *Bifidobacterium* species and lactic acid bacteria, mainly *Lactobacillus* species have been used extensively as probiotics in foods and beverages as probiotic due to their generally recognized as safe (GRAS) status (Franz, Stiles, Schleifer, & Holzapfel, 2003; Gibson, 2004; Bendali, Durand, Hebraud, & Sadoun, 2011; Nyanzi, & Jooste, 2012). Some of the potential benefits of probiotics include prevention of constipation, colon cancer, lactose intolerance, infantile diarrhoea, travellers' diarrhoea, antibiotic induced diarrhoea, hypercholesterolaemia, vaginitis and intestinal infections (Marchand, & Vandenplas, 2000; Holzapfel, & Schillinger, 2002; Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Franz, Stiles, Schleifer, & Holzapfel, 2003; Rousseau, Lepargneur, Roques, Remaud-Simeon, & Paul, 2005).

Several probiotic strains antagonise enteropathogens and the mechanisms (although not fully determined yet) may involve competition for receptor sites and nutrients, aggregation with bacterial pathogens and production of hydrogen peroxide, presence of

antimicrobial substances, stimulation of the immune system (Shalev, Battino, Weiner, Colodner, & Keness, 1996; Atanassova et al., 2003; Strus, Kucharska, Kukla, Brzychczy-Wloch, Maresz, & Heczko, 2005; Gueniche et al., 2010; Bendali, Durand, Hebraud, & Sadoun, 2011). There are several reports about antimicrobial activity of lactic acid bacteria (LAB) (Ström, Sjogren, Broberg and Schnurer, 2002). Most of the antimicrobial activities of the probiotic organisms are attributed to organic acids, low pH and protein-like substances (Atanassova et al., 2003; De Muynck, Leroy, De Maeseneire, Arnaut, Soetaert, & Vandamme, 2004; Strus, Kucharska, Kukla, Brzychczy-Wloch, Maresz, & Heczko, 2005). Organic acids such as 3-phenyl-L-lactic acid and caproic acid produced by *Lb. plantarum* and *Lb. sanfrancisco* respectively had antifungal activity (Ström, Sjogren, Broberg, & Schnurer, 2002; De Muynck, Leroy, De Maeseneire, Arnaut, Soetaert, & Vandamme, 2004). From *Lb. plantarum*, compounds such as cyclo-(glycyl-L-leucyl), mevalonolactone, benzoic acid and methylhydantoin, which are synergists of lactic acid, were isolated and inhibit the growth of *Fusarium avenacum* (Atanassova et al., 2003).

Antimicrobial activity of probiotic supernatants and ethyl acetate extracts of supernatants have been reported (Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti, & Gobbetti, 2000). However, constituents of extracts of probiotic bacterial cells have not been extensively investigated for their antimicrobial activity.

Not a lot has been focused on antifungal activity of LAB (Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti, & Gobbetti, 2000). The need to have natural food preservatives and antipathogenic (antibiotics) compounds with fewer side effects motivates the investigation of LAB (De Muynck, Leroy, De Maeseneire, Arnaut, Soetaert, & Vandamme, 2004). Moulds and yeasts have increasing resistance against antibiotics and preservatives such as sorbic acid and benzoic acid (Voulgari, Hatzikamari, Delepoglou, Georgakopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2010).

Candida albicans and other *Candida* species which are pathogenic to humans are becoming resistant to antifungal agents such as amphotericin B and the triazole antifungals (Sanglard, & Odds, 2002). Vaginal candidiasis (VC) affects 75% of women at least once in their lifetime and 5-10% of women have chronic-recurrent vaginal candidiasis (Sudbery, 2011). *Candida*'s antifungal-resistance results in clinical treatment failures and persistence in immunocompromised individuals such as the HIV/AIDS patients in whom it causes oropharyngeal and other forms of candidiasis (Sanglard, & Odds, 2002). The problem of *C. albicans* is compounded by continuous intake of antibiotic drugs, anticancer agents, oral contraceptives, HIV protease inhibitors and compromised immune system (Koga-Ito et al., 2011). Plant extracts have been shown to have anticandidal activity (Shai et al.,2008, 2009). Cytotoxicity can however be a concern and although probiotic extracts may have a lower activity they are known to be safe.

Consumers prefer fresh healthy and natural foods without chemical preservatives (Tharmaraj, & Shah, 2009). It appears that there is a need to have natural biopreservatives in probiotic foods and beverages which can also impart health benefits to the consumer. Benefits can include suppression of pathogens which are the causative agents of several infections. Even though there are some reports regarding antimicrobial activity (attributed to organic acids and bacteriocins) of LAB, a lot is lacking about the constituent compounds in probiotic extracts. This study aimed at investigating possible antifungal activity and characterization of constituents in probiotic methanol extracts from selected *Lactobacillus* strains.

2. Materials and methods

2.1. General

Infrared (IR) spectra were recorded on a Bruker Alpha FT-IR spectrometer (Optik GmbH, Germany). NMR spectra were recorded with a Varian spectrometer at 400 MHz. Chemical shifts (δ) were quoted in parts per million (ppm) from internal standard tetramethylsilane (TMS). A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to determine and generate accurate ion-fragment mass data. Column chromatography was performed on MN silica gel 60 (0.063-0.2 mm / 70-230) mesh. Precoated plates of TLC silica gel 60 F_{254} (Merck, Germany) were used during comparative TLC and monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with 30% H_2SO_4 followed by heating up to 110°C.

2.2. Probiotic Cell materials

2.2.1. Probiotic cultures and preservation

Lactobacillus strains were isolated from dairy food products, pharmaceutical probiotic supplements and some were obtained from supplier companies. In total, 13 *Lactobacillus* strains of the 32 isolates, were selected on the basis of their enhanced acid tolerance, antibiotic resistance profile and antimicrobial activities against indicator bacterial pathogens (*Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 14028) and *Candida albicans* strains (unpublished data). Preliminary and/or phenotypic identification of the *Lactobacillus* strains was done using API 50 CHL galleries as well as API WEB (V3.2 and V5.1) software (API system, Biomerieux, France). The probiotic cultures were preserved in a freezing medium as described by Nyanzi (2007).

2.2.2. Confirmation of identity of bacterial isolates

The identity of the isolates was confirmed by applying molecular typing techniques and phylogenetic analysis using sequences of the 16S rRNA gene, and *rpoA* and *pheS* protein-coding genes. The 27F and 1492R universal primers were used to amplify the 16S rRNA gene of all the probiotic isolates according to the procedure of Guo, Kim, Nam, Park and Kim (2010). The primer combination, *pheS*-21-F / *pheS*-23-R, was used for the amplification of the *pheS* gene while the *rpoA* gene of the bacterial isolates was amplified using *rpoA*-21-F / *rpoA*-23-R primer pair (Naser et al., 2005).

After gel electrophoresis of the PCR products, the amplicons were sequenced in a genetic analyser ABI PRISM^{TM} 3100 (Applied Biosystems, USA). The resulting sequences were edited using Chromas Lite 2.0 and BioEdit v. 7.0.9 software (http://en.bio-soft.net/format.html/BioEdit). Identification of the isolates was done by comparing study sequences to those in the nucleotide database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/blastn/) using *blastn* search option.

2.3. Bacterial cell mass growth

Two litres of De Man, Rogosa and Sharpe broth (MRSB) (in 2 l volume Duran Schott bottle) were inoculated with 4% of overnight broth culture and incubated in a water bath at 37°C for 18 to 20 h. After incubation, the MRSB culture was centrifuged (Sorvall Rc, USA) at 7000 rpm at 4°C for 10 min to obtain a cell pellet. The cell pellet was washed twice with filter-sterilise phosphate buffered saline (PBS) and then suspended in sterilised distilled water. The suspension was frozen at -80°C for at least 4 h and then freeze-dried) at -85°C for at least 48 h or until dry.

2.3.1. Particle size analysis and extraction of powdered cells

The freeze-dried bacterial cell mass was then subjected to physical breakage into powdered material using a mortar and pestle. The particle size of the resultant powder was determined using Particle Size Analyzer (MicrotracFlex Ver. 10.5.4, USA). The powdered bacterial cell mass was stored in sterile Schott bottles ready for extraction. The disintegrated cell pellet was serially extracted using acetone and methanol. Acetone was used because it is volatile, harmless to the bacteria cells and its constituents and it is miscible with polar and non-polar solvents (Eloff, 1998a). Acetone should be effective in breaking cell membranes and to extract more non-polar constituents while methanol should extract more polar constituents. The cell powder (2 g) and the extractant (10 ml) were mixed, vigorously shaken and sonicated (Bandelin electronic sonicator, Pro-Nr 780.06021340.008, Berlin, Germany) at 100% power (120/240 W, 1.2 A, 230 V, 35 KHZ frequency) for 15 min before filtering using Whatman No. 1 filter paper. The filtrate was dried under in a fume cupboard at room temperature and the mass of the resulting extract was determined.

2.3.2. Preparation of fungal culture and determination of the minimum inhibitory concentrations (MIC) value of extracts

The American Type Culture Collection (ATCC) *Candida albicans* (ATCC 10231) was used. Its culture was taken from 24 h fresh agar culture plate and inoculated in fresh Sabouraud dextrose broth (SDB) (Fluka, Switzerland) prior to conducting the assay. The turbidity of the fungal suspension was adjusted to a McFarland standard 0.5 equivalent to a concentration of 1-5 x 10^7 cfu/ml and was further diluted (1:100) in SDB to obtain a final inoculum of approximately 1.5×10^5 cfu/ml.

A two-fold serial microdilution with *p*-iodonitrotetrazolium violet (INT) as indicator of fungal growth described by Eloff (1998b) and modified by Masoko, Picard and Eloff (2005) was used to determine the MIC values of samples. A 100 µl of extracts (80 mg/ml) dissolved in acetone was serially diluted two-fold with sterile distilled water in 96-well microtitre plates and 100 µl of freshly prepared fungal culture in SDB was added to each well. Acetone (25%) and amphotericin B (4 µg/ml) were used as negative and positive controls, respectively. A 40 µl of 0.2 mg/ml of INT was added to each well and microtitre plates were sealed in plastic bags and incubated at 37^{o}C for 60 h at 100% relative humidity. The MIC was recorded after 24, 48 and 60 h as the lowest concentration inhibiting microbial growth, indicated by a decrease in the intensity of the red colour of the formazan.

2.3.3. Isolation of constituents from the probiotic extracts

The methanol extract from probiotic strain M (1.6 g) was subjected to silica gel column chromatography eluted with an isocratic mixture (v/v) of ethyl acetate / methanol / water (8:1.5:0.5) to afford 24 fractions of 50 ml each which were combined based on similarity of TLC chromatograms into 4 fractions. Compound **1** (110 mg) was purified after crystallization and filtration of fraction III in acetone followed by re-crystallization in dichloromethane/methanol (1:1). Fraction II was subjected to Sephadex LH-20 eluted with dichloromethane/methanol (1:1) to obtain mainly compound **2** (4.7 mg). Fraction IV was oily, was soluble in diethyl ether but not in hexane and not investigated further due to its complex mixture indicated by TLC. However, further silica gel column chromatography was carried out for other probiotic cell extracts (O, P and N) and compound **1** was commonly found in most of the extracts while compounds **2** were present in traces and identified only with co-TLC.

2.3.3.1. Lactic acid (**1**)

$C_3H_6O_3$; colourless crystals, IR: ν_{max} 3344, 2984, 1721, 1631, 1588, 1222 cm^{-1}; ^1H NMR data (400 MHz, D_2O): δ 3.99 (*q*, 8.0 Hz, H-2) and 1.15 (*d*, 8.0 Hz, 3-Me); ^{13}C NMR data (100 MHz, D_2O): δ 181.3 (C-1), 67.8 (C-2) and 19.7 (C-3); MS-ESI (+): *m/z* 91 $[M+H]^+$.

2.3.3.2. 6-O-(α-D-Glucopyranosyl)-1,6-di-O-pentadecanoyl-α-D-glucopyranose (**2**)

$C_{42}H_{78}O_{13}$; white amorphous powder; $[\alpha]_D$ (not determined); IR: ν_{max} 3370, 2980, 1730, 1580, 1210, 1121 cm^{-1}; ^1H (400 MHz) and ^{13}C (100 MHz) NMR data (methanol-d_4) (see Table 1), TOF-MS-ESI (-): *m/z* 797.3239 $[M+Li]^-$ (Calc. 797.3192); ESI-MS: *m/z* 566 $[M+H-225]^+$, 341 $[M+H-450]^+$.

Table 1: ^1H (400MHz) and ^{13}C (100 MHz) NMR (Methanol-d_4) data of **2**

Position	**^{13}C**	**^1H**	**HMBC**	**Position**	**^{13}C**	**^1H**	**HMBC**
1	173.6			1'''	173.3		
2	33.7	2.32 (*m*)	C-1, 3, 4	2'''	33.6	2.32 (*m*)	C-1''', 3''', 4'''
3	24.7	1.60 (*m*)	C-1, 2, 4	3'''	24.6	1.60 (*m*)	C-1''', 2''', 4'''
4-14	31.7-28.5	1.29-1.26 (br.*s*)	-	4'''-14'''	31.7-28.5	1.29-1.26 (br.*s*)	-
15	13.1	0.89 (*t*, 7.2)	C-13, 14	15'''	13.1	0.89 (*t*, 7.2)	C-13''', 14'''
1'	96.7	5.04 (*d*, 3.2)	C-2'	1''	96.2	5.01 (*d*, 3.2)	C-5'''
2'	72.4	3.52 (*m*)	-	2''	71.9	3.68 (*m*)	C-1''',
3'	76.2	3.57 (*dd*, 3.2, 10.0)	C-1', 2'	3''	70.0	3.32 (*m*)	C-2'''
4'	70.1	5.23 (*m*)	-	4''	69.7	3.78 (*m*)	C-3'''
5'	69.9	3.87 (*m*)	C-1', 4', 6'	5''	71.1	4.08 (*m*)	C-1''', 4''', 6'''
6'	62.5	4.47 (*dd*, 2.8, 12.4)	-	6''	61.4	3.78 (*m*)	C-4'''
		4.21 (*dd*, 6.4, 12.4)	C-1''', 4'			3.68 (*m*)	C-1''', 5'', 4''

3. Results and Discussion

3.1. Confirmation of identity of bacterial isolates

All the isolates investigated were identified according to their 16S rDNA sequences and confirmed using sequences of the *rpoA* and *pheS* genes (Table 2). The *rpoA* and *pheS*

Table 2: Confirmation of the identity of the probiotic isolates used in the study

Bacterial isolates	**16S rDNA sequence identification**	***pheS* gene sequence identification**	***rpoA* gene sequence identification**
U	*Lb. acidophilus*	*Lb. acidophilus*	*Lb. acidophilus*
V	*Lb. acidophilus*	*Lb. acidophilus*	*Lb. acidophilus*
W	*Lb. acidophilus*	*Lb. acidophilus*	*Lb. acidophilus*
Z	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*
M	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
Y	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
D	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
C	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
O	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
N	*Lb. casei*	*Lb. casei*	*Lb. casei*
B	*Lb. casei*	*Lb. casei*	*Lb. casei*
P	*Lb. casei*	*Lb. casei*	*Lb. casei*
X	*Lc. lactis* subsp. *lactis*	*Lc. lactis* subsp. *lactis*	*Lc. lactis* subsp. l*actis*

genes have been reported by other workers to be of importance in confirming identities of lactic acid bacteria (Naser et al., 2005). The superiority of *rpoA* and *pheS* gene sequencing over the 16S rRNA sequencing in discrimination and identification of LAB strains was confirmed in a separate study (Nyanzi, Jooste, Cameron, & Witthuhn, 2013).

3.2. Particle size of the powdered freeze-dried LAB cells extracted

The particle size of all of the powdered freeze-dried cells of bacterial strains was in the range 57.19 – 70.22 µM except for *Lb. acidophilus* strain V which had particle size averaging 44.85 µM. This reflected, on average, a difference of 13 µM between particles of different powdered cells of bacterial strains. Extractability would therefore be expected to be relatively similar for all powder samples of bacterial strains.

Table 3: Minimum inhibitory concentrations (MIC) value of extracts from probiotic strains against *Candida albicans*

Probiotic bacterial strains	**MIC values (mg/ml)**		
	Time of incubation		
	24 h	**48 h**	**60 h**
Lb. acidophilus strain U	1.25	5	10
Lb. acidophilus strain V	1.25	5	10
Lb. acidophilus strain W	1.25	5	10
Lb. helveticus strain Z	1.25	2.5	5
Lb. rhamnosus strain M	1.25	2.5	5
Lb. rhamnosus strain Y	1.25	2.5	5
Lb. rhamnosus strain O	1.25	2.5	2.5
Lb. rhamnosus strain D	1.25	1.25	2.5
Lb. rhamnosus strain C	1.25	2.5	5
Lc. lactis subsp. *lactis* strain X	2.5	10	10
Lb. casei strain P	1.25	2.5	2.5
Lb. casei strain B	5	10	10
Lb. casei strain N	1.25	1.25	2.5
Amphotericin B	0.5 µg/ml	1.0 µg/ml	1.0 µg/ml

3.3. The MICs of antifungal methanol extracts from selected probiotic bacterial cells

The minimum inhibitory concentrations (MIC) values of methanol extracts from selected probiotic *Lactobacillus* strains against *Candida albicans* ATCC 10231 is presented in Table 3. The MICs of acetone extracts were not determined due to low yields (0.21% - 0.60%) that were obtained. The yield of the methanol extracts was in the range 4.14% to 42.08% (Nyanzi, 2013). All the methanol extracts had MICs of 1.25 mg/ml except extracts from bacterial strains B and X which had MICs of 5 and 2.5 mg/ml respectively against *Candida albicans* after 24 h of incubation at 37°C. After 48 h of incubation, the antifungal activity of extracts from *Lb. helveticus* strain Z and *Lb. rhamnosus* strains (M, Y, O, D and C) (MIC 1.25 – 2.5 mg/ml), were better than extracts from *Lb. acidophilus* strains (U, V and W), (MIC 5 mg/ml). The MIC values of extract from *Lb. casei* strain N and P were in the range 1.25 – 2.5 mg/ml for the entire period of incubation.

The anti-*Candida* activity should be representative of the probiotic strain from which the methanol extract was obtained. Generally, *Lb. helveticus* strain Z, *Lb. rhamnosus* strains (M, Y, O, D and C) and *Lb. casei* strain P and N can therefore be recommended for further research based on their antifungal activities. The activity of the extracts appeared to be fungistatic and not fungicidal based on the increased MICs after extended incubation periods. This conclusion was also confirmed by the confluent *Candida* growth after 100 µl each from row 'A' wells of the microtitre plates was aseptically inoculated separately on potato dextrose agar (PDA) (Merck, Germany) petri-plates by employing the spread plate technique. As would be expected, the fungistatic potential of the extracts also depended on the *Candida* population since higher population levels never had their growth hindered.

The approach in this study deviated from what some workers consider to be the norm. Normally, most workers test the antifungal activity of probiotic strains by using supernatants,

cell-free supernatants in form of neutralized, neutralised and filtered or unadjusted pH (Bendali, Durand, Hebraud, & Sadoun, 2011). In most of these studies, the antifungal activity is attributed to protein-like substances (De Muynck, Leroy, De Maeseneire, Arnaut, Soetaert, & Vandamme, 2004) and organic acids such as 3-phenyl-L-lactic acid and caproic acid produced by *Lb. plantarum* and *Lb. sanfrancisco* respectively (Ström, Sjogren, Broberg, & Schnurer, 2002; De Muynck, Leroy, De Maeseneire, Arnaut, Soetaert, & Vandamme, 2004; Tharmaraj, & Shah, 2009; Voulgari, Hatzikamari, Delepoglou, Georgakopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2010). It was reported that organic acids, such as lactic acid, have antifungal activity based on their ability to lower the pH attributed to lactic acid (Voulgari, Hatzikamari, Delepoglou, Georgakopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2010). In this study, a microplate INT method developed by Eloff (1998b) was used to determine the antifungal activity of methanol extracts from selected probiotic *Lactobacillus* strains. This method is quantitative unlike the frequently-used less-quantitative disc agar method and well-diffusion method (Eloff, 1998b).

In the present investigation, against *Candida albicans*, the MICs of extracts from *Lb. helveticus* strain Z and *Lb. rhamnosus* strains (M, Y, O, D and C) were in the range 1.25 – 2.5 mg/ml, while extracts from *Lb. acidophilus* strains (U, V and W) had an MIC of 5 mg/ml after 48 h of incubation at 37°C. Arguably, 48 h of incubation might be the optimal period since there can be a delay in growth of the yeast within 24 h. Some workers have reported antifungal activities of LAB but not necessarily in quantitative terms. *Lactobacillus plantarum* MiLAB 393 was reported to release fungistatic substances against *Aspergillus nidulans* (Tharmaraj, & Shah, 2009). Peptides from *Lactobacillus pentosus* had fungistatic activity against *Candida albicans* (Atanassova et al., 2003; Tharmaraj, & Shah, 2009; Voulgari, Hatzikamari, Delepoglou, Georgakopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2010). Other lactobacilli including, *Lb. plantarum* (Lavermicocca, Valerio, Evidente,

Lazzaroni, Corsetti, & Gobbetti, 2000; Ström, 2005), *Lb. salivarious*, *Lb. coryneformis* Si3, and *Lb. sake* (Voulgari, Hatzikamari, Delepoglou, Georgakopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2010) were reported to have antifungal activity. Most of the above reports indicate that lactobacilli were fungistatic, however, in most of the cases quantities were not indicated as is the case in the present study. This study illustrated that certain concentrations of methanol extracts from certain probiotic *Lactobacillus* cells have anti-*Candida* activity. This is a contribution to address what Ström, Sjogren, Broberg and Schnurer (2002) referred to as 'scanty available information' regarding antifungal activity of LAB.

The MICs of extracts from most of our probiotic strains against *Candida albicans* ATCC 10231 were in the range 1.25-5 mg/ml. These MICs are much higher than the <0.1 mg/ml MIC values from considered interesting plant extracts (Eloff, 2004; Ríos & Recio, 2005). It should be recalled that probiotic products and/or extracts are not medicinal products. Natural plant extracts may lead to the development of prescription drugs. Hence the requirement that MICs of plants extracts to be lower than 0.1 mg/ml to be interesting.

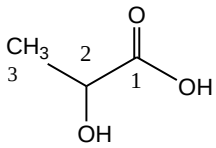
The MICs we found against *Candida albicans* ATCC 10231 in extracts from in probiotic cells were however much lower than results found for compounds isolated form probiotics by other authors against fungi. In a study by Ström (2005), the MIC value of cyclo (L-Phe-Pro), a cyclic dipeptide from *Lb. plantarum* MiLAB 393 against *A. fumigatus* and *P. roqueforti* was 20 mg/ml and when 3-phenyllactic acid (5 mg/ml) was included as an additional inhibitor, the MIC value changed to 10 mg/ml. The MIC value of 3-phenyllactic acid (alone) against *A. fumigatus* and *P. roqueforti* was 7.5 mg/ml (Ström, Sjogren, Broberg, & Schnurer, 2002). This implied that the isolated compounds can be synergistic in their effect. In a study by Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti, and Gobbetti (2000), phenyllactic acid and 4-hydroxy-phenyllactic acid was obtained from *Lb. plantarum* 21B. The former compound was the most active and its MIC value against *Eurotium* spp.,

Penicillium expansum, *Endomyces* spp., *Aspergillus* spp., *Monilia* spp. and *Fusarium* spp. was 50 mg/ml. However, against *P. roqueforti* IBT18687 and *P. corylophilum* IBT6978, the MIC value was 166 mg/ml (Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti, & Gobbetti, 2000) which was a much lower activity than that of 3-phenyllactic acid (7.5 mg/ml) against *P. roqueforti* reported by Ström, Sjogren, Broberg and Schnurer (2002).

Because the MIC of the methanol extracts of probiotic cells had a much higher activity against fungi than isolated compounds from probiotic growth media, it made much sense to try to isolate the bioactive compounds from the methanol extracts.

3.4. Characterization of constituents from methanol extracts of probiotic cells

Probiotic cell extracts were subjected to repeated silica gel column chromatography followed by Sephadex LH-20 to produce ~~afford~~ two compounds: lactic acid (**1**), and 6-*O*-(*α*-D-glucopyranosyl)-1,6-di-*O*-pentadecanoyl-*α*-D-glucopyranose (**2**) (Figure 1) with yields of 6.88 and 0.3% respectively. The structures of the compounds were elucidated using experimental and published spectroscopic data.



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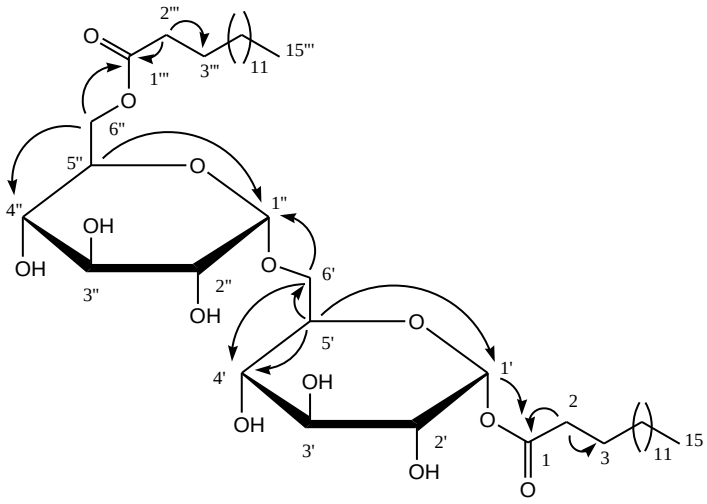

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Figure 1: Chemical structures of isolated compounds (**1** and **2**) from probiotic cells and key HMBC (H C) correlations of **2**.

Compound **1** was obtained as colourless crystals from a mixture of dichloromethane: methanol (1:1). All its spectroscopic data were in agreement with those of lactic acid (**1**), a common secondary metabolite or by-product of lactic acid bacteria metabolism (Guoxun, Hua, Jeffrey, Ciaran, & Xinhua, 2012). This compound is isolated here for the first time from the extracts of probiotic bacterial cells.

Compound **2** was obtained as white amorphous powder. Its molecular formula, $C_{42}H_{78}O_{13,}$ was determined by negative ion from the TOF-MS-ES (at *m/z* 797.3239 $[M+Li]^-$) in conjunction with the NMR data (Table 1). The ^1H and ^{13}C NMR spectra of **2** (Table 1) had signals consistent for two glucosyl and two fatty acid ester moieties. The signals observed on the ^1H NMR spectrum at δ 5.04 (*d*, *J* = 3.2 Hz, 1H) and 5.01 (*d*, *J* = 3.2 Hz, 1H) and on the ^{13}C NMR spectrum at δ 96.7 (CH), 76.4 (CH), 72.4 (CH), 70.1 (CH), 69.9 (CH), 62.5 (CH_2), 96.2 (CH), 71.9 (CH), 71.1 (CH), 70.0 (CH), 69.7 (CH) and 61.4 (CH_2) were characteristic for the presence of two anomeric protons from a disaccharide moieties with α configurations [(*J* = 3.2 Hz)] in both cases. The signals at δ 173.6 (C=O), 173.3 (C=O), 33.7 (CH_2) / 33.6 (CH_2), 24.7 (CH_2) / 24.6 (CH_2), 31.7-28.5 [2 x $(CH_2)_n$] and 13.1 [2 x (CH_3)] observed on the ^{13}C NMR spectrum together with the absorption band at ν_{max} 1730 cm^{-1} on the IR spectrum revealed the presence of two fatty acid esters groups in **2**. This was substantiated by the occurrence of some characteristic signals on the ^1H NMR spectrum at δ 2.32 (*m*, 4H), 1.60 (*m*, 4H), 1.29-1.26 (br.*s*, 44H) and 0.89 (*t*, *J* = 7.2 Hz, 6H) attributable to protons H-2/H-2''', H-3/H-3''', H-(4-14)/H-(4'''-14'''), and H-15/H-15''', respectively. The HMBC correlations (Figure 1) between the proton at δ 5.04 (H-1') and carbon at δ 173.6 (C-1) and between the proton at δ 3.68 (H-6'') and carbon at δ 173.3 (C-1''') were indicative for the fatty acid ester groups to be sited at positions 1' and 6'', respectively. Fragments observed on the ESI-MS at *m/z* 566 $[M+H-225]^+$ and 341 $[M+H-450]^+$ were relevant to identify the fatty acid moieties as

two pentadecanoyl groups. The disaccharides present in **2** were determined to be two glucosyl groups based on their NMR data in comparison with published values (Kim et al., 2010). The interconnection C-6'-*O*-C-1" between the two glucosyl groups was established by further HMBC correlation among the proton at δ 4.21 (H-6') and carbon at δ 96.2 (C-1"). From the above data and by comparison with published data (Kim et al., 2010; Qi, Wu, Ma, & Luo, 2003), the structure of **2** was assigned to be 6-*O*-(α-D-glucopyranosyl)-1,6-di-*O*-pentadecanoyl-α-D-glucopyranose. This is a novel compound isolated and characterised here for the first time.

Workers have endeavoured to isolate compounds from LAB. Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti and Gobbetti (2000) subjected ethyl acetate extracts to preparative silica gel thin layer chromatography and column chromatography and spectroscopic analysis to obtain phenyllactic acid and 4-hydroxy-phenyllactic acid. In a study carried out by Ström (2005), antifungal compounds, cyclo (L-Phe-*trans*-4-OH-L-Pro) and cyclo (L-Phe-L-Pro) were obtained from *Lb. coryniformis* Si3 and *Lb. plantarum* MiLAB 393 respectively. Ström (2005) also observed that in addition to the cyclic dipeptides, *Lb. plantarum* L4 released 3-hydroxydodecanoic acid and 3-hydroxy-5-*cis*-dodecenoic acid categorised as hydroxylated fatty acids. Most reports have indicated that compounds isolated from LAB have antimicrobial activity (Voulgari, Hatzikamari, Delepoglou, Georgakopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2010). Phenyl-lactic acid and 4-hydroxy-phenyl-lactic acid from *Lb. plantarum* 21B had fungicidal activity (Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti and Gobbetti, & 2000; Atanassova et al., 2003; Tharmaraj, & Shah, 2009). Atanassova et al. (2003) reported that *Lb. casei* subsp. *casei* LC-10 and *Lb. casei* subsp. *pseudoplantarum* LB1931 were sources of 2-pyrrolidone-5-carboxylic acid (PCA) that had activity against *Pseudomonas putida*, *Bacillus subtilis*, and *Enterobacter cloacae*

(Atanassova et al., 2003). A peptide of about 43 kDa isolated from *Lb. paracasei* subsp. *paracasei* M3 and partially characterised had fungistatic and antibacterial activity (Atanassova et al., 2003; Voulgari, Hatzikamari, Delepoglou, Georgakopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2010). The compound was active against *Candida pseudointermedia* NBICC 1812, *C. albicans*, *C. blankii* NBIMCC 85, *Saccharomyces cerevisiae* NBICC 1812, and had also bactericidal activity against *Helicobacter pylori* NCIPD 230 and *Bacillus subtilis* ATCC 6633 (Atanassova et al., 2003). Needless to highlight that most of the compounds reported here were isolated from concentrates of cell-free supernatants or ethyl acetate extracts from culture supernatants (Lavermicocca, Valerio, Evidente, Lazzaroni, Corsetti and Gobbetti, & 2000; Atanassova et al., 2003).

Our finding reports, for the first time, the occurrence of fractions and/or compounds within the cells of studied *Lactobacillus* strains with the potential to inhibit pathogens. However, the anti-*Candida* activity of the new fatty acid derivative (**2**) isolated could not be determined due to the low quantity obtained. Nevertheless, Ström (2005) reported that the MIC values of 3-hydroxy fatty acids from *Lb. plantarum* were in the range of 5-100 µg/ml against moulds and yeasts. This is indicative of the possibility of adequate amounts of isolated compounds from methanol extracts of tested *Lactobacillus* strains to illustrate antimicrobial activity.

4. Conclusions

This study aimed at determining quantitatively the antifungal activity of methanol extracts and constituents in cells of probiotic from selected *Lactobacillus* strains. The methanol extracts had antifungal activity against *Candida albicans* with MICs of extracts from *Lb. helveticus* strain Z and *Lb. rhamnosus* strains (M, Y, O, D and C) ranging from 1.25 to 2.5 mg/ml, while extracts from *Lb. acidophilus* strains (U, V and W) had MIC of 5 mg/ml

after 48 h of incubation. This is the first time that the occurrence of lactic acid and a fatty acid glycoside derivative are illustrated within the cells of studied *Lactobacillus* strains. It is also the first description of this novel fatty acid glycoside discovered in nature. It may be worthwhile isolating larger quantities of this compound to determine its antimicrobial activity.

The methanol extracts, however, need further investigation for use as biopreservatives in food and beverages in addition to the need for their non-cytotoxicity to be confirmed. Furthermore, the elucidation of lipid fractions from the extracts which were insoluble in hexane but soluble in di-ethyl ether and acetone may in future provide a better understanding of constituents' technological functionality.

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