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Extraction, Evaluation and Structure Elucidation of Bioactive Metabolites of *Lactobacillus helveticus* CNRZ 32

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Abstract: There is no previous work that utilizes the multi-solvent extraction and structure elucidation of *Lactobacillus helveticus* cell-free supernatant (CFS). In this study, the CFS of *Lb. helveticus* CNRZ 32 was extracted by ethyl acetate, diethyl ether, dichloromethane, and n-hexane solvents. The extracts of considerable antimicrobial activities were characterized through GC/MS clarify metabolic profiles, TLC for compounds separation, and bio-autography to determine the number and R_f of effective compounds. Ethyl acetate extract possessed the strongest effect on all tested pathogens with inhibition diameter reached 38 mm in the case of *Staphylococcus sciuri*, while Diethyl ether and Dichloromethane extracts came secondly. The extract of Ethyl acetate mainly included butyl lactate with area % (59.45), while 9,12-Octadecadienoic acid (Z,Z)-, methyl ester and different health beneficial compounds were identified in both Diethyl ether and Dichloromethane extracts. Due to the strong synergism among Chitosan Nanoparticles and different extracts, the MIC values were lowered by about 20 – 50%.

Keywords: *Lactobacillus helveticus* CNRZ 32; GC/MS; Bio-autography; Chitosan Nanoparticles; MIC.

1. Introduction

Extraction of natural bioactive compounds using organic solvents was found to be more interested in higher plants [1]. Lactic acid bacteria have a great contribution to improving human health. Many researchers have evidenced the positive contribution in microbial interactions with many foodborne pathogenic and spoilage microorganisms. Authors reported the inhibitory behavior of LAB either through the production of antimicrobial metabolites, what so-called "metabolic secretion" [2], or in the form of starter cultures through competitive exclusion against bacteria and fungi [3, 4]. Regarding the immunomodulation effect, [5] demonstrated LAB-induced synergistic activation of intestinal Plasmacytoid dendritic cells (pDCs) and the induction of the expression of anti-viral genes in the lung by the oral administration of LC-Plasma in combination with emulsifiers *in vivo*. Also, their metabolic

secretion includes the production of a wide variety of metabolites such as organic acids, bacteriocins [6], some volatile compounds, and antifungal esters [7]. The extraction of antimicrobial substances may be conducted through a wide range of organic solvents. By using Ethyl acetate extraction, [8] successfully isolated 2- (2-1 mino-1- hydroxyethoxy) ethyl 2-methylpropanoate that have a broad antibacterial activity from a strain of *Lactobacillus plantarum*. Also, [9] obtained a bacteriocin-like compound that effectively inhibited gram-positive and gram-negative tested organisms. They applied the sorption–desorption method, butanol extraction, and SEC-HPLC method. A 70% (v/v) 2-propanol and 0.1% (v/v) trifluoroacetic acid (TFA) extraction system was applied to get the antimicrobial compound mixture from *Lactococcus lactis* fermentate. The produced mixture showed inhibitory behavior against all of gram-positive such as *Bacillus subtilis, Listeria innocua, Streptococcus pneumoniae* and gram-negative like *Pseudomonas aeruginosa* [10]. Besides, [11] used *Streptomyces lydicus* metabolites to control the growth of *Staphylococcus aureus* and *Bacillus cereus* successfully. After extraction with different solvent systems, they found that aqueous fraction (methanolic) possessed the observed activity, while Ethyl acetate extract only exhibited a dose-dependent antioxidant activity. More recently, [12] applied the extraction of different antibacterial, antifungal, anticancer, hepato-protective substances from *Lactobacillus helveticus* using Head Space-Gas Chromatography/Mass Spectroscopy for extraction and analysis.

Considering the application of chitosan in dairy products, [13] used chitosan at the nanoscale that previously loaded with Natamycin for the packaging of cheese samples. They noted that treated samples significantly lowered their mold and yeast content than that of free-state Natamycin. In another assessment on milk-borne *Staphylococcus aureus*, [14] found that the activity of Nisin-loaded NPs exceeded the free Nisin by 50%. Also, [15] found that Nisin-loaded NPs effectively reduced *Staphylococcus aureus* and *Listeria monocytogenes* populations by at least five-fold log CFU than the free Nisin.

The main aim of this study was the chromatographic profiling of different extracts obtained from *Lb. helveticus* CNRZ 32. After that, the antimicrobial potential of each extract was assessed through disc diffusion and Minimal inhibitory concentration.

2. Materials and Methods

2.1. Materials.

Nutrient agar was provided from Panreac Quimica, Spain; MRS agar was purchased from SRL, India; M17 agar was supplemented by CONDA, Spain; and Malt extract agar was imported from Biolife, Italy; Nisin (1100 IU/mg equivalent to 27.5 µg/mg) was imported from Luoyang Chihon Biotechnology Co., Ltd, China; Natamycin (50%) was imported from Quimicas, Spain; Chitosan (M.W: 100.000 – 300.000) was purchased from ACROS ORGANICS, UK.

2.2. Preparation of the cell-free supernatants (CFSs).

About 250 ml of MRS bottles were inoculated with 2% of LAB pure strains [4], and incubated at 30^{o}C for 72 hours. CFSs were prepared by centrifugation of fermented media for 20 min at 7000 rpm and kept at 4^{o}C till use.

2.3. Characterization of the CFSs.

2.3.1. Antimicrobial activity.

2.3.1.1. Pathogenic and spoilage foodborne isolates.

Pathogenic and food spoilage microorganisms; *Escherichia coli* strain E11 (accession number KY780346.1), *Salmonella enterica* strain SA19992307 (accession number CP030207.1), *Pseudomonas aeruginosa* strain Kasamber5 (accession number KY549641.1), *Bacillus cereus* strain 151007-R3-K09-40-27F (accession number KY820914.1), and *Staphylococcus sciuri* strain 2-6 (accession number MH491952.1), *Penicillium chrysogenum* strain J127 (KF572447.1) and *Candida parapsilosis* strain F2-17 (KP852497.1) were isolated and identified by Al-gamal *et al.* [4] from Egyptian cheese at Dairy Microbiological Lab., National Research Centre, Egypt.

2.3.1.2. Disc diffusion technique.

Antibacterial assay: the assay was performed as recommended in BSAC guidelines [16]. Briefly, from the overnight incubated culture, a typical colony was picked and introduced in a 5 ml of tryptone soy broth. The broth culture was incubated at 35ºC until visible turbidity reached 0.5 “McFarland" standard solution. Then, nutrient agar plates (25 ml agar / 9cm plate or equivalent) were inoculated with sterile cotton swabs in three directions to finally give a semi-confluent growth after overnight incubation. Within 15 minutes, discs with tested substances were applied on the dried surface of the inoculated agar plates. After incubation at 35ºC for 20 h, inhibition zone diameters (mm) were recorded.
Antifungal assay: Mold and yeast strains were plated onto Malt Extract Agar (MEA) and incubated for 3 days at 30ºC. The spore suspension of each fungus was prepared in 0.01% “tween 80” solution by comparing it with the 0.5 “McFarland standard. Petri dishes containing MEA medium were inoculated as described above, and results were recorded after 48 h.
Positive and negative controls: Dimethyl sulfoxide (DMSO) solution was considered as a negative control. Nisin (100 mg/ml) was used as the positive antibacterial control, while Natamycin (100 µg/ml) as the positive antifungal control.

2.3.1.3. Determination of the Minimal Inhibitory Concentrations (MICs).

Different extracts either in Free State or Nanoparticles were tested for MIC value. MICs were performed by the agar dilution method according to EUCAST protocol [17]. Briefly, molten agar tubes were allowed to cool in the water bath at 50ºC, and then supplemented with the accurately prepared dilution series of the tested substance, vortexed well, and poured in sterile pre-labeled Petri dishes. After complete dryness of the agar surface at room temperature, 1µl of 10^7 CFU/ml of microbial suspension was inoculated. As recommended for disc diffusion, plates were incubated, and results were recorded.

2.3.2. Isolation and structure elucidation of bioactive compounds.

2.3.2.1. Sample preparation and Gas Chromatography/Mass Spectroscopy analysis.

The analysis was performed in chromatographic laboratory, central laboratories network, National Research Centre, Dokki, Egypt using a GC-MS system (7890A-5975C, Agilent Technologies Inc., Santa Rosa, CA, USA) equipped with an HP-5 MS capillary column

(30 m × 0.25 mm, 0.25 mm, Agilent Technologies Inc., Santa Rosa, CA, USA). The injection volume of the sample was 1 µL. Helium (99.999%) was used as the carrier gas at 1 ml/min as flow-rate. The temperature of the injection port was 280 ºC, and the column temperature program was 40 ºC for 5 min, followed by an increase to 150 ºC at a rate of 5ºC/min, and an increase to 210 ºC at the rate of 10ºC/min. The MS conditions Capillary column and 5975B Inert XL MS system under electron ionization at 70 eV and Quadrupole mass analyzer. The MS source and Quadrupole were held at 230 °C and 150 °C, respectively. Helium was used as carrier gas at 1 ml/min. as a flow rate.

2.4. Preparation and characterization of loaded-nanoparticles.

Extracts-loaded Chitosan nanoparticles (Ch.-NPs) were prepared by dissolving 2 Grams of chitosan in 1% acetic acid solution. After complete dissolution, the chitosan solution was added drop wisely to the vigorously stirred Sodium Tripolyphosphate (TPP) solution (0.03%). The resulted suspension was then subjected to sonication (DAIGGER Sonicator Model GEX 750, USA; sonication power, 750 Watts, frequency, 20 kHz and amplitude 50%, in Marine Toxins Lab., National Research Centre) for 30 minutes at 25°C. Nanoparticles were stabilized by the addition of 0.4% Cetyltrimethylammonium bromide (CTAB) as a cationic surfactant.

The nanostructure of the samples was examined for suspensions of the corresponding nano-composites in water using a JEOL JEM-1400 transmission electron microscope (TEM) (at Research Park, Faculty of Agriculture, Cairo University) with an acceleration voltage of 80 kV. The microscopy probes of the nano-composite samples were prepared by adding a small drop of the water dispersions onto a Lacey carbon film-coated copper grid, then allowing them to dry in air.

2.5. Statistical analysis.

Statistical significance was determined using Statistica Version 9 (State Soft, Tulsa, Okla., USA). The means were determined by analysis of variance test (ANOVA, two-way analysis) ($p<0.05$) [18].

3. Results and Discussion

3.1. Evaluation of different solvent extracts of Lb. helveticus CFS.

In order to get the concentrated bioactive compounds, CFS was introduced for extraction with four organic solvents; n-Hexane, Dichloromethane, Ethyl acetate, and Diethyl ether. Furthermore, the solvent residues were then excluded through rotary evaporation. After complete evaporation of the solvent residues, extracts were tested for their antimicrobial potential through disc diffusion assay.

3.1.1. Disc diffusion technique.

The results presented in Table 1 summarize the antimicrobial effects of different extracts through disc diffusion. Ethyl acetate extract possessed the strongest effect on all tested pathogens with inhibition diameter ranged from 38 mm for *Staphylococcus sciuri* to 12 mm in the case of *Candida parapsilosis*, resembling about 80% of the positive antifungal control;

Natamycin and 506% of the antibacterial positive control activities. The both of Diethyl ether and Dichloromethane extracts came secondly, while n-Hexane showed a very slight inhibition.

Table 1. Inhibitory potential of different solvent extracts on cheese-borne microorganisms.

Crude extracts	Inhibition zone diameters (mm)						
	Staph. sciuri	***B. cereus***	***Sal. enterica***	***E. coli***	***Ps. aeruginosa***	***P. chrysogenum***	***C. parapsilosis***
Ethyl acetate	38 ± 2.30 ^{H}	36 ± 2.50 ^{H}	31 ± 2.50 ^{K}	23 ± 2.10 ^{B}	14 ± 1.00 ^{C}	15 ± 0.05 ^{C}	12 ± 0.25 ^{D}
Diethyl ether	21 ± 1.05 ^{B}	25 ± 1.75 ^{B}	15 ± 0.75 ^{C}	10 ± 0.05 ^{G}	18 ± 1.05 ^{F}	12 ± 0.05 ^{D}	10 ± 0.02 ^{G}
n-Hexane	6 ± 0.05^{A}	6 ± 0.05^{A}	ND	ND	6 ± 0.05^{A}	ND	ND
Dichloromethane	22 ± 1.50 ^{B}	21 ± 2.00 ^{B}	15 ± 0.50 ^{C}	10 ± 0.09 ^{G}	13 ± 0.49 ^{D}	16 ± 0.55 ^{C}	13 ± 0.75 ^{C}
Nisin	7.5 ± 0.05 ^{E}	7 ± 0.05 ^{E}	ND	ND	ND	NT	NT
Natamycin	NT	NT	NT	NT	NT	17 ± 1.00 ^{C}	15 ± 1.00 ^{C}
Negative Control	ND	ND	ND	ND	ND	ND	ND

Data expressed as Mean ± Standard error; all columns or rows of the different letter are significantly different at P <0.05; **ND**: non-detected inhibition; and **NT**: Not Tested

3.1.2. Transmission Electron Microscope imaging of chitosan nanoparticles.

The size and morphology of prepared chitosan Nanoparticles were shown in Figure 1. The particles appeared to be spherical, with size ranged from 14.6 nm to 18.5 nm.

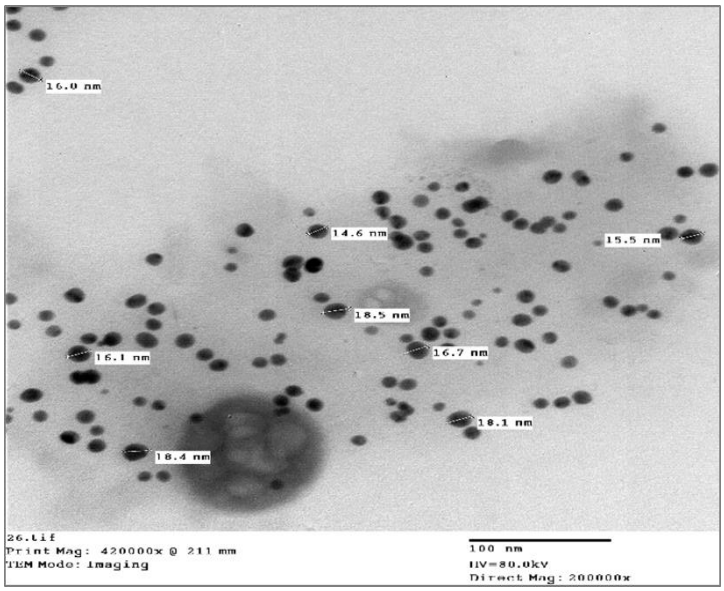


Figure 1. TEM micrograph of Natamycin loaded-Chitosan Nanoparticles.

3.1.3. Estimation of the minimal inhibitory concentrations (MICs).

This assay aimed to estimate the MICs for the most potent crude extracts; ethyl acetate, diethyl ether, and dichloromethane either in Free State or as loaded on Chitosan Nanoparticles. Tables 2 & 3 summarize the extract-specific MIC values against the cheese-borne indicator microorganisms, all in comparison with Nisin as the positive antibacterial control and Natamycin as the positive antifungal control.

As presented in Tables 2 & 3, the loading of active extracts on Ch. NPs. lowered the MIC values against hosted bacteria from 0.25 mg/ml to 0.2 mg/ml (~20%) for both Ethyl acetate and Diethyl ether extracts, while reached to 40 – 50% in case of Dichloromethane extract. Only in the case of *Salmonella enterica*, the reduction of MIC values reached 50, 60, and 70% for Ethyl acetate, Diethyl ether extracts, and dichloromethane extracts, respectively. It is also noted that the effective Nisin concentration was lowered from 50 to 62.5%. The observed synergistic effect of Nisin or such antibacterial cationic peptide and Chitosan Nanoparticles combination was agreed by many previous researches [19]. By following up *Staphylococcus aureus* count, Lee *et al.* [20] observed about 29% count reduction caused by Nisin loaded chitosan NPs more than free Nisin. This may be clarified by the increased contact surface of densely charged cationic chitosan NPs which bind the anionic cell membrane causing disruption of its function, consequently lowered the concentration of Nisin that causes inhibition of wall synthesis and formation of pores in the cell membrane. As a result, cellular components leak out, causing cell death. The ineffective contribution of Free Nisin to gram-negative bacterial inhibition was established by Vukomanović *et al.* [21]. They also attributed the current noticed inhibitory effect of Nisin (Table 3) against both *Escherichia coli* and *Pseudomonas aeruginosa* to the synergism between Nisin and Nanoparticles. The nanostructure gives spherical Nisin, increasing contact area within cell membranes. In addition, the high concentrations of Nisin were proved to induce cell membrane distortion through the high surface-bound density of Nisin that increases the membrane tension.

For antifungal MIC values, the greater reduction in MIC (70%) was observed in Ch. NPs.-loaded Natamycin. Both ethyl acetate and Diethyl ether extracts showed 50% reduced MICs in the case of *Penicillium chrysogenum*, while dichloromethane gave only about 17% reduction. *Candida parapsilosis* that possessed a slight reduction in MICs for loaded extracts (17 – 25%) even for the positive control (0%), appeared more resistant than *Penicillium chrysogenum*.

Table 2. MIC values of crude extracts in free states against indicator strains.

Tested bacteria	Free Extract (mg/ml)			Control positive (mg/ml)
	Ethyl acetate	Diethyl ether	Dichloromethane	
Staphylococcus sciuri	0.25 ± 0.01 ^{A}	0.25 ± 0.01 ^{A}	0.5 ± 0.02 ^{C}	0.6 ± 0.05 ^{C}
Bacillus cereus	0.25 ± 0.02 ^{A}	0.25 ± 0.01 ^{A}	1 ± 0.05 ^{B}	0.8 ± 0.10 ^{D}
Salmonella enterica	0.25 ± 0.01 ^{A}	0.25 ± 0.01 ^{A}	0.5 ± 0.05 ^{C}	ND
Escherichia coli	0.25 ± 0.02 ^{A}	0.25 ± 0.01 ^{A}	1 ± 0.10 ^{B}	ND
Pseudomonas aeruginosa	0.25 ± 0.02 ^{A}	0.25 ± 0.01 ^{A}	1 ± 0.05 ^{B}	ND
Penicillium chrysogenum	3 ± 0.25 ^{A}	2 ± 0.00 ^{C}	3 ± 0.25 ^{A}	0.01 ± 0.00^{E}
Candida parapsilosis	6 ± 0.00 ^{B}	4 ± 0.00 ^{D}	4 ± 0.00 ^{D}	0.005 ± 0.00^{B}

Data expressed as Mean ± Standard error; all columns or rows of the different letter are significantly different (P<0.05), **ND**: Not detected.

Table 3. MIC values of crude extracts-loaded Ch. NPs., against bacterial indicator strains.

Tested bacteria	Extract-loaded Ch. NPs. (mg/ml)			Control positive (mg/ml)
	Ethyl acetate	Diethyl ether	Dichloromethane	
Staphylococcus sciuri	0.2 ± 0.00 ^{A}	0.2 ± 0.00 ^{A}	0.3 ± 0.00 ^{D}	0.3 ± 0.00 ^{D}
Bacillus cereus	0.2 ± 0.00 ^{A}	0.2 ± 0.00 ^{A}	0.6 ± 0.00 ^{E}	0.3 ± 0.00 ^{D}
Salmonella enterica	0.125 ± 0.00 ^{B}	0.1 ± 0.00 ^{C}	0.15 ± 0.00 ^{N}	ND
Escherichia coli	0.2 ± 0.00 ^{A}	0.2 ± 0.00 ^{A}	0.5 ± 0.00 ^{G}	20 ± 0.00 ^{R}
Pseudomonas aeruginosa	0.2 ± 0.00 ^{A}	0.2 ± 0.00 ^{A}	0.6 ± 0.00 ^{E}	30 ± 1.00 ^{S}
Penicillium chrysogenum	2 ± 0.05 ^{A}	1 ± 0.10 ^{C}	2.5 ± 0.10 ^{E}	0.003 ± 0.00^{F}
Candida parapsilosis	5 ± 0.10 ^{B}	3.25 ± 0.20 ^{D}	3 ± 0.05 ^{D}	0.005 ± 0.00^{G}

Data expressed as Mean ± Standard error; all columns or rows of the different letter are significantly different (P<0.05), **Ch. NPs**: Chitosan nanoparticles.

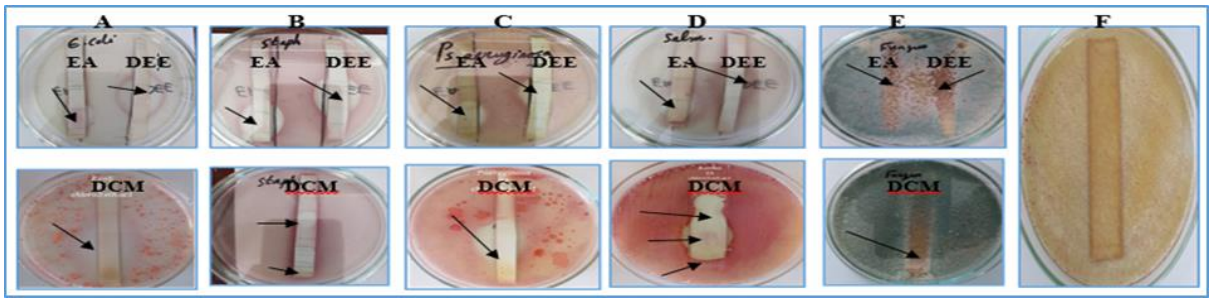
The enhanced effect of Natamycin upon loading on NPs. was noticed by [22] to reach 2.5 times free Natamycin. As explained formerly, the high density of positively-charged Ch. NPs. enables particles to bind effectively with the negatively-charged fungal cell membranes. Furthermore, the small size of NPs. allows them to diffuse easily into microbial cells and get their way to the nuclear area causing denaturation of both DNA and RNA [23].

3.2. Bioautographic studying of crude extracts.

Crude extracts were separately subjected to Thin Layer Chromatographic (TLC) silica gel plates to get partially separated. After complete dryness, plates were taken out to distinguish bands by visual detection under UV light (254 nm). The developed bands were marked using a lead pencil, and the R_f values were calculated. R_f value can be defined as the distance traveled by a certain band, divided by the total distance from the base-line to the end-line.

The inhibitory range of bio-autographic TLC plates on microbial growth was presented in Figure 2 and Table 4. They present the selected solvent system, number, and R_f of bands/spots of every crude extract with their target microorganisms. After separation of the 3 active extracts on TLC plates, the bands were visualized and marked under UV light (254 nm).

As shown, each extract included at least two antimicrobial active bands, at the same time, all pathogens were inhibited by two or more bands except *Candida parapsilosis* that inhibited only by the extracts as it is. These results can be explained when the components of the extracts get identified via instrumental facilities.



A: *E. coli***; B:** *Staph. sciuri***; C:***Ps. aeruginosa***; D:** *Salmonella enterica***; E:** *Penicillium chrysogenum***; F:** *Candida parapsilosis***; EA:** ethyl acetate extract**; DEE:** diethyl ether extract**; DCM:** dichloromethane extract

Figure 2. The inhibition of microbial growth on bio-autographic TLC plates.

Table 4. The inhibitory range of bio-autographic TLC plates on microbial growth.

Extract 1	Solvent system	No. of active bands	R_f	Target organism
Ethyl Acetate	*Chloroform : EA (1:1)*	5	0.45, 0.49, 0.54, 0.58, 0.63	*Pseudomonas aeruginosa*
		4	0.45, 0.49, 0.54, 0.58	*Escherichia coli & Salmonella enterica*
		4	0.54, 0.58, 0.63, 0.74	*Bacillus cereus*
		5	0.45, 0.49, 0.54, 0.58, 0.63	*Staphylococcus sciuri*
		2	0.63, 0.74	*Penicillium Chrysogenum*
		ND	---	*Candida Parapsilosis*

3.3. GC/MS analysis of crude extracts.

In this part, the volatile profiles of the three effective extracts; Ethyl acetate, Diethyl ether, and dichloromethane, were illustrated. GC/MS analysis was used to inspect the components of crude extracts. The GC/MS chromatogram, as well as mass spectra of the most abundant compounds for each extract, was also demonstrated.

Metabolic profiling was summarized in Table 5 that shows the names of the abundant components along with their retention times, area %, and molecular formulas.

Table 5. Metabolic profiling of the crude extracts using GC/MS.

Extract	Peak	RT	Name	Area sum%	Formula
Ethyl acetate	1	5.553	Pentanoic acid, 3-methyl-	2.13	$C_6H_{12}O_2$
	2	5.786	Butanoic acid, 3-methyl-	1.78	$C_5H_{10}O_2$
	3	10.07	Butyl lactate	59.45	$C_7H_{14}O_3$
	4	12.197	Isoamyl lactate	1.21	$C_8H_{16}O_3$
	5	17.059	Benzoic acid	7.94	C7H6O2
	6	26.827	Butylated Hydroxytoluene	9.86	$C_{15}H_{24}O$
	7	37.756	6-Tridecene, (Z)-	1.97	$C_{13}H_{26}$
	8	42.192	Hexadecane, 1-chloro-	1.81	$C_{16}H_{33}Cl$
Diethyl ether	1	5.553	Butanoic acid	9.31	$C_4H_8O_2$
	2	5.786	Butanoic acid, 3-methyl-	1.78	$C_5H_{10}O_2$
	3	10.07	Butanoic acid, 2-methyl-	2.47	$C_5H_{10}O_2$
	4	12.197	Hexanoic acid	2.62	$C_6H_{12}O_2$
	5	17.059	Benzoic acid	33.47	$C_7H_6O_2$
	6	26.827	9,12-Octadecadienoic acid,	1.68	$C_{19}H_{34}O_2$
Dichloromethane	1	5.0034	Butanoic acid	15.8	$C_4H_8O_2$
	2	9.149	Pentanoic acid, 4-methyl-	2.92	$C_6H_{12}O_2$
	3	22.339	Hydrocinnamic acid	2.47	$C_9H_{10}O_2$
	4	42.67	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	23.51	$C_{11}H_{18}N_2O_2$
	5	47.205	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	1.25	$C_{19}H_{34}O_2$

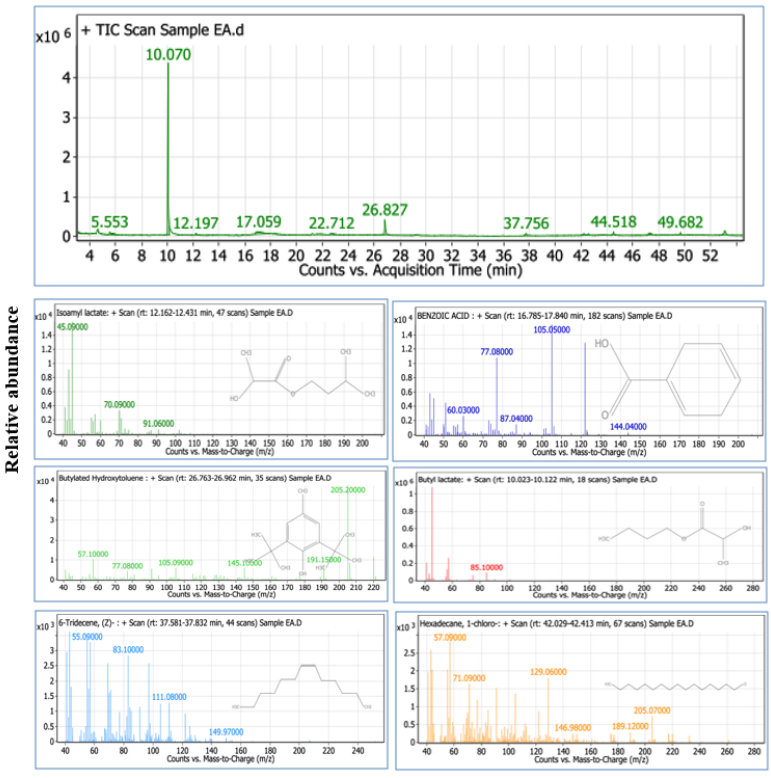


Figure 3. GC/MS Spectral Chromatogram and mass spectra of *ethyl acetate* extract.

As observed in Table 5, Ethyl acetate extract included butyl lactate with area % (59.45), butylated hydroxytoluene (9.86%), benzoic acid (7.94%), pentanoic acid, 3-methyl- (2.13%), 6-tridecene, (Z)- (1.97%), hexadecane, 1-chloro- (1.81%), butanoic acid, and 3-methyl-

(1.78%), isoamyl lactate (1.21%). The GC/MS chromatogram and mass spectra of the most common compounds were illustrated in Figure 3.

For diethyl ether extract, the commonly detected compounds included benzoic acid (33.7%), butanoic acid (9.31%), hexanoic acid (2.62%), butanoic acid, 2-methyl- (2.47%), butanoic acid, 3-methyl- (1.78%), and 9,12-octadecadienoic acid, methyl ester (1.68%). The GC/MS chromatogram and mass spectra of the common compounds were shown in Figure 4.

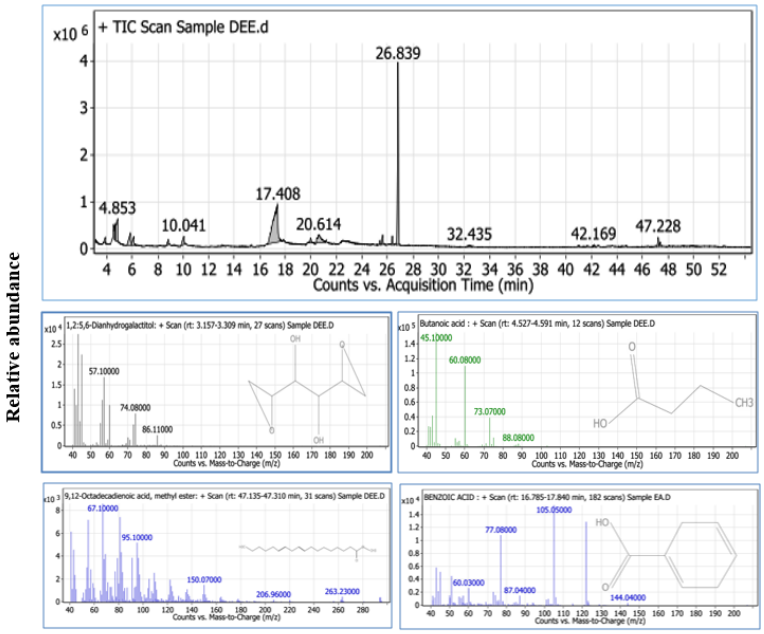


Figure 4. GC/MS Spectral chromatogram and mass spectra of *diethyl ether* extract.

As seen in Figures 5, dichloromethane extract was reported to contain pyrrolo [1,2-a] pyrazine -1,4-dione,hexahydro-3- (2-methylpropyl)- (23.51%), butanoic acid (15.8%), pentanoic acid, 4-methyl- (2.92%), hydrocinnamic acid (2.47%), and 9,12-octadecadienoic acid (Z,Z)-, methyl ester (1.25%).

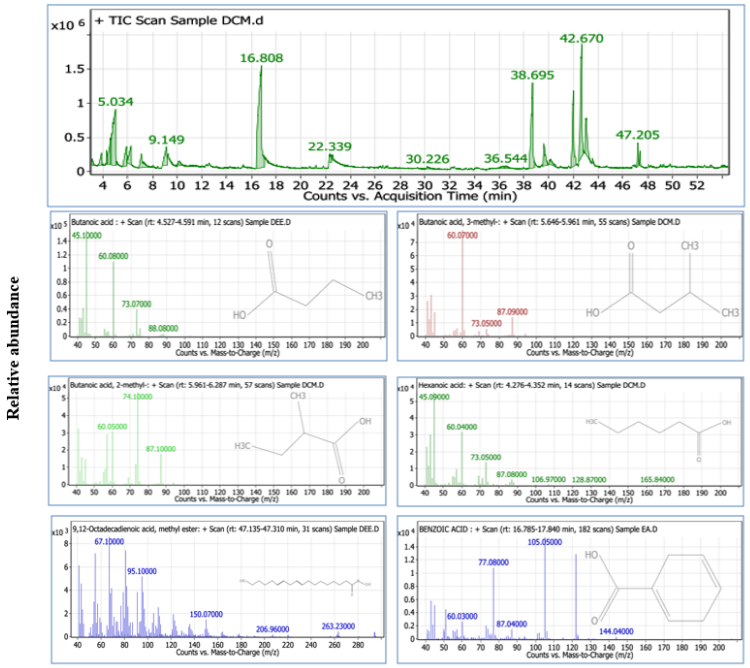


Figure 5. GC/MS Spectral Chromatogram and mass spectra of *dichloromethane* extract.

The bioactivity of these compounds, either as antimicrobial, or health promoters, was described in the literature by many researchers. Kavčič *et al.* [24] reported the excellent activity of n-butyl lactate, a form of lactic acid produced by Lactic acid bacteria against many potential pathogenic bacteria and spoilage yeasts and fungi. Their results revealed that n-butyl lactate could completely inhibit the mycelial growth of such *Aspergillus niger* and *Penicillium sp.*, while having a high inhibitory potential against *Escherichia coli*, *Pseudomonas sp.*, and *Bacillus cereus*. In addition, Zhang *et al.* [25] confirmed the role of Butylated Hydroxytoluene in retarding of pathogenic and spoilage growth, along with acting as a good antioxidant. Deepa *et al.* [26] reviewed the great contribution of Butanoic acid to retard the growth of *Salmonella sp.* in broiler chicken production stations. In addition, Adeyemi *et al.* [27] referred to the significant health beneficial role of 9,12- Octadecadienoic acid (Z,Z)-, methyl ester. As reported, this compound showed a good anti-inflammation, anticancer, hypocholesterolemic, hepato-protection, and antimicrobial activity. It is important to state that the highest content of this health-beneficial compound was currently observed in the Diethyl ether extract. In addition, all extracts were seen to be more effective and wider spectra either against bacteria, gram-positive and gram-negative or against yeasts and fungi than Nisin alone and Natamycin alone. Furthermore, these bio-protective LAB extracts were proven to contain diverse biologically active compounds.

The collective view found that the greatest inhibition zones were obtained in the case of gram-positive pathogens (Table 1), the multi-active bio-autographic bands in gram-positive bacterial pathogens (Figure 2) among all pathogenic bacteria, fungi, and yeasts. Inclusion of bacteriocins in all extracts may come in the front of all suggestions. Lv *et al.* [28] successfully extracted a bacteriocin from *Lactobacillus plantarum* using ethyl acetate solvent. In addition, the considerable content of organic acids supports the antimicrobial activity.

4. Conclusions

Lactobacillus helveticus CNRZ 32 exhibited inhibitory activity against different microbial pathogens and food spoilers. In addition to bacteriocins, this bacterium could synthesize a wide range of metabolites that antagonize many microbial concerns. The results suggest the synergistic relationship between Ch. NPs. and *Lactobacillus helveticus*-based antimicrobials that lowered the MIC value by 20 – 50%, but still less than the loaded Natamycin. The antibacterial, antifungal, and health-promoting potentials of *Lactobacillus helveticus* CNRZ 32, and its metabolic reservoir could make them an efficient alternative to both Nisin and Natamycin in dairy industries.

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Conflicts of Interest

The authors declare no conflict of interest.

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