

# Attempts to Improve Antimicrobial Efficiency by Mixed-*Lactobacillus* Extracts as Crude or Nano-formulated Against Pathogenic and Food Spoilage Bacteria, Molds and Yeasts

Mohamed F. El-Ssayad<sup>1,\*</sup> , Gamal A. Ibrahim<sup>2</sup> , Osama M. Sharaf<sup>3</sup> 

<sup>1</sup> Dairy Science Dept., (Dairy Microbiol. Lab.), National Research Centre, 33 El-Bohouth St. (Former El-Tahrir St.) Dokki, Giza, Egypt; sayad.nrc2012@gmail.com (M.F.E.); gamalwahab2015@gmail.com (G.A.I.); sharafosama@yahoo.com (O.M.S.);

\* Correspondence: sayad.nrc2012@gmail.com (M.F.E);

Scopus Author ID: 57073976000

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**Abstract:** Over the world, there are millions of disease cases, in addition to thousands of deaths due to food-based outbreaks every year. In order to diminish the risk of foodborne infection and intoxications and control microbial food spoilage, there is a great need to improve modes of food preservation. As well-known, nature-dependent preservation enables consumers to keep their safety while eating highly qualified food. This work aims to enhance the safety and quality of common popular food products by introducing new preservative applications suggestions. The idea is to use different *lactobacilli*-based fermentations; in single or mixed-status and study the obtained extracts to evaluate their influence to manage microbial contamination of food. A set of five *Lactobacillus* strains was utilized by this study, methanol extract of cold acetone precipitate, Diethyl ether extract, and both were tested as crude or Nano-formulated against common microbial pathogens and food spoilers. Methanol extract of all *lactobacillus* strains shows antibacterial-specific activity with inhibition zone ranging from 7 mm in the case of *Lactobacillus rhamnosus* against *Salmonella enterica*, to 24 mm in the case of *Lactobacillus helveticus* against *Escherichia coli*. Diethyl ether extracts based on *Lactobacillus helveticus* and *Lactobacillus plantarum* show a considerable inhibition to all utilized microbial concerns, including *Saccharomyces cerviceae*. Upon estimating three forms of mixed-*Lactobacillus* that are based on *Lactobacillus helveticus* (h), *Lactobacillus plantarum* (p), and *Lactobacillus rhamnosus* GG (G), the ph-dependant combination showed uprising inhibition that reached maximally 58% over that of single *Lactobacilli*. Nano formulation of mixed-based total extracts maximally upraised the inhibition to 39%, 32%, and 100% against gram-positive, gram-negative, and the fungus; *Penicillium chrysogenum*, respectively.

**Keywords:** antimicrobial activity; nanoparticles; pathogenic and spoilage microorganisms; single and mixed-*Lactobacillus* fermentation; food biopreservation.

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## 1. Introduction

Because there is no zero-risk system, foodborne diseases and intoxications are in progress. The Council for Agricultural Science and Technology reported that foodborne pathogens cause an estimated 6.5-33 million cases of human illness and up to 9000 deaths annually, the main foods implicated being meat, poultry, eggs, seafood, and dairy products [1, 2]. Awareness in bio-preservation has increased worldwide, supported by research results that protective cultures or

their inhibitory metabolites may have bio-preservative potential to render pathogenic populations in foods [3,4]. These pathogens that account for most of these cases include *Salmonella*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* [5, 6]. Fungi, the public food spoilers, are common concerns in dairy products, where a favorable niche for their growth exists. There is great attention to control fungal spoilage through searching for efficient solutions [7-9]. Many conventional methods called traditional hurdle technologies are applied in combinations to achieve real preservation. Microbial control and prevention include good manufacturing practices, proper hygiene, inactivation treatments, temperature control, and modified atmosphere packaging [7, 10, 11].

Lactobacilli are known to produce antibacterial compounds, including bacteriocins and bacteriocin-like peptides [12-15]. Most of the bacteriocins produced by *Lactobacillus* species are small, thermally stable proteins, known as type II bacteriocins [16] which efficiently affect some antibiotic-resistant microbes [17]. These compounds can induce rupture of the cell membrane, causing leakage of cell contents and playing a role in sterilization [18].

*Lactobacilli* produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin during lactic fermentations. All of these can antagonize the growth of some spoilage and pathogenic bacteria in foods [19]. In particular, there has been a renewed interest in the antimicrobial activity of lactobacilli, which has been important for centuries in the preservation of food. Probiotic *Lb. rhamnosus* strain shows a broad spectrum of activity against *gastrointestinal tract* pathogens and food spoilage organisms [20]. *Lactobacilli* drive the inhibitory process through the production of organic acids (primarily lactic and acetic acid), hydrogen peroxide, bacteriocins, and other substances [21-23], showing strong antagonistic activity against spoilage and pathogenic bacteria and fungi [24].

Nanoparticles can be applied to control the delivery of antimicrobial compounds in food and nonfood microbial safety applications [25]. Antimicrobial compounds can be attached to cores of nanoparticles and delivered into bacterial cells. The antibacterial mechanisms of NPs are not completely understood, but oxidative stress induction, metal ion release, and non-oxidative theory are the most accepted [26-28].

The aim of the present investigation was to assess the perfect combination of nano or crude lactobacilli cell-free supernatant (*Lactobacillus helveticus*, *Lb. plantarum*, *Lb. reuteri*, *Lb. rhamnosus*, *Lb. rhamnosus* GG) that gives rise to an antimicrobial mixture useful to control pathogenic and spoilage bacteria, molds, and yeasts.

## 2. Materials and Methods

### 2.1. Bio-protective strains.

*Lactobacillus plantarum* DSA 20174 is provided by Cairo MIRCEN, Faculty of Agriculture, Ain Shams University, Egypt; *Lactobacillus rhamnosus* NRRL B-442 and *Lactobacillus reuteri* NRRL B-14171 are obtained from the Northern Regional Research Laboratory, Illinois; USA. *Lactobacillus helveticus* CNRZ 32 Collection of dairy Microbiological Laboratory (supplemented from Centre National de Recherche Zootechnique, Jouy-en-Josas, France). *Lactobacillus rhamnosus* GG was supplemented by Afify *et al.*, [29] from the collection of the Food sciences & Nutrition dept., NRC.

## 2.2. Pathogenic and food spoilage microorganisms.

*Escherichia coli* (*E. coli*) strain E11 (accession number KY780346.1), *Salmonella enterica* (*S. enterica*) strain SA19992307 (accession number CP030207.1), *Pseudomonas aeruginosa* (*Ps. aeruginosa*) strain Kasamber5 (accession number KY549641.1), *Bacillus cereus* (*B. cereus*) strain 151007-R3-K09-40-27F (accession number KY820914.1) were isolated and identified by Al-Gamal *et al.*, [30]. *Listeria monocytogenes* (*L. monocytogenes*) strain was supplemented from the collection of Dairy Microbiological Lab., NRC, Egypt; *Staphylococcus aureus* (*S. aureus*) is a clinical isolate, while *Aspergillus flavus* (*Asp. flavus*)3357 and *Saccharomyces cereviceae* (*Sac. Cerviceae*) Y-2223 were provided by the Northern Regional Research Laboratory Illinois, USA (NRRL).

## 2.3. Materials.

Skimmed milk was purchased from animal production Research Institute, Agricultural Research Centre, Egypt; MRS broth is purchased from SRL, India; Nutrient agar is provided from Panreac Quimica, Spain; Malt extract agar is imported from Biolife, Italy; Chitosan (M.W: 100.000 – 300.000) is purchased from ACROS ORGANICS, UK; Acetone is purchased from ADWIC, Egypt; Methanol (HPLC grade) is supplemented by Fisher Scientific, UK.

## 2.4. Precipitation of active peptides by cold acetone.

The antimicrobial peptides are concentrated from 150 ml of Cell-free supernatant via cold acetone extraction. Four times the sample volume of cold (-20°C) acetone is added to the cooled sterile supernatant sample (4°C), mixed for 15 min, and incubated 60 min at 4°C. The pellet is collected by further centrifugation and extracted in 50 ml methanol with stirring for 2 hours [31]. Evaporation of methanol is carried out using a rotary evaporator until peptide dry film is obtained.

## 2.5. Characterization of Methanol extract

### 2.5.1. Fourier-Transform Infrared Spectroscopy (FT-IR) analysis.

The absorbance FT-IR spectra of the samples are documented using an FT-IR Perkin–Elmer spectrometer. The spectra are collected within a scanning range of 400 - 4000  $\text{cm}^{-1}$ , Central Lab. for Services, NRC, Dokki, Giza, Egypt.

### 2.5.2. GC/MS analysis of diethyl ether extract.

The analysis was performed in a 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). Exactly, 1  $\mu\text{L}$  of the sample was injected using Helium (99.999%) - as the carrier- at 1 ml/min. The injection port temperature was 280 °C, and the column temperature cycle was 40 °C for 5 min, followed by an increase to 150 °C at a rate of 5°C/min, and an increase to 210 °C at the rate of 10°C/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, using Helium as carrier gas as follows.

#### 2.5.3. Preparation and characterization of loaded-nanoparticles.

Chitosan nanoparticles (Ch.-NPs) are prepared by dissolving 2 Grams of chitosan in 1% acetic acid solution. After complete dissolution, chitosan solution is added drop-wisely to the vigorously stirred Sodium Tri-polyphosphate (TPP) solution (0.03%). The resulted suspension is then subjected to sonication (sonication power, 750 Watts, frequency, 20 kHz and amplitude 50%, for 30 minutes at 25°C. Nanoparticles are stabilized by the addition of 0.4% Cetyltrimethylammonium bromide (CTAB) as a cationic surfactant. Nanoemulsions were prepared by mixing one part of extract into two parts of both H<sub>2</sub>O and Tween 80.

The particle size distribution and zeta potential were estimated in central laboratories network, National Research Centre, Dokki, Egypt using Particle Sizing Systems, Inc. Santa Barbara, Calif., USA.

#### 2.5.4. Antimicrobial assay.

Firstly, the antimicrobial activity of the crude peptide extract that had been dissolved in 0.2 ml of DMSO and extract-loaded chitosan NPs. is evaluated through disc diffusion assay as recommended in the British Society for Antimicrobial Chemotherapy guidelines [32]. Briefly, a typical colony was picked and introduced in a 5 ml of tryptone soy broth from the overnight incubated culture. The broth culture was incubated at 35°C 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 give a semi-confluent growth after overnight incubation finally. Within 15 minutes, discs with tested substances were applied on the dried surface of the inoculated agar plates. After incubation at 35°C for 20 h, inhibition zone diameters (mm) were recorded.

#### 2.5.5. 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) ( $p < 0.05$ ) [33].

### 3. Results and Discussion

#### 3.1. Antimicrobial activity.

##### 3.1.1. Methanol extract of cold acetone precipitates.

*Lactobacillus fermentates* were treated with cold acetone and underwent centrifugation. The resulting precipitates were then extracted with methanol. The disc diffusion method estimated the inhibitory activity of lactobacillus extracts against the common food microbial concerns, and the results are presented in Table 1. It is clear that the tested extracts have a strictly antibacterial effect with no activity against molds nor yeasts. Among all LAB cultures, the tested peptides of

*Lb. helveticus* and *Lb. rhamnosus* GG show higher antimicrobial activity against all bacterial strains.

**Table 1.** Inhibition zone (mm) of single Lactobacilli methanol extract.

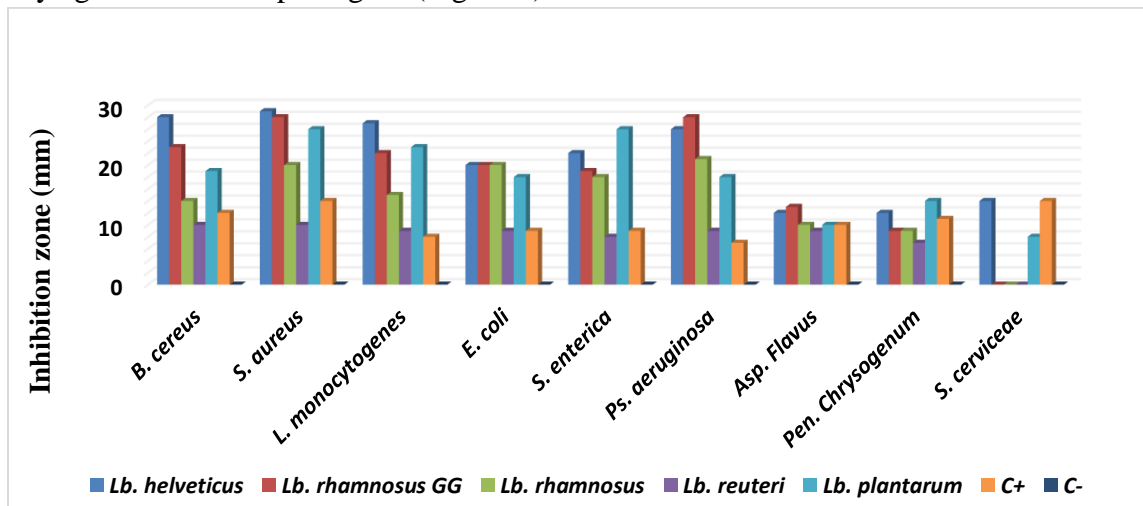
	<i>Lb. helveticus</i>	<i>Lb. rhamnosus</i> GG	<i>Lb. rhamnosus</i>	<i>Lb. reuteri</i>	<i>Lb. plantarum</i>	C+	C-
<i>B. cereus</i>	15.0 ± 0.96 <sup>A</sup>	14.0 ± 0.96 <sup>A</sup>	11.0 ± 0.00 <sup>D</sup>	11.0 ± 0.00 <sup>D</sup>	11.0 ± 0.28 <sup>D</sup>	13.0 ± 0.00 <sup>M</sup>	0.0 ± 0.00 <sup>H</sup>
<i>S. aureus</i>	15.0 ± 0.28 <sup>A</sup>	10.0 ± 0.00 <sup>C</sup>	10.0 ± 0.00 <sup>C</sup>	0.0 ± 0.00 <sup>H</sup>	8.0 ± 0.00 <sup>F</sup>	17.0 ± 0.00 <sup>B</sup>	0.0 ± 0.00 <sup>H</sup>
<i>L. monocytogenes</i>	18.0 ± 0.28 <sup>B</sup>	12.0 ± 0.48 <sup>D</sup>	11.0 ± 0.48 <sup>D</sup>	10.0 ± 0.00 <sup>C</sup>	7.0 ± 0.00 <sup>G</sup>	9.0 ± 0.00 <sup>F</sup>	0.0 ± 0.00 <sup>H</sup>
<i>E. coli</i>	24.0 ± 0.96 <sup>K</sup>	13.0 ± 0.00 <sup>M</sup>	9.0 ± 0.00 <sup>F</sup>	14.0 ± 0.48 <sup>A</sup>	18.0 ± 0.96 <sup>B</sup>	20.0 ± 0.00 <sup>E</sup>	0.0 ± 0.00 <sup>H</sup>
<i>S. enterica</i>	12.0 ± 0.00 <sup>D</sup>	16.0 ± 0.00 <sup>A</sup>	7.0 ± 0.00 <sup>G</sup>	18.0 ± 0.48 <sup>B</sup>	12.0 ± 0.96 <sup>D</sup>	12.0 ± 0.00 <sup>D</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Ps. aeruginosa</i>	11.3 ± 0.28 <sup>D</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Asp. flavus</i>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	9.0 ± 0.00 <sup>F</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Pen. chrysogenum</i>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	12.0 ± 0.00 <sup>D</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Sac. cereviceae</i>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>	12.0 ± 0.00 <sup>D</sup>	0.0 ± 0.00 <sup>H</sup>

Data expressed as Mean ± Standard error; all columns or rows of the different letter are significantly different at P < 0.05

The tested extracts of all studied Lactobacilli caused a considerable inhibition to all bacterial pathogens. Inhibition zone diameter ranged from 7 mm to 24 mm, depending on both pathogenic and Lactobacillus strains. At P < 0.05, among all Lactobacilli, the extract of *Lb. helveticus* only affected the growth of *Ps. aeruginosa* (11.3 mm).

### 3.1.2. Diethyl ether extracts (DEE) of single Lactobacilli fermentates.

Disc diffusion assay of the Diethyl ether extracts was performed to define their inhibitory activity against different pathogens (Figure 1).



**Figure 1.** Inhibitory effect of Diethyl ether extract of single Lactobacilli fermentate.

As observed, the growth of all pathogenic and food spoilage strains was inhibited by DEE of studied Lactobacilli. The diameter of inhibition zones came in the range of 7 mm to 29 mm. Only *Saccharomyces cereviceae* showed no response (not inhibited) to all Lactobacilli except *Lactobacillus helveticus* and *Lactobacillus plantarum*, which induced inhibition diameter of 9 mm and 18 mm respectively.

### 3.1.3. Total extract of single Lactobacilli fermentates.

Data in Table 2 describe the degree of antimicrobial effect due to the application of lactobacilli total extract (Methanol extract + DEE).

**Table 2.** Inhibition zone (mm) of total extracts of single Lactobacilli

	<i>Lb. helveticus</i>	<i>Lb. rhamnosus</i> GG	<i>Lb. rhamnosus</i>	<i>Lb. reuteri</i>	<i>Lb. plantarum</i>	C+	C-
<i>B. cereus</i>	39 ± 1.44 <sup>E</sup>	33 ± 0.96 <sup>G</sup>	35 ± 0.48 <sup>K</sup>	35 ± 1.44 <sup>K</sup>	30 ± 0.48 <sup>H</sup>	12 ± 0.00 <sup>M</sup>	0.0 ± 0.00 <sup>D</sup>
<i>S. aureus</i>	43 ± 0.00 <sup>F</sup>	40 ± 0.00 <sup>E</sup>	40 ± 0.00 <sup>E</sup>	28 ± 0.50 <sup>B</sup>	30 ± 0.00 <sup>H</sup>	13 ± 0.00 <sup>M</sup>	0.0 ± 0.00 <sup>D</sup>
<i>L. monocytogenes</i>	29 ± 0.70 <sup>H</sup>	32 ± 0.00 <sup>G</sup>	32 ± 0.00 <sup>G</sup>	23 ± 0.00 <sup>C</sup>	30 ± 0.00 <sup>H</sup>	13 ± 0.00 <sup>M</sup>	0.0 ± 0.00 <sup>D</sup>
<i>E. coli</i>	33 ± 0.00 <sup>G</sup>	30 ± 0.00 <sup>H</sup>	23 ± 0.00 <sup>G</sup>	18 ± 0.00 <sup>A</sup>	27 ± 0.50 <sup>B</sup>	0.0 ± 0.00 <sup>D</sup>	0.0 ± 0.00 <sup>D</sup>
<i>S. enterica</i>	33 ± 0.00 <sup>G</sup>	36 ± 0.00 <sup>K</sup>	28 ± 0.00 <sup>H</sup>	28 ± 0.00 <sup>H</sup>	30 ± 0.00 <sup>H</sup>	18 ± 0.00 <sup>A</sup>	0.0 ± 0.00 <sup>D</sup>
<i>Ps. aeruginosa</i>	35 ± 0.69 <sup>K</sup>	32 ± 0.00 <sup>G</sup>	24 ± 1.00 <sup>C</sup>	20 ± 0.00 <sup>Z</sup>	32 ± 0.00 <sup>G</sup>	16 ± 0.00 <sup>R</sup>	0.0 ± 0.00 <sup>D</sup>
<i>Asp. flavus</i>	12 ± 0.96 <sup>M</sup>	14 ± 1.44 <sup>M</sup>	9 ± 0.48 <sup>S</sup>	9 ± 0.48 <sup>S</sup>	11 ± 0.96 <sup>M</sup>	12 ± 0.28 <sup>M</sup>	0.0 ± 0.00 <sup>D</sup>
<i>Pen. chrysogenum</i>	15 ± 0.96 <sup>R</sup>	15 ± 1.44 <sup>R</sup>	8 ± 0.48 <sup>S</sup>	16 ± 1.44 <sup>R</sup>	13 ± 0.96 <sup>M</sup>	10 ± 0.48 <sup>S</sup>	0.0 ± 0.00 <sup>D</sup>
<i>Sac. cereviceae</i>	15 ± 0.00 <sup>R</sup>	0.0 ± 0.00 <sup>D</sup>	0.0 ± 0.00 <sup>D</sup>	0.0 ± 0.00 <sup>D</sup>	10 ± 0.00 <sup>S</sup>	15 ± 0.00 <sup>R</sup>	0.0 ± 0.00 <sup>D</sup>

Data expressed as Mean ± Standard error; all columns or rows of the different letter are significantly different at P < 0.05

As observed, there is a general extension and enhancement of inhibition ranging from 9 mm (in the case of *Lactobacillus rhamnosus* and *Lactobacillus reuteri*) to 43 mm (in case of *Lactobacillus helveticus*) against all pathogenic and spoilage strains. In the case of *Saccharomyces cereviceae*, just *Lactobacillus helveticus* and *Lactobacillus plantarum* still inhibiting the yeast growth by 15 mm and 10 mm respectively.

#### 3.1.4. Different extracts of Mixed-Lactobacilli fermentates.

As their antimicrobial behavior is promising, all of *Lactobacillus helveticus*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* GG have undergone a set of combinations to inspect the effect of mixed-Lactobacillus fermentation on inhibitory potential against the group of pathogenic and food spoiler organisms. Three combinations, including *Lactobacillus helveticus* : *Lactobacillus plantarum* (ph 1:1); *Lactobacillus helveticus* : *Lactobacillus plantarum* : *Lactobacillus rhamnosus* GG (phG 1:1:1); and *Lactobacillus helveticus* : *Lactobacillus rhamnosus* GG (hG 1:1) were generated. The resulted data of different extracts from mixed-Lactobacillus fermentation are presented in Table 3. Regarding the total extract, there is a general increment-except small declining against some pathogens- in the inhibitory effect of all extracts belonging to the three combinations. Significantly, there is no difference in activity against *B. cereus* among the three Lactobacilli combinations.

At P < 0.05, the ph-based combination shows a significant enhancement in the activity, ranging from 16.7% and 13.3% with molds; *Asp. flavus* and *Pen. chrysogenum* to ~58% in the case of *Listeria monocytogenes*. The observed decline in the activity is seen with *S. aureus*, *Ps. aeruginosa*, and *Sac. cereviceae* as 21%, 5.7%, and 46.7%, respectively. Within hG-based combination, inhibitory activity is still improving to reach ~33.3% with *Pen. chrysogenum*, 28.6% with *Ps. aeruginosa*, and 33.33% with *E. coli*, but activity is reduced by 24.6% in the case of *S. aureus*. Upon evaluation of phG-based mixture, the resulted activity is severely decreased, especially with *S. aureus* (30.2%) and *Ps. aeruginosa* (20%).

**Table 3.** Inhibitory potential of different extracts from mixed-Lactobacillus fermentation.

	ph Combination			pHG Combination			hG Combination			C+	C-
	DEE extract	Methanol extract	Total extract	DEE extract	Methanol extract	Total extract	DEE extract	Methanol extract	Total extract		
<i>B. cereus</i>	36 ± 1.44 <sup>A</sup>	26 ± 0.00 <sup>C</sup>	43 ± 0.00 <sup>T</sup>	34 ± 0.00 <sup>A</sup>	22 ± 0.00 <sup>C</sup>	42 ± 0.00 <sup>T</sup>	39.3 ± 1.47 <sup>R</sup>	23 ± 0.00 <sup>C</sup>	43.7 ± 0.99 <sup>T</sup>	14 ± 0.00 <sup>E</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Staph. aureus</i>	32.7 ± 1.54 <sup>A</sup>	18 ± 0.00 <sup>D</sup>	34 ± 0.00 <sup>A</sup>	30 ± 0.00 <sup>B</sup>	16 ± 0.00 <sup>E</sup>	30 ± 0.00 <sup>B</sup>	33 ± 0.00 <sup>A</sup>	18 ± 0.00 <sup>D</sup>	32.4 ± 1.54 <sup>A</sup>	15 ± 0.00 <sup>E</sup>	0.0 ± 0.00 <sup>H</sup>
<i>L. monocytogenes</i>	30 ± 0.00 <sup>B</sup>	27 ± 1.00 <sup>C</sup>	46 ± 1.73 <sup>P</sup>	32 ± 0.00 <sup>A</sup>	24 ± 0.00 <sup>C</sup>	42 ± 0.00 <sup>T</sup>	35 ± 0.00 <sup>A</sup>	29 ± 0.70 <sup>C</sup>	47 ± 1.73 <sup>P</sup>	10 ± 0.00 <sup>G</sup>	0.0 ± 0.00 <sup>H</sup>
<i>E. coli</i>	35 ± 0.00 <sup>A</sup>	16 ± 0.00 <sup>E</sup>	38 ± 0.00 <sup>R</sup>	27 ± 0.00 <sup>C</sup>	20 ± 0.00 <sup>D</sup>	37.7 ± 1.47 <sup>A</sup>	31 ± 0.00 <sup>B</sup>	13 ± 0.00 <sup>G</sup>	44 ± 0.83 <sup>T</sup>	8 ± 0.00 <sup>K</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Sal. enterica</i>	37 ± 0.00 <sup>A</sup>	22 ± 1.11 <sup>D</sup>	39.5 ± 0.00 <sup>R</sup>	33 ± 0.00 <sup>A</sup>	22 ± 0.00 <sup>C</sup>	34.8 ± 0.48 <sup>A</sup>	36 ± 0.00 <sup>A</sup>	21 ± 0.00 <sup>D</sup>	39 ± 0.99 <sup>R</sup>	10 ± 0.00 <sup>G</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Ps. aeruginosa</i>	31.6 ± 1.11 <sup>B</sup>	16 ± 0.00 <sup>E</sup>	33 ± 0.00 <sup>A</sup>	29 ± 0.99 <sup>C</sup>	18 ± 0.00 <sup>D</sup>	28 ± 0.28 <sup>C</sup>	35 ± 0.00 <sup>A</sup>	18 ± 0.00 <sup>D</sup>	45 ± 0.00 <sup>P</sup>	0 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Asp. flavus</i>	23 ± 1.73 <sup>C</sup>	0.0 ± 0.00 <sup>H</sup>	14 ± 0.48 <sup>E</sup>	17 ± 0.00 <sup>F</sup>	0.0 ± 0.00 <sup>H</sup>	12 ± 1.47 <sup>G</sup>	19 ± 1.44 <sup>D</sup>	0.0 ± 0.00 <sup>H</sup>	11 ± 0.99 <sup>G</sup>	11 ± 0.00 <sup>G</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Pen. chrysogenum</i>	25.7 ± 0.73 <sup>C</sup>	0.0 ± 0.00 <sup>H</sup>	17 ± 0.48 <sup>F</sup>	23 ± 0.00 <sup>C</sup>	0.0 ± 0.00 <sup>H</sup>	14 ± 1.44 <sup>E</sup>	26 ± 0.00 <sup>C</sup>	0.0 ± 0.00 <sup>H</sup>	20 ± 0.00 <sup>D</sup>	10 ± 0.00 <sup>G</sup>	0.0 ± 0.00 <sup>H</sup>
<i>Sac. cereviceae</i>	15.3 ± 0.73 <sup>E</sup>	0.0 ± 0.00 <sup>H</sup>	8 ± 0.00 <sup>K</sup>	15 ± 0.00 <sup>E</sup>	0.0 ± 0.00 <sup>H</sup>	8 ± 0.00 <sup>K</sup>	20.7 ± 1.21 <sup>D</sup>	0.0 ± 0.00 <sup>H</sup>	12 ± 0.00 <sup>G</sup>	16 ± 0.00 <sup>E</sup>	0.0 ± 0.00 <sup>H</sup>

Data expressed as Mean ± Standard error; all columns or rows of the different letter are significantly different at P <0.05

**Table 4.** Influence of Nano-formulation on the inhibitory range of mixed-Lactobacilli total extract

	Total extract			C+	C-
	ph	pHG	hG		
<i>B. cereus</i>	56 ± 1.77 <sup>A</sup>	50 ± 0.00 <sup>B</sup>	54 ± 0.00 <sup>A</sup>	14 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>M</sup>
<i>S. aureus</i>	39 ± 0.94	36 ± 0.15 <sup>C</sup>	45 ± 0.75 <sup>D</sup>	15 ± 0.70 <sup>H</sup>	0.0 ± 0.00 <sup>M</sup>
<i>L. monocytogenes</i>	53 ± 1.48 <sup>B</sup>	49 ± 2.45 <sup>B</sup>	54 ± 1.00 <sup>A</sup>	10 ± 0.00 <sup>K</sup>	0.0 ± 0.00 <sup>M</sup>
<i>E. coli</i>	44 ± 0.00 <sup>D</sup>	45 ± 1.00 <sup>D</sup>	49 ± 0.36 <sup>B</sup>	8 ± 0.00 <sup>E</sup>	0.0 ± 0.00 <sup>M</sup>
<i>S. enterica</i>	52.5 ± 0.50 <sup>B</sup>	46.3 ± 1.50 <sup>D</sup>	44.4 ± 2.00 <sup>D</sup>	10 ± 0.00 <sup>K</sup>	0.0 ± 0.00 <sup>M</sup>
<i>Ps. aeruginosa</i>	36 ± 0.00 <sup>C</sup>	37 ± 1.00 <sup>C</sup>	48 ± 0.74 <sup>B</sup>	0 ± 0.00 <sup>M</sup>	0.0 ± 0.00 <sup>M</sup>
<i>Asp. Flavus</i>	14 ± 0.35 <sup>H</sup>	12 ± 0.35 <sup>G</sup>	12 ± 0.65 <sup>G</sup>	11 ± 0.77 <sup>G</sup>	0.0 ± 0.00 <sup>M</sup>
<i>Pen. Chrysogenum</i>	25 ± 0.94 <sup>F</sup>	28 ± 1.00 <sup>F</sup>	29 ± 1.15 <sup>F</sup>	10 ± 0.20 <sup>K</sup>	0.0 ± 0.00 <sup>M</sup>
<i>S. cereviceae</i>	8 ± 0.00 <sup>E</sup>	8 ± 0.14 <sup>E</sup>	12 ± 0.00 <sup>G</sup>	16 ± 0.00 <sup>H</sup>	0.0 ± 0.00 <sup>M</sup>

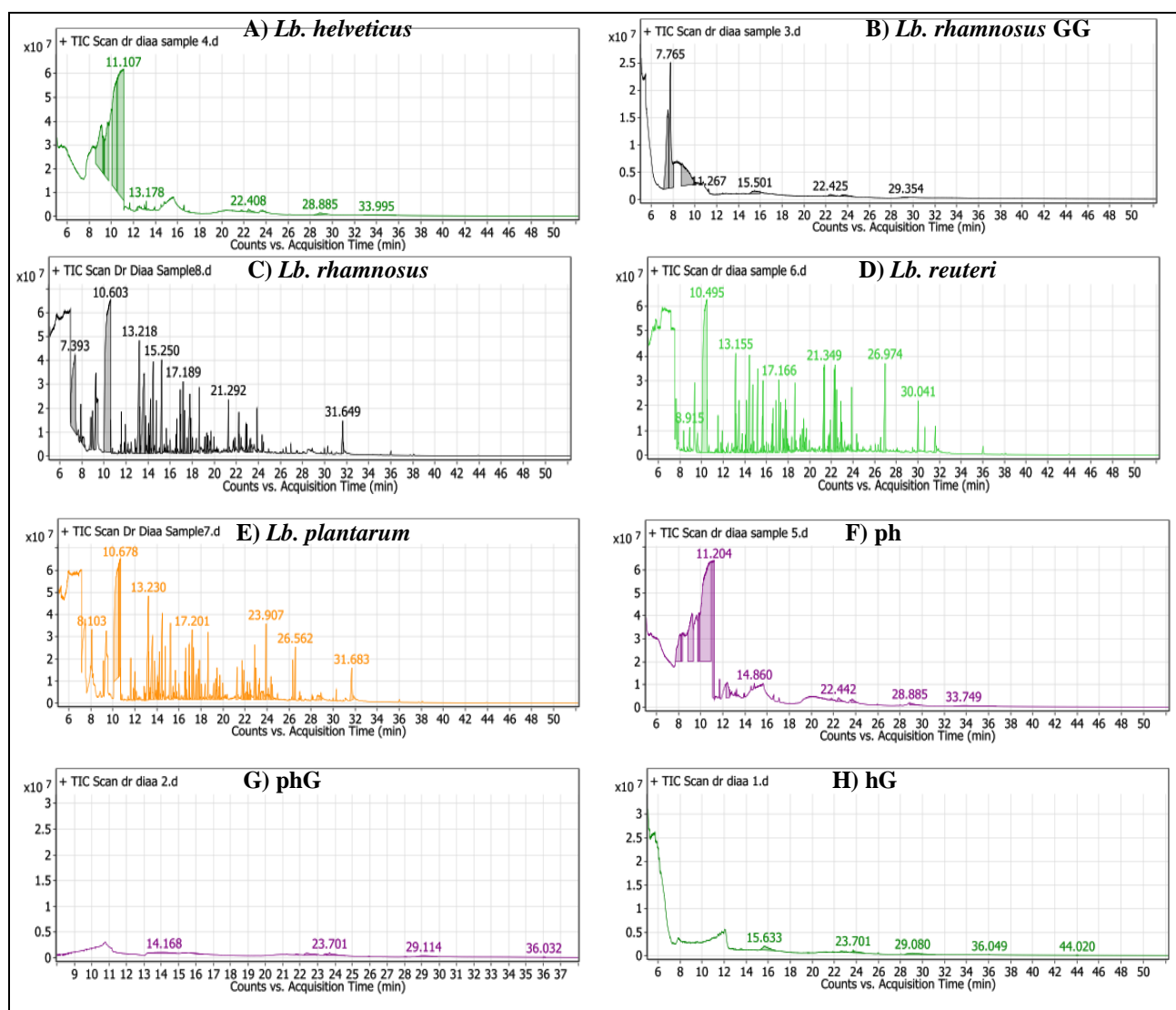
Data expressed as Mean ± Standard error; all columns or rows of the different letters are significantly different at P <0.05.

**Table 5.** CG/MS-identified antimicrobial components of single and mixed-lactobacillus extracts.

Compound	RT	Area %	Application
2-Hydroxyisocaproic acid, derivative	13.23	6.42	Broad spectrum bactericidal, fungicidal (Candida and Aspergillus)
3-Phenyllactic acid, derivative	18.66	2	Broad and effective antibacterial & antifungal activity
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	23.77	1.11	Potential antifungal
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	23.66	1.12	Potential antifungal
3-Phenyllactic acid, derivative	18.65	1.39	Broad and effective antibacterial & antifungal activity
3-Phenyllactic acid, derivative	18.63	1.79	Broad and effective antibacterial & antifungal activity
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	23.69	1.63	Potential antifungal
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	23.70	31.82	Potential antifungal
9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans-	28.03	0.49	Antimicrobial
Lactic acid	15.63	61.49	Wide range of applications

### 3.1.5. Nano-formulation of the mixed-Lactobacilli total extract.

Total extracts of mixed-Lactobacilli fermentates were loaded on chitosan nanoparticles and undergone an evaluation of inhibitory activity through disc diffusion. Results presented in Table 4 show the influence of applying mixed-Lactobacilli total extracts in Nano form. Other than *Asp. flavus* and *Sac. cereviceae*, there is significant progress in the inhibitory potential with all pathogens and food spoiler microbes. The activity against gram-positive bacteria upturns by 15 - 30%; 17 - 20%; and 15 - 39% in case of ph; pHG, and with hG - dependent combinations, respectively. Gram-negative bacteria are excessively inhibited as 9 - 32%, 19 - 32%, and 7 - 13% by ph; pHG, and with hG - dependent combinations, respectively. The highest increment is measured in the case of *Pen. Chrysogenum* was extra inhibited by 47%, 100%, and 45% by ph; pHG, and with hG - dependent combinations, respectively.



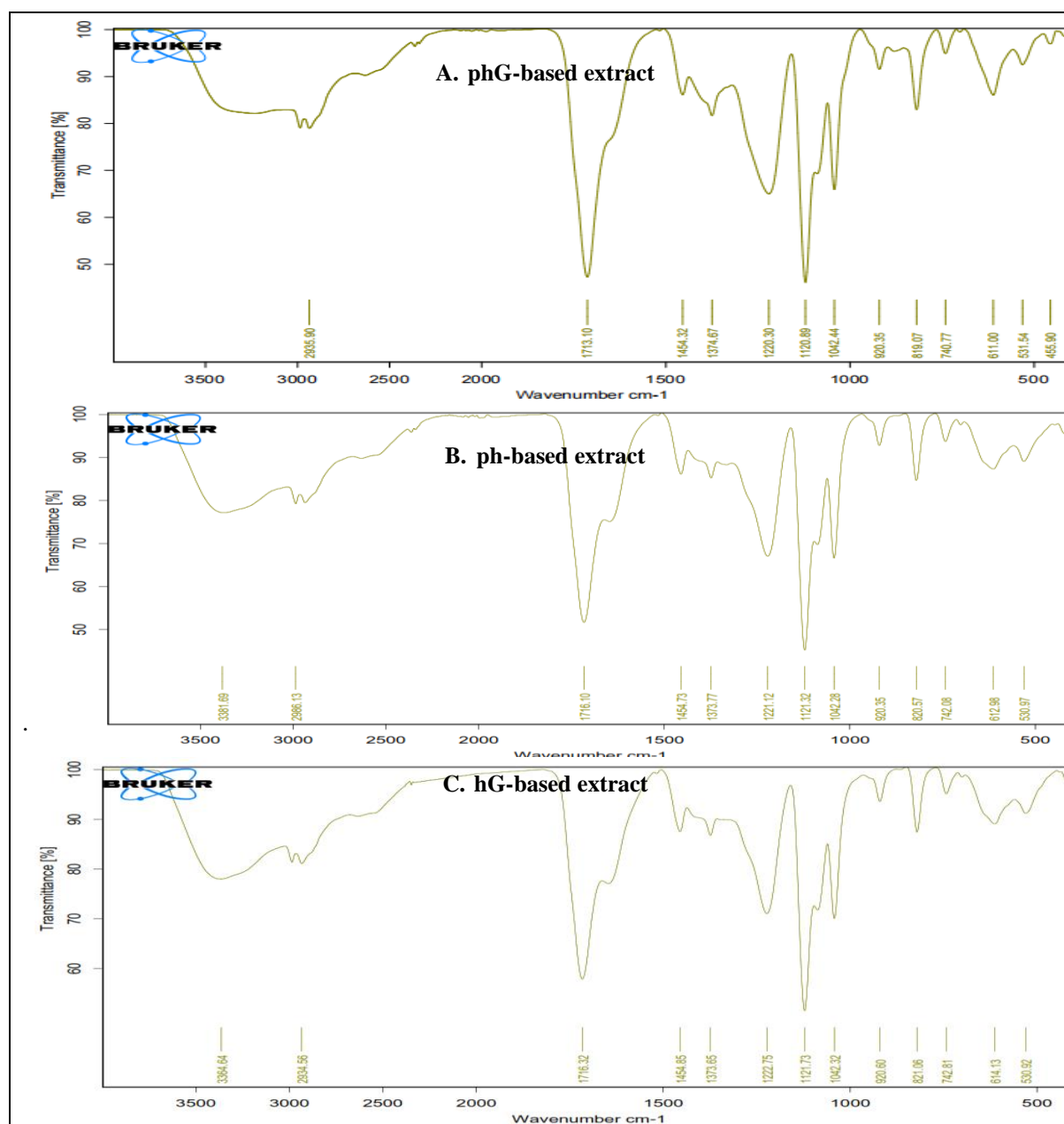
**Figure 2.** GC/MS chromatograms of different Lactobacilli extracts.

### 3.2. Instrumental characterization of bioactive extracts.

In order to understand the mechanism of antimicrobial action of all obtained active extracts, a set of instrumental investigations were performed to inspect the main components that cause the biological activity of each extract.

### 3.2.1. GC/MS analysis of diethyl ether extract.

Volatile profile was inspected to explain or understand the effect of each extract against different microbial concerns and resulted in data are supplemented in Table 5 and Figure 2.



**Figure 3.** FT-IR spectra of Mixed-Lactobacilli methanol extracts.

Results show that each single or mixed-Lactobacilli DEE extract contains at least a single active compound, with a potential antimicrobial application, besides lactic acid as the main metabolite. In detail, extract of *Lb. plantarum* includes 2-Hydroxyisocaproic acid, derivative, and 3-Phenyllactic acid, a derivative that is also produced by both of *Lb. rhamnosus* and *Lb. reuteri*. Also, Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) - is contained within extracts of *Lb. helveticus*, *Lb. rhamnosus*, ph, and phG-based combinations. A 9-Octadecenoic acid (2-phenyl-1, 3-dioxolan-4-yl) methyl ester trans- was also extracted from phG-based mixture fermentate, while hG-dependent extract only contains Lactic acid.

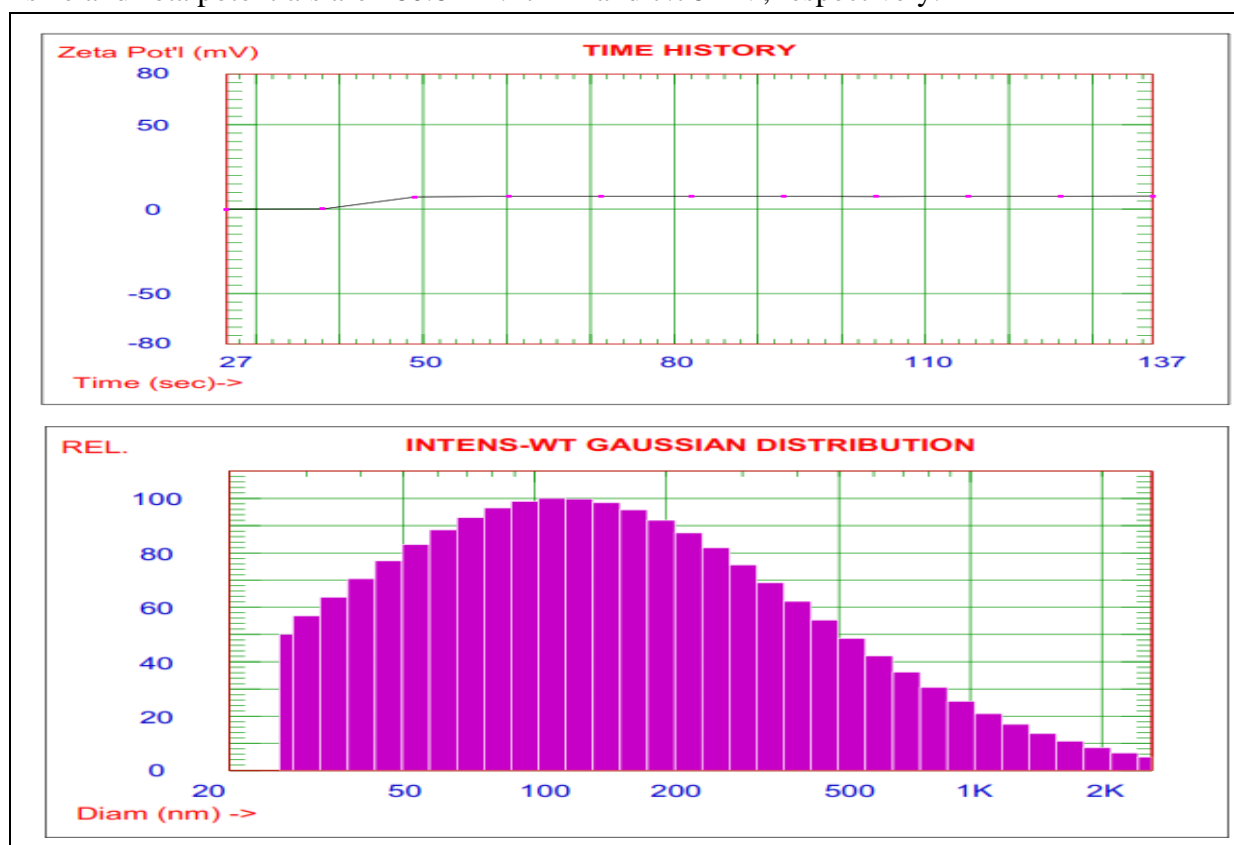
### 3.2.2. FT-IR spectroscopic analysis of methanol extract.

Methanol extract of mixed-Lactobacilli fermentations was tested by Fourier transform infrared spectroscopic analysis to predict functional groups of the bioactive components contained within each, and spectra were shown in Figure 3.

Fourier Transform Infra-Red was applied to detect vibration and functional groups contained in methanol extract. The obtained bands at wavenumbers  $1650\text{ cm}^{-1}$ ,  $1520\text{ cm}^{-1}$ , and  $1310\text{ cm}^{-1}$ , respectively, can be attributed to the presence of amide I, amide II, and amide III of protein while at wavenumber  $1454\text{ cm}^{-1}$  there is asymmetric bending of methyl or methylene group of protein. The bands at the area of  $1454\text{ cm}^{-1} - 1221\text{ cm}^{-1}$  confirmed the existence of the carboxyl group of protein. The presented results of both Table 1 and Figure 3 revealed that the methanol extract seems to bacteriocin-like compound.

### 3.2.3. Zeta potential and particle size distribution of prepared Nanoparticles.

Total extracts of mixed-Lactobacilli fermentation were characterized via zeta potential and particle size distribution facilities. Results are shown in Figure 4. As estimated, the average size and zeta potentials are  $260.8 \pm 1.27\text{ nm}$  and  $7.76\text{ mV}$ , respectively.



**Figure 4.** Particle size distribution and zeta potential of pHG-based total extract.

### 3.3. Discussion.

The current study is in the way to improve tools of food preservation that based on nature, utilizing five Lactobacillus strains. Different systems extracted fermented Cell-free supernatants, and the obtained extracts were evaluated and characterized.

The results presented in Table 1 summarize the antimicrobial effects of methanol extracted acetone precipitate through disc diffusion. The negative response of fungal populations is that the tested extract (methanol extract of cold acetone precipitate) is strictly

antibacterial and has no activity against molds and yeasts. From FT-IR spectra that are involved in Figure 3, the obtained bands at wavenumbers  $1650\text{ cm}^{-1}$ ,  $1520\text{ cm}^{-1}$ , and  $1310\text{ cm}^{-1}$ , respectively, can be attributed to the presence of amide I, amide II, and amide III of protein, while at wavenumber  $1454\text{ cm}^{-1}$  there is asymmetric bending of methyl or methylene group of protein. The bands at the area of  $1454\text{ cm}^{-1}$ – $1221\text{ cm}^{-1}$  confirmed the existence of the carboxyl group of protein [34]. The presented results of both Table 1 and Figure 3 revealed that the methanol extract seems to be bacteriocin-like compound.

From methanol extract to Diethyl ether extract, activity was increased, as well, range of inhibited microbes was extended (Figure 1). As listed in Table (5), this enhancement may be due to the inclusion of at least one antimicrobial compound other than lactic acid. In detail, both *Lactobacillus helveticus* and *Lactobacillus rhamnosus* GG with their based-combinations possess the production of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- which is known as a strong antifungal agent [35]. The observed effect was reported by Rocha-Ramírez *et al.*, [36].

In addition, 3-Phenyllactic acid and derivatives were detected within extracts of *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus reuteri*. This compound was applied as a high potential antibacterial and antifungal compound [37].

The considerable potential of *Lactobacillus plantarum* as both antibacterial and antifungal can be attributed to the co-production of 2-Hydroxyisocaproic acid and derivatives that are considered a great bactericidal and fungicidal compound [38]. The results in this study come agreed by Arena *et al.*, [39]; they used various strains of *Lb. plantarum* to control serious food pathogens like *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* O157:H7, and *Staphylococcus aureus*. Also, *Lb. plantarum* is commonly used to inhibit fungal growth, especially that invade cereals-based products. The results obtained by Arena *et al.* [40] reported the promising applications of *Lb. plantarum* is a probiotic strain to be used in different fields.

Moreover, 2-Hydroxyisocaproic acid and 9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans- contained within ph and pHG-based *Lactobacillus* combinations may be responsible for their increased antimicrobial activity [41]. Nikolova *et al.*, [42] investigated the inhibitory potential of *Lactobacillus helveticus* strain 50 p1, and results reflected a strong effect against clinical isolates like *Pseudomonas aeruginosa* and *Bacillus sp.* Moreover, there is an agreement with the results of Gomez *et al.*, [43]. They used a biofilm of mixed-lactic acid bacteria to retard the growth of different food-originated human pathogens, such as; *Salmonella typhimurium* and *E. coli* O157:H7.

Recent progress in nanotechnology with applicable uses in the food sector, which is rather recent compared with their use in biomedical and pharmaceutical applications. Nano-formulated materials have applications in various food science sectors, including new packaging materials and encapsulated food components [44, 45]. Seeing the application of chitosan in dairy-based foods, Santonicola, *et al.*, [46] used Natamycin-loaded Nano chitosan to pack cheese samples. They reported a significant reduction of mold and yeast content of Nano-treated samples than that of free-state Natamycin. Also, [47, 48] studied the activity of bioactive compound-loaded NPs against some pathogenic bacteria. El- Sayed *et al.* [49] reported that Nano-encapsulation of bioactive material has strong antimicrobial activity and can extend cheese shelf life during the storage period in all treatments. As recorded in Table (4), the application of mixed-Lactobacilli as Nano-formulated stimulates the increase in their antimicrobial activity by 39%, 32%, and 100% over their crude state in the case of Gram-

positive, gram-negative, and *Penicillium chrysogenum*, respectively. The main cause of inhibition enhancement is thought to be the damage of cell membranes after direct contact between Nanoparticles and bacterial membranes [50]. Nanoparticles were reported to show a biocidal effect that covered both gram-positive and gram-negative bacteria [51]. Finally, as seen in the current study, several studies recommended the green production of antimicrobial nanoparticles to protect the environment [52].

The Lactobacillus extracts, particularly the mixed-based, are considered a good tool for controlling or managing pathogenic and food spoilage microorganisms. The inhibitory effect can be magnified if used as Nano-formulated for food preservation and safety maintenance for both food and consumers. This study will help many researchers do further studies to develop the food bio-preservation sector.

#### 4. Conclusions

It can be concluded that the current findings recommend the usefulness of the Mixed-based Lactobacillus extract that depends on *Lb. helveticus*, *Lb. plantarum* and *Lb. rhamnosus* GG to inhibit the growth of pathogenic food spoilage bacteria and molds. Additional simulation studies need to validate the effect of this formulation to extend the shelf life of food products. Food preservation by natural methods is an appropriate way to solve economic losses caused by microbial spoilage and reduce foodborne poisoning, including infections and intoxications.

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#### Conflicts of Interest

The authors declare no conflict of interest.

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