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The potential of Fermented *Asparagus sprengeri* extract by *Lactobacillus plantarum* DMS 20174 on Antioxidant Properties and Memory Retention in *vitro* and in *vivo*.

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Abstract

Asparagus sp. are a superior gift from nature to promote a disease-free, healthy lifestyle. Through Gut-Brain Axis contact, Lactobacillus strains perform a therapeutic role in neurodegenerative illnesses, particularly Alzheimer's disease (AD). This study was aimed to determine the effect of asparagus extract on *Lactobacillus plantarum* DMS 20174 (*L. plantarum*) resistance to acid, bile salt, gastric and intestinal environments. Also, the impact of fermented asparagus extract on antimicrobial, antioxidant and anti-cholinesterase activities was evaluated. Furthermore, the study aimed to investigate the dietary treatment effect of fermented Asparagus extract on anti-Alzheimer and microbiological properties in high fat diet (HFD) fed albino rats. *L. plantarum* cells gained some resistance towards harsh gastric and intestinal conditions with asparagus extract. Various short chain fatty acids (SCFA) were detected by HPLC in cell free extract (CFE) of the fermented extract. Its antimicrobial activity, scavenging of DPPH and anti-cholinesterase activity were improved. Treatment of high fat diet (HFD) groups with *L. plantarum* and asparagus extract decreased acetyl cholinesterase (AChE) and malondialdehyde (MDA). Furthermore, treatment with DMS 20174, asparagus extract, and the combined mixture had greater counts of Lactobacilli and lower counts of total aerobes, coliform bacteria, staphylococci, and fungi with HF-fed mice. In conclusions: fermented asparagus extract may be useful in reducing risk of neurodegenerative diseases.

Keywords: Fermentation; *Asparagus sprengeri*; *Lactobacillus plantarum*; Alzheimer; antioxidants; anticholinesterase activity.

1. Introduction

Excessive generation of reactive oxygen species (ROS) within the brain has been linked to late-onset neurodegenerative illnesses, including AD (Anwar, 2022). Alzheimer's disease is the leading cause of dementia and one of the most common neurodegenerative conditions. There is a lot of evidence indicating dietary risk factors in AD and cognitive impairment as people get older. As a result, diet and nutrition are critical in reducing AD risk (Sarkar & Chegu Krishnamurthi, 2022). Phytochemical ingredients obtained from medicinal plants provide a huge pool of varied substances that can be used to develop new medications (Khoba et al., 2022). In view of this, the fermented asparagus extract was employed as a natural antioxidant to have beneficial effects in the reduction of AD (anti-cholinesterase activity).

Asparagus is a herbaceous plant with a wide range of bioactivities that has been used as medicine and food since antiquity. Asparagus includes phytochemical components such as polysaccharides, anthocyanins, polyphenols, and saponins, which have been shown *in vitro* and *in vivo* to have anticancer, antioxidant, antitumor, hypoglycemic, anti-hypertensive, immunomodulatory, and anti-epileptic properties (Guo et al., 2020). In Egypt, the asparagus genus represented by *A. sprengeri* is not widely grown (Hassan et al., 2014).

Enhancing *A. sprengeri's* biogenic activity may rely on standardized and marketable products with well-known or innovative applications. For this

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purpose, bioprocessing of plant materials by microbial fermentation has piqued attention due to its superior ability to exploit natural bioactivities compared to industrial enzymatic procedures. The effect of *L. plantarum* fermentation on the health-promoting properties of asparagus fructan has only been studied in a few research (Sakr, 2022). Furthermore, there have been numerous research on asparagus plants (Jiménez-Sánchez et al., 2016), however, there is relatively little knowledge about the biological activity of *A. sprengeri* metabolites produced during fermentation.

Lactic acid bacteria (LAB) fermented asparagus is gaining popularity as a novel asparagus-related product due to its high nutritional content and physiologically active components (Tabaszewska et al., 2018; Zhang et al., 2018). In comparison to non-fermented asparagus fructan, fructan obtained from fermented asparagus (ASF) showed greater pathogenic inhibitory, antioxidant, fibrinolytic, anticoagulant, anticancer, and anti-obesity effects (Sakr, 2022).

Diets high in fat and (or) fructose contribute prominently to impaired cognition. Simple sugars and saturated fats are major components of the diet that promote obesity and insulin resistance. The effects of such diets on the brain are poorly understood. Addition of fructose to the water of rats fed a high-fat, high-glucose diet led to impaired hippocampal synaptic plasticity and cognition in middle-aged rats (Stranahan et al., 2008). In this work, the effects of asparagus extract fermentation with *L. plantarum* as a supplementary nutrient for Alzheimer's disease treatment were assessed *in vitro* and *in vivo*.

Because of its vast variety of health-promoting benefits, *L. plantarum* has recently gained more scientific attention. In this study, the goal was to clarify the functional food properties of fermented *A. sprengeri* extract, the *in vitro* antimicrobial, antioxidant and anti-cholinesterase features were determined compared with control deMan, Rogosa, and Sharpe media (MRS). In addition, the stress tolerance of *L. plantarum* DMS 20174 was tested (antibiotics, pH, bile salt, and mimic gastrointestinal tract). Furthermore, this study was aimed to evaluate the anti-Alzheimer activities of asparagus extract and *L. plantarum* DMS 20174 against high fat-induced Alzheimer's disease in albino rats.

1. Material and methods

2.1. Collection of *A. sprengeri* tuber

Fresh *A. sprengeri* tubers were collected from Egyptian plant nursery during harvest. The harvested tubers were cleaned, washed with tap water to remove remaining soil, manually peeled then cut into little pieces, and the whole pulp was homogenized using a food processor. The homogenized plant was filtered, and the filtrate was dehydrated in a cabinet drier for 24 hr. The dried samples were ground in electrical mill to pass through 60 mesh sieve before used (Sakr, 2022).

2.2. Bacterial strain and culture conditions.

Pure culture of *L. plantarum* DMS 20174 was purchased from Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain-shams University, Egypt. Strains were propagated in de Man, Rogosa, and Sharpe (MRS) broth (Biolife, Italy) and sub-cultured at twice time by 1% (v/v) inoculum in MRS broth at 37 °C for 24 hr before inoculation.

2.3. Impact of asparagus extract on the characteristics of *L. plantarum* DMS 20174 as probiotic bacteria *in vitro*.

2.3.1. Antibiotic resistance

The antibiotic susceptibility of *L. plantarum* DMS 20174 was determined using the antibiotic disc diffusion method. MRS (without asparagus) and asparagus extract-based MRS agar plates were then smeared with 100 µL of freshly made MRS broth medium and allowed to dry. Antibiotic discs were placed on the seeded plates and incubated at 37ºC for 24 hr. The diameter of the inhibition zone along each disc was measured. The results were divided into three categories: susceptible (S), intermediate (I), and resistant (R). The test was carried out in accordance with the Clinical and Laboratory Standards Institute's guidelines (CLSI, 2017).

2.3.2. Acid and bile salt tolerance properties

The *L. plantarum* DMS 20174 was grown in sterile MRS broth at 37°C for 24 hr, and centrifuged at 6000 rpm for 10 min at 4ºC. For acid challenge investigations in the centrifuged samples, the cell pellet was diluted in sterile MRS (without asparagus) and asparagus extract-based media with pH levels of 6.5, 5.0, 4.0, 3.0, and 2.0. Bile salt tolerance was tested in a medium containing 0, 0.15, 0.30, and 0.45 % bile salts and incubated at 37°C. After an acid challenge and a bile salt tolerance test, the number of viable cells was counted at 0, 3, 6, and 12 hr (Huang et al., 2015).

2.3.3. Resistance to gastric and intestinal juice

The harvested cells of *L. plantarum* were washed in sterile 0.1 % NaCl solution and adjusted to 10^8 CFU/mL. For 0, 2, and 4 hr, the cells were suspended in gastric juice (GJ) containing sterile 0.1 % NaCl solution (pH adjusted to 2.5) containing 3 mg/mL pepsin and in intestinal juice (IJ) containing

sterile 0.1 % NaCl solution (pH adjusted to 8) containing 0.3 % bile salt and 1 mg/mL pancreatin. Furthermore, the bacterial cells and asparagus extract were added to the same previous media. The cells were plated in MRS agar and colony forming units were determined after incubation period at 37 ºC. As a control, cells were suspended in 0.1 % saline at pH 7 (Osmanagaoglu et al., 2010).

2.4. Fermentation of *A. sprengeri* by *L. plantarum* DMS 20174

Five gram of asparagus extract was added to flask containing 2 g of peptone to prepare one hundred of the required fermentation medium (Campanella et al., 2017). The pH value of the medium was adjusted to 6 ± 0.5 using 1N NaOH before autoclaving at 121 °C for 15 min then cooled to ambient temperature and kept at 4°C for up to 24 hr. The sterilized fermentation medium and MRS medium (without asparagus extract, control) was incubated with $1x10^8$ CFU/mL of activated *L. plantarum* DMS 20174 at 37°C for 48 hr. After fermentation, samples were taken to perform the viable cell counts (log CFU/mL) onto MRS agar. Also, the pH was measured using a pH meter (pH meter; Beckman, USA).

2.4.1. Preparation of cell free extract (CFE)

The CFE of MRS and asparagus extract-based medium inoculated previously with *L. plantarum* DMS 20174 was obtained by centrifugation at 6000 rpm for 10 min at 4ºC. The syringe filter (0.45 µm filter, CHMLAP Group) was used to sterilize CFE. Different experiments were performed on bacterial CFE.

2.4.2. SCFA analysis using HPLC

10 µL of CFE was injected in high performance liquid chromatography (HPLC, Agilent 1260 series) (Bozkurt & Gürbüz, 2008). An Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm) was used with the mobile phase consisted from of 10 mM potassium dihydrogen phosphate at pH 2.4 (A) and acetonitrile (B) at flow rate 1 ml/min. The mobile phase was designed in a linear gradient as follows: 80% A (0 min), 66.7 % A (0–1 0 min), and 80% A (10-12.6 min), SCFA were monitored at 210 nm, the multi-wavelength detector. Lactic, acetic, and butyric acid calibration curves were used to determine the amount of organic acids in the CFE.

2.4.3. Bioactivities of the fermented asparagus extract

2.4.3.1. Antimicrobial activity

The antibacterial potential of CFE against pathogenic microorganisms was tested using the agar well diffusion method (Bagewadi et al., 2019). *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 98082, and *Bacillus pumilis* ATCC 14884 were provided from the American Type Culture Collection (ATCC, USA). *Staphylococcus aureus* 91161, *Listeria monocytogenus*, and *Salmonella typhimurium* were provided by BFEL, Germany. The studied microorganism's suspension (10^6 CFU/mL) was planted on nutrient agar plate (Sigma-Aldrich, Spain). A sterile cork borer with a diameter of 7 mm was used to cut uniform wells in the dried agar plate. Each well was then filled with 50 µL of the prepared CFE. The inhibitory efficiency of fermented asparagus extract was exhibited on the basis of the clear zone diameter and compared to the probiotic CFE clear zone after incubation at 37ºC for 24 hr.

Using serial dilution procedures in 96-well plates, the MIC of the CFE of fermented asparagus extract and *L. plantarum* DMS 20174 was measured (Tarrah et al., 2019). At 10^6 CFU/well, pathogens were introduced to each well. Then, using MRS broth, the previously prepared CFE was serially diluted by half, and the diluted samples were placed into plate wells at final concentrations ranging from 3.13 to 50%. The MIC was established to be the lowest concentration with no observable growth after incubation at 37 ºC for 24 hr.

2.4.3.2. Antioxidant activity

The DPPH radical scavenging assay was used to determine the scavenging radicals of CFE (Xing et al., 2015). One mL of each prepared CFE was mixed thoroughly with 1.0 mL of methanolic DPPH radical solution (0.0034 g/L; Sigma, Germany) and incubated for 30 min in the dark (room temperature). As a reference standard, Methanol was employed. DPPH (%) = $(A_{blank} - A_{sample}) / A_{blank}$ X 100% was determined by measuring the absorbance at 517 nm. The absorbance of ascorbic acid was also determined.

2.4.3.3. Anti-cholinesterase activity

Ellman's microplate assay was used to assess the inhibitory activity of the investigated samples against acetyl cholinesterase. In a 96-well microplate, 140 µL of 0.1 M sodium phosphate buffer (pH 8) was first introduced to each well, followed by 20 µL of samples (CFE) and 20 µL of 0.09 unit/mL AChE. 10 µL of 10 mM DTNB was injected to each well after a pre-incubation at room temperature, followed by 10 µL of 14 mM acetylthiocholine iodide as the substrate. After 30 min of enzymatic reaction beginning, the absorbance of the colored end product was measured at 412 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT). As a reference standard, donepezil was used. By subtracting the absorbance of their corresponding blank, the absorbencies of the test samples were adjusted. The 50 % inhibitory concentration was calculated using a set of eight concentrations (IC_{50}). The following formula was used to estimate the percentage inhibition: Percentage inhibition = absorbance of control − absorbance of CFE / absorbance of control × 100% (Obregon et al., 2005).

2.5. *In vivo* protective potential of asparagus extract and *L. plantarum* supplementation against high fat-induced Alzheimer disease.

2.5.1. Preparation of *L. plantarum* culture

L. plantarum had the ability to produce acetylcholine neurotransmitter (Stephenson et al., 1947). Preparation and treatment of *L. plantarum* was done according to the previously reported studies (12×10^8 CFU/mL; 10 mL /kg body weight) (Peng et al., 2014).

2.5.2. Animals and experimental design

Forty-eight male Sprague-Dawley albino rats (120-150 g) were obtained from Helwan farm of Serum and Vaccine, Cairo, Egypt. The animals were housed individually in metallic cages and they were kept under controlled room temperature with a fixed12:12-h artificial light/dark period and a suitable humidity of 45–60%. Water and basal diet were received ad-libitum for one week as an adaptation period basing on AIN-93 recommendations (Reeves et al., 1993).

Following one week of acclimatization, rats were randomly divided into two groups (n = 24 rats / group) and fed either a basal diet (BD) or HFD containing 15% tallow + 7.5% corn oil + 1% cholesterol for 20 weeks. On the week 13th, both of BD or HFD groups fed rats were subdivided into four groups and treated with 1mL saline (negative control), asparagus extract, *L. plantarum* (10 ml/kg body weight; 12×10^8 CFU/mL) or mixture of both, respectively.

At the end of the experimental period, all rats were fasted overnight; blood sample was collected from the abdominal femoral arteries of the rats, and centrifuged at 3000 rpm for 20 min to obtain the serum, which was kept frozen at −80 °C until further analysis. After decapitation, caecum and cerebellum samples were taken at the time of sacrifice. Cerebellum tissue was immediately excised and homogenized using a homogenizer surrounded with an ice jacket and with 10% potassium chloride in a dilution 1:10 of tissue homogenate, followed by centrifugation in cooling centrifuge at 4°C for 20 min at 5000 r.p.m. The homogenates were used for the determination of AChE and MDA.

2.5.2.1. Assay for serum lipids

Serum TC, HDL-C and TAG concentrations were determined enzymatically according to the colorimetric method (Allain et al., 1974; Burstein et al., 1970; Fossati & Prencipe, 1982), respectively, using enzymatic colorimetric Stanbio kits. Serum LDL-C value was calculated according to the equation [LDL-C=TC-(HDL-C + TAG/5)] (Fridewald, 1972).

2.5.2.2. Determination of tissue MDA (nmol/g tissue) by HPLC

For determination of MDA levels; the samples were analyzed on an Agilent HP 1200 series HPLC apparatus (USA) as described above. The analytical column was Supelcosil C18 (5 µm particle and 80 A^o pore size) (250 x 4.6 ID). The mobile phase was 82.5:17.5 (v/v) 30 mM monobasic potassium phosphate (pH 3.6) methanol and the flow rate was 1.2 ml/min, wavelength 250 nm was applied for detection. MDA standard was prepared by dissolving 25 µL 1,1,3,3 tetraethoxypropane (TEP) in 100 mL of water to give a 1 mM stock solution. Working standard was prepared by hydrolysis of 1 mL TEP stock solution in 50 mL sulfuric acid (1%) and incubation for 2 hr at room temperature. The resulting MDA standard of 20 nmol/ml was further diluted with 1% sulfuric acid to yield the final concentration of 1.25 nmol/ml to get the standard for the estimation of total MDA (Karatepe, 2004).

2.5.2.3. Determination of Acetylcholinesterase (AChE) activity in brain tissue homogenate

The procedure used for the determination of acetylcholinesterase activity in the brain hippocampus samples of rats is a modification of Ellman et al., (1961) method as described by Gorun et al., (1978). AChE activity was determined after extraction using following protocol: The brain hippocampus tissue samples were weighed and homogenized in a 20-mmol-phosphate buffer (pH 7.6). 0.01 mL of tissue homogenate or serum were added to 0.14 mL phosphate buffer 20 mmol (pH 7.6), and 0.05 mL of 5-mmol-acetylthiocholine iodide. After 10 min of incubation at 38 °C, the reaction was stopped with 1.8 mL of DTNB – phosphate and measured at 412 nm using Shimadzu spectrophotometer UV –1601. Omitting the enzyme from the incubation mixture made the control samples. After addition of the color reagent, appropriate amount of tissue homogenates was added to the control. The cholinesterase activity was determined as µmol SH from a standard curve.

2.5.2.4. Microbiological analysis

The faeces samples were obtained from the intestinal section of the caecum and 0.1 g of the cecal content was weighed, transferred into a tube with 0.9 mL of peptone water diluent, and homogenized by vortexing. To determine the number of distinct taxonomic categories of microorganisms, subsequent 10-fold serial dilutions (from 10^{-2} to 10^{-8}) of each sample were plated in triplicates in selective culture medium. MRS agar was used for Lactobacillus sp.., nutrient agar medium was used for total aerobes, sabouraud agar was used for microscopic fungi, macConkey agar was used for coliform bacteria, and mannitol salt agar was used for staphylococci. All plates were incubated at 37°C for 48 hr for counting

except plates of fungi were incubated at 30°C for 5 days. The viable counts were calculated using the log 10 of colony-forming units (CFU/g) of faeces.

2.6. Statistical analysis

The data were interpreted as the mean ± (standard deviation). One-way analysis of variance was used to conduct statistical analyses using the protocol of the SPSS 16 statistical package (SPSS Inc., Chicago, IL, USA). $P \leq 0.05$ was used to assess if differences were significant and the Duncan test was used to determine significance.

2. Results and discussion

3.1. Impact of asparagus extract on the characteristics of *L. plantarum* DMS 20174 as probiotic bacteria *in vitro.*

3.1.1. Antibiotic susceptibility

L. plantarum DMS 20174 demonstrated phenotypic resistance to tetracycline, ampicillin, streptomycin, and gentamicin among the tested antibiotics (**Table 1**), but was sensitive to chloramphenicol and tigecycline. Except for oxytetracycline, adding asparagus extract to the medium showed no influence on the sensitivity of *L. plantarum* DMS 20174 to these antibiotics. Antibiotic resistance in Lactobacilli is a double-edged sword today. Antibiotic resistance in some probiotics can be beneficial for patients who have an unbalanced gut microbiota as a result of antibiotic use, but it can also serve as a breeding ground for resistance genes to spread to other bacteria (Imperial & Ibana, 2016). Lactobacilli species were also highly susceptible to chloramphenicol and tetracycline (Wang et al., 2010). This finding is also in line with a previous study that found the chloramphenicol resistance gene in the majority of lactobacillus strains that were susceptible to the antibiotic (Campedelli et al., 2019). *L. plantarum* was previously found to be resistant to aminoglycoside antibiotics (gentamicin, streptomycin, and kanamycin) as well as ciprofloxacin (Yang et al., 2019). Lactobacillus sp. has also been found to be resistant to aminoglycosides, glycopeptides, and quinolone antibiotics (Charteris et al., 2001). Gentamicin resistance is thought to be inherent, and it has been investigated extensively in lactobacilli (Campedelli et al., 2019).

3.1.2. Tolerance of *L. plantarum* DMS 20174 for low pH and bile salt condition

Probiotics' tolerance for acidic pH and bile salts are significant traits (de Albuquerque et al., 2018). **Figure 1** shows the effect of a low pH environment on *L. plantarum* DMS 20174. This strain was resistant at pH 2.0 and 3.0 in both treatments (with and without asparagus extract), with loss in viable cells. The viable cells of *L. plantarum* cultivated with and without asparagus extract decreased by 4.09 and 4.38 Log CFU/mL at pH 2.0, respectively, whereas, the viable cells decreased by 3.47 and 3.86 Log CFU/mL at pH 3.0, respectively. This was in a line with a previous report (Mahboubi & Kazempour, 2016). The log CFU/mL of *L. plantarum* DMS 20174 increased with time at pH 4.0, 5.0, and 6.5. In accordance with our study, Wang et al. (2017), found that increasing pH boosted *L. plantarum* cell viability. When asparagus extract was added to probiotic bacteria growing medium, the log CFU/mL of *L. plantarum* DMS 20174 increased from 6.19 to 7.69 (**Fig. 1a**). These findings support prior research that showed potentially probiotic bacteria were able to maintain viability when exposed to pH ranges of 2.0 to 4.0 (Argyri et al., 2013; Plessas et al., 2017).

The findings of the bile salts tolerance test revealed that *L. plantarum* DMS 20174 was resistant to bile salts at concentrations of 0.15 – 0.45% (w/v) and that there were differences in log CFU/mL of probiotic bacteria in the presence of bile salts compared to the control group **(Fig. 1b)**. The viable cell numbers dropped as the bile salt concentration was increased. The results of our study was in agreement to various investigations. For example, Wang et al. (2017), reported that an increase in bile concentration from 0.1 % to 0.5 % resulted in a significant drop in *L. plantarum* B1. When the bile concentration was increased from 0.5 % to 1 %, the viability of the *L. plantarum* DSM2648 was lowered by 2 log units (Anderson et al., 2010). In comparison to media without asparagus extract, adding the asparagus extract to the bile salts-containing media enhanced the log CFU/mL of *L. plantarum* DMS 20174 throughout time. The log CFU/mL of *L. plantarum* was increased from 8.61 to 9.58 at 0.15% bile salt after 12 hr. The results of this investigation corroborate the findings of several other studies, which found that *L. plantarum* could live in a wide range of bile concentrations (0.05–4.0) over periods ranging from 3 to 24 hr (Balasingham et al., 2017; Giri et al., 2018).

3.1.3. Survival to simulation on gastrointestinal tract (GIT) transit

The major stress that could reduce the survival of ingested probiotics is exposure to stomach and intestinal fluids along the digestive tract (Liong & Shah, 2005). As a result, resistance of *L. plantarum* was studied using a protocol that mimicked stomach and intestinal conditions in the presence and absence of asparagus extract. The cell count reached to about 70.3 and 73.95% in the absence and presence of asparagus extract after 4 hr of exposure to artificial gastric juice with pepsin, demonstrating that the organism remains resistant to gastric juice **(Fig. 2)**.

In the presence of bile and pancreatin, the viable cell counts of *L. plantarum* were 74.6 and 80.34 % in the presence and absence of asparagus extract when compared with control, respectively

(Fig. 2). *L. plantarum* will reach the colon after passing through the GIT tract and exhibit their probiotic effects, as established by the previous studies (Ding et al., 2017; Ghatani & Tamang, 2017). As a result, LAB must withstand the presence of stomach and small intestine digestive enzymes in order to proliferate, adhere to the GI tract, and provide effective probiotic function (Argyri et al., 2013; Plessas et al., 2017).

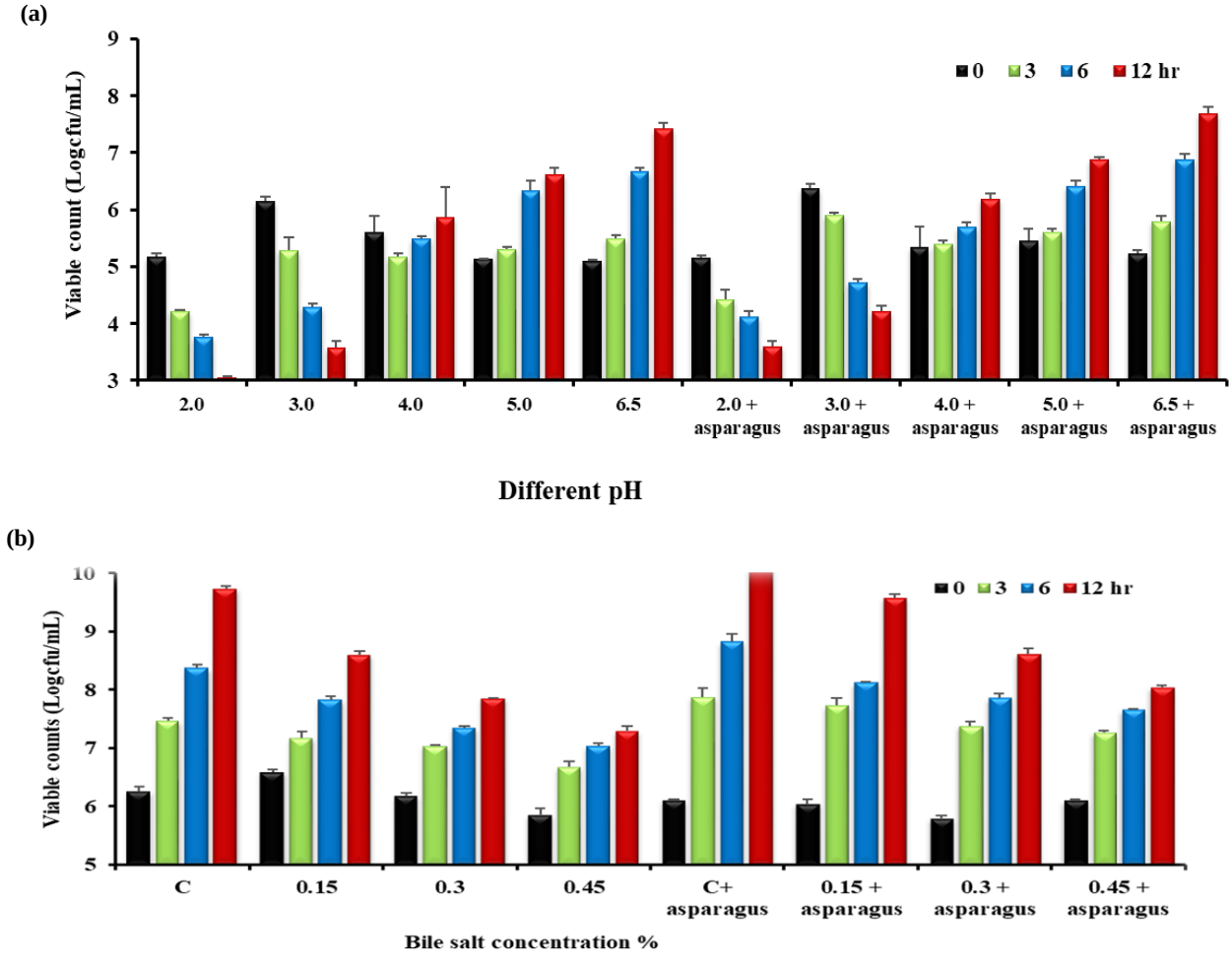

Fig. 1. Survival of *L. plantarum* DMS 20174 in MRS (without asparagus) and asparagus extract-based media at various pH values (a), varying bile salt concentrations (b).

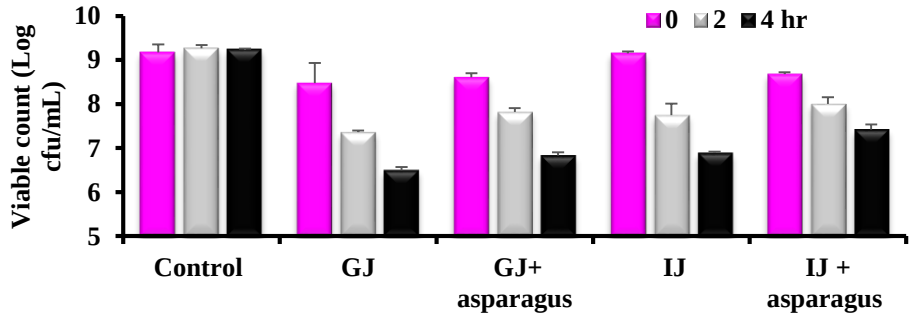

Fig. 2. Survival of cell counts of *L. plantarum* DMS 20174 in transit under the simulated gastrointestinal tract with and without asparagus extract. GJ = gastric juice; and IJ = intestinal juice.

Table 1 : Antibiotic susceptibility of *L. plantarum* DMS 20174 to antibiotics using the disc diffusion method.

Antibiotics used	Abbreviation	Conc.	Diameter of inhibition zone (mm)	
			MRS media without asparagus extract	MRS media with asparagus extract
Tetracycline	TE	30	15 (R)	15(R)
Oxytetracycline	OT	30	20 (I)	21(S)
Ceftriaxone	CRO	30	15 (R)	16 (I)
Trimethprime / Sulphamethoxazole	SXT	25	20 (I)	20 (I)
Cefobid	CFP	30	17 (I)	18 (I)
Chloramphenicol	C	30	26 (S)	26 (S)
Duricef	CFR	30	15 (R)	16 (I)
Ampicillin	AM	10	14 (R)	14 (R)
Oxacillin	OX	1	18 (I)	17 (I)
Tigecycline	TGC	15	22 (S)	21 (S)
Impenem	IPM	10	17 (I)	17 (I)
Streptomycin	S	10	12 (R)	13 (R)
Gentamicin	CN	10	13 (R)	13 (R)
Amikacin	AK	30	18 (I)	20 (I)

S, susceptible (≥21 mm); I, intermediate (16–20 mm) and R, resistance (≤15 mm).
Conc.; concentration.

3.2. Asparagus extract effect during fermentation by *L. plantarum* DMS 20174

L. plantarum DMS 20174 grew well on asparagus extract (5 %, w/v), and the cell density of the strain was 10.33 log CFU/mL, compared to 11.18 log CFU/mL on MRS media. The pH values for asparagus extract-based medium and MRS medium after 48 hr of fermentation declined from 6.0 to 4.02 ± 0.15 and 3.78 ± 0.42, respectively, Asparagus extract contained enough fermentable carbohydrates to support bacterial growth. Asparagus comprises various significant compounds and essential nutrients, including oligosaccharides (Fukushi et al., 2000), dietary fiber (Sun et al., 2010), amino acid derivatives (Kasai & Sakamura, 1981), vitamins and minerals (Kim et al., 2009)**,** and lignans (Thompson et al., 1991). Asparagus also includes flavonoids (mostly rutin) and phenolic compounds, both of which are powerful antioxidant (Makris & Rossiter, 2001). The findings revealed that strain DMS 20174 could digest simple sugars in an asparagus extract-based medium, leading to the production of organic acids and a pH drop (Panda et al., 2017). As a result, the asparagus extract-based media employed in this investigation is an excellent medium for *L. plantarum* fermentation.

2.2.1. Effect of asparagus extract on SCFA production *in vitro*

The concentrations of organic acids in SCFA are presented in **Fig. (3)** and identified by HPLC. Lactic acid was shown to be the highest concentration of organic acids in CFE without asparagus extract amounted 3.176 mg/mL, whereas in asparagus extract fermented with *L. plantarum*, it appeared as the lowest concentration amounted 3.115 mg/mL. The acetic acid content was 3.339 mg/mL in asparagus extract that was similar with found in the control (3.33 mg/mL). Propionic acid concentrations in the fermented asparagus were lower (3.719 mg/mL) than in the control (3.754 mg/mL). All CFE contained butyric acid that were 5.029 mg/mL to 5.051 mg/mL. There were also unknown acids (unidentified). LAB creates organic acids, mainly lactic acid, which is the typical fermentative product and lowers the pH of the substrate to a level that inhibits pathogenic, putrefactive, and toxigenic bacteria from growing (Prakash, 2016). SCFAs are generated directly by bacteria by fermentation of non-digestible components, resulting in a wide range of metabolites (Postler & Ghosh, 2017). Furthermore, The acidic environment created by LAB fermentation works as a catalyst for food matrix modification, resulting in the production of bioactive compounds (Xiao et al., 2020).

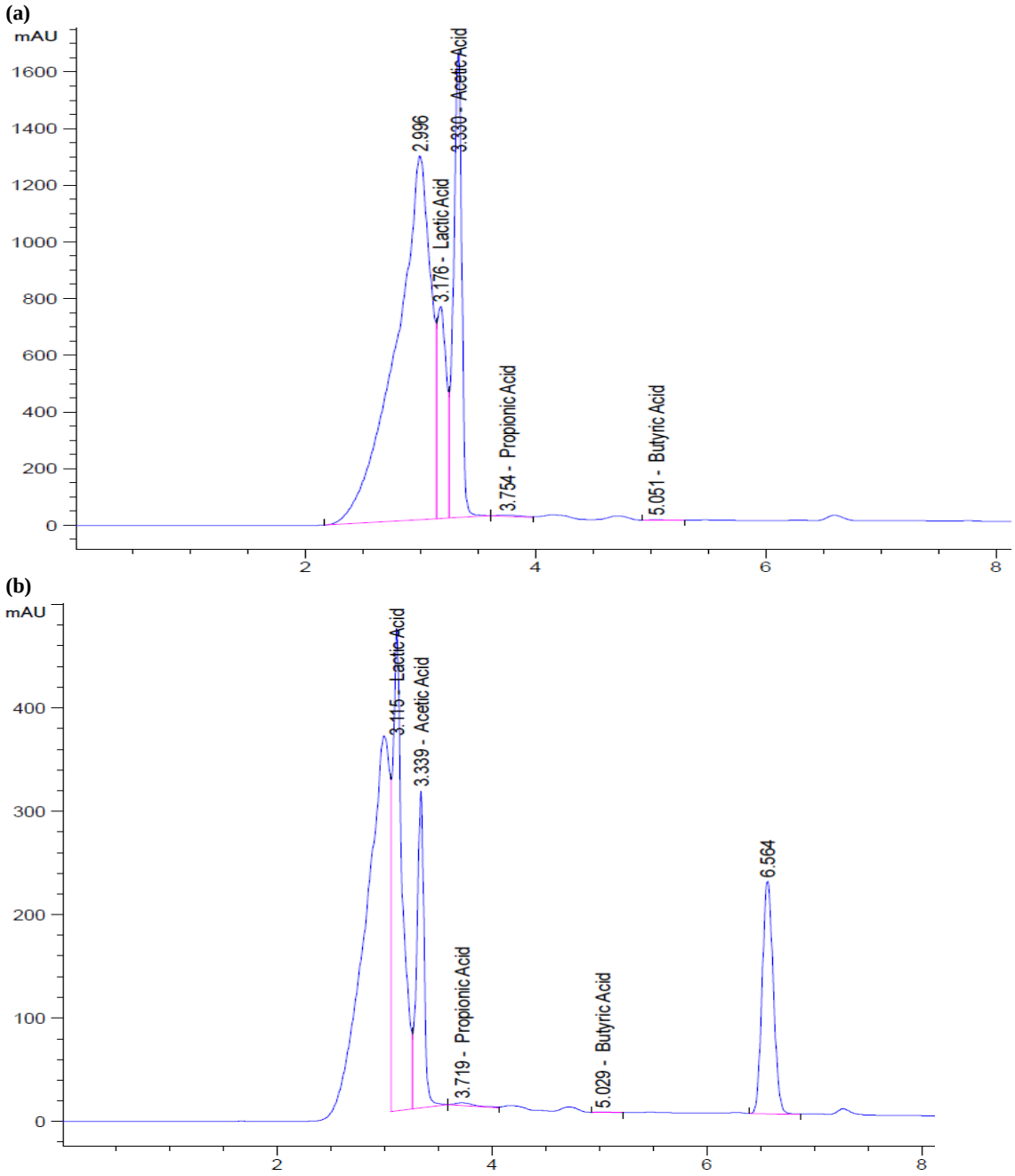

Fig.3: Organic acid profile in *L. plantarum* DMS 20174 fermented MRS broth (a) and asparagus extract (b).

2.2.2. Asparagus extract fermentation effect on microbial activity

The results showed that the fermented asparagus extract possess significant antibacterial activity depending on the bacterial species. The inhibition of various indicator strains was given in **Table 2**. The CFE of *L. plantarum* grown on MRS and asparagus extract media showing clear zones of inhibition of tested bacterial growth ranged from 19.20±0.28 to 27.10±0.14 mm and from 20.45±0.05 to 26.65±0.15 mm diameters, respectively. The antibacterial activity of the fermented asparagus extract was high against both the Gram-positive and Gram-negative bacteria, but low against *E. coli*. *S. typhimurium* and *Ps. aeregenosa* were the most resistant to the fermented asparagus extract. The media without asparagus (control) had the highest inhibitory activity against *St. aureus*. The antibacterial activity against *B. pumilis* was the lowest. Lactobacilli sp. had antibacterial properties against pathogens such as *E. coli*, *L. monocytogenus*, and *St. aureus* (Saif & Sakr, 2020). Abou El Nour & Sakr, (2020) also found that the cell free supernatant of Lactobacillus sp. inhibited pathogenic growth effectively.

Table 2: Antimicrobial activity and MIC of *plantarum* DMS 20174 cell-free extract against pathogenic bacteria.

Indicators	Antibacterial activity (inhibition zone, mm)		MIC (%)	
	CFE of *L. plantarum*	CFE of Asparagus extract-based media	CFE of *L. plantarum*	CFE of Asparagus extract-based media
St. aureus	27.10±0.10^{a}	22.40±0.10^{d}	6.25	12.50
L. monocytogenus	22.65±0.15^{c}	23.15±0.15^{c}	12.50	6.25
B. pumilis	19.20±0.20^{d}	21.30±0.20^{e}	25.00	12.50
E. coli	22.70±0.20^{c}	20.45±0.25^{f}	12.50	12.50
Ps. aeruginosa	24.55±0.05^{b}	26.65±0.15^{a}	6.25	6.25
S. typhimurium	22.40±0.40^{c}	26.30±0.20^{b}	12.50	6.25

Means having different superscript letters in the same column differ significantly (P<0.05).
CFE of *L. plantarum:* cell free extract of *L. plantarum* grown in MRS media.

The antimicrobial activity of LAB is due to the production of hydrogen peroxide, oxygen metabolites, exopolysaccharide, saturated fatty acids acting as a biosurfactant, and other factors (Moradi et al., 2019). The ability to limit pathogenic bacteria's growth aids in the treatment of infectious disorders caused by *St. aureus*, *L. monocytogenus*, *B. pumilis*, *E. coli*, *Ps. aeregenosa*, and *S. typhimurium*. By enhancing beneficial bacteria, the LAB can create extracellular bacteriostatic and bactericidal substances against infections, as well as balance the host's gut microbiota (Brunt & Austin, 2005). The prebiotic component (inulin) in asparagus extract-based media improved the log CFU of *L. plantarum* DMS 20174 and enhanced the antibacterial effectiveness of *L. plantarum* DMS 2017. Gram-negative bacteria were more sensitive to CFE of fermented asparagus than Gram-positive bacteria. This is because CFE contains a number of antimicrobial compounds that may limit the pathogenicity of bacteria, which can work together to cause an infection in AD by impairing the bacterial lipopolysaccharide. The concept of antimicrobial protection in AD was proposed in light of these observations (Moir et al., 2018).

The antimicrobial activity of CFE was also estimated as MIC (**Table 2**). The highest MIC of *L. plantarum* DMS 20174 was 25% against *B. pumilis* with CFE of *L. plantarum*. While the lowest MIC was 6.25% against *St. aureus* and *Ps. aerogenosa* with CFE of MRS media and against *L. monocytogenus*, *Ps. aerogenosa* and *S. typhimerium* with CFE of fermented asparagus media. There are no studies on the antimicrobial activity of the fermented asparagus extract by *L. plantarum* DMS 20174 in the literature.

2.2.3. Asparagus extract fermentation effect as antioxidant activity

Oxidative stress is a brain damaging process that plays a key part in AD pathogenesis and can possibly be regarded a major element in the disease's pathogenesis (Bai et al., 2022). The result of inhibition percentage of fermented asparagus extract was lower than that reported by *L. plantarum*. The IC_{50} of fermented asparagus extract had the maximum activity (54.81 ± 0.43 µL/mL), while the IC_{50} of *L. plantarum* had the lowest activity (179.8 ± 6.3 µL/mL).The antioxidant capacity increased in a concentration-dependent way. The IC_{50} of ascorbic acid was discovered to be 14.2 ± 0.6 µg/mL, which was greater than the tested CFE (**Fig. 4a**). According to Kwaw et al. (2018), who demonstrated a substantial increase in the scavenging activities of DPPH of mulberry juice after fermentation. Our findings demonstrated that following fermentation, *L. plantarum* can strengthen the antioxidant capacity of asparagus extract, which could be linked to the release of phenolic chemicals (Johnson et al., 2011). In addition, as compared to non-fermented asparagus fructan, fermented asparagus fructan had the highest antioxidant activity (Sakr, 2022).

2.2.4. Fermented asparagus extract inhibitory effect on cholinesterase activity

Inhibition of acetylcholinesterase enzyme, is considered as a promising strategy for the treatment AD which breakdowns acetylcholine and can be provided by the medicinal plants. AD is a progressive neurological illness marked by memory and cognitive impairment as well as behavioural abnormalities (Chen et al., 2022). In AD, high acetylcholinesterase activity is linked to cholinergic impairment and memory loss (Garcia-Ayllón et al., 2010). This is related to the accelerated hydrolysis of acetylcholine, a key neurotransmitter involved in nerve impulse transmission from one neuron to the next.

As a result, inhibiting acetylcholinesterase increases acetylcholine availability, improving neuronal transmission and memory performance (Äolovia & Krstia, 2013). The enzyme inhibitory action of fermented asparagus extract, however, has yet to be determined.

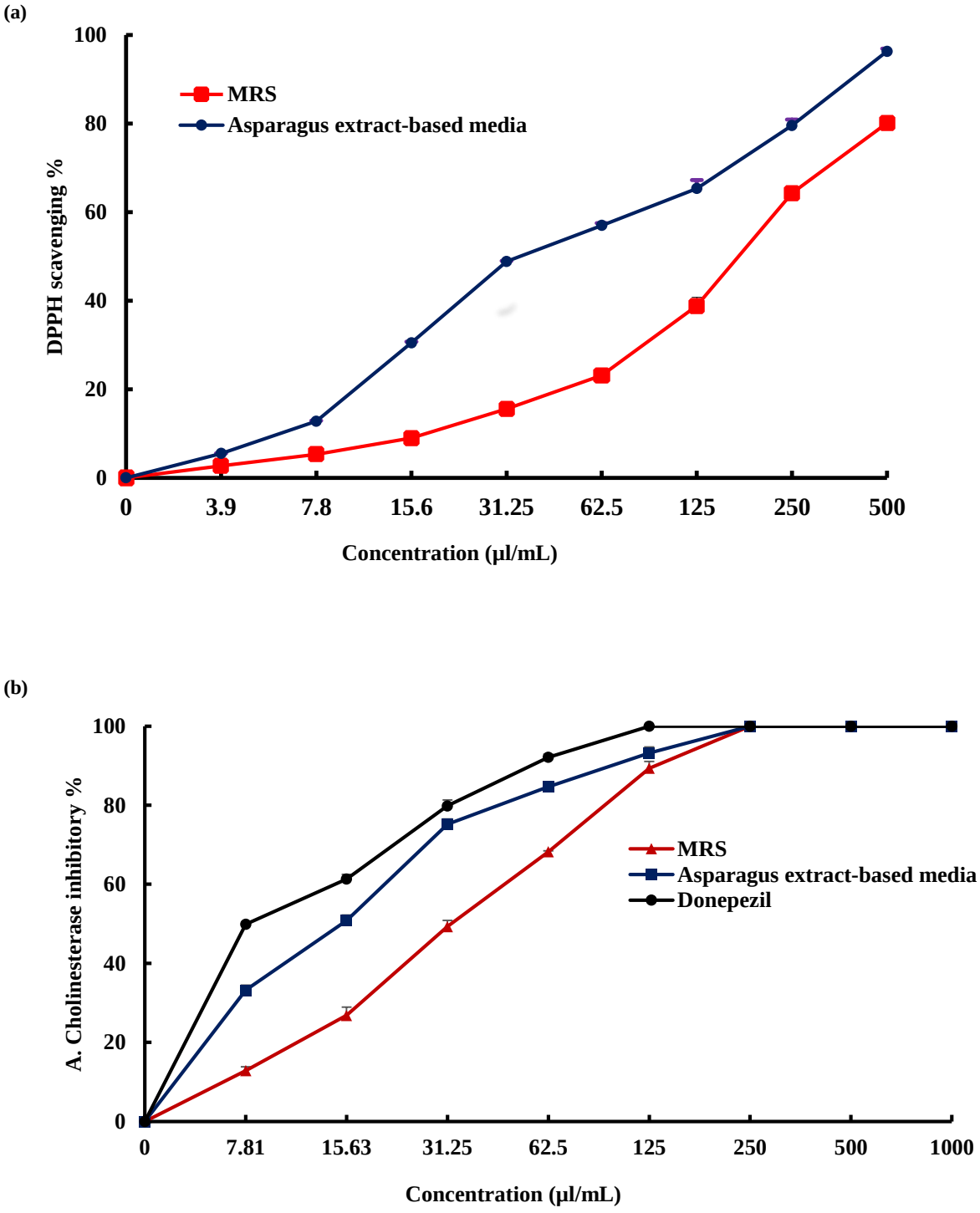

Fig. 4 Radical scavenging (a) and anticholinesterase (b) activities of *L. plantarum* DMS 20174 fermented MRS broth and asparagus extract.

Figure 4b shows the impact of both CFE on AChE inhibitory activities in this investigation. Fermented asparagus extract had minimal activity when compared to the standard. In comparison to donepezil (7.89 ±1.3 µL/mL), it had an IC_{50} of 15.38±1.6 µL/ml. Both CFE were found to inhibit AChE in a dose-dependent manner **(Fig. 4b**), with inhibition increasing with fermentation. Overall, these findings suggest that fermentation increases the AChE inhibitory action of fermented asparagus extract.

2.3. *In vivo* protective potential of asparagus extract and *L. plantarum* supplementation against high fat-induced Alzheimer disease

2.3.1. Cholinergic parameters

Obtained data showed that the HFD decrease cholinergic parameters resembling at the accelerated level of AChE and markedly ameliorate from HFD group for each treated group asparagus extract and *L. plantarum* but the mixed group showed the best amelioration but still varied from control group. In addition, the same trend MDA increased at HFD and significant differs from positive group and the best treatment was *L. plantarum* or mixed group which decrease the phospholipids deterioration (**Table 3)**.

Antioxidant enzymes were stimulated and oxidative stress markers were lowered in the brain when probiotic strains were administered (Divyashri et al., 2015). Several other animal studies support the Lactobacilli and Bifidobacteria's medicinal potential (Athari Nik Azm et al., 2018; Kobayashi et al., 2017; Musa et al., 2017). According to Musa et al. (2017), probiotics may reduce LPS-induced memory loss and neuroinflammation through anti-inflammation by suppressing AChE and antioxidant activities. Furthermore, the ethanolic extract of *Asparagus racemosus* roots is an excellent natural nootropic for improving learning and memory impairment linked with neurodegenerative illnesses (AChE), particularly AD (Uddin et al., 2016). Aqueous extract of *A. cochinchinesis* also helps to reduce neuronal cell damage in brain mice during the pathological advancement stage of AD by reducing oxidative stress (Lee et al., 2018).

2.3.2.Lipid profile

Among the lipid profile of the HFD group showed significant increase of TC, LDL and disrupt other parameters. In contrast, treated group significant ameliorate LDL and HDL which decrease the bad lipid and increase the good lipid and the best TC was asparagus extract group (**Table 4**). The oral administration of *L. plantarum* of HFD group resulted in higher decreases of TC and LDL cholesterol by 19.39% and 36.79%, respectively. The saponin content of asparagus extract may be responsible for its hypolipidemic effect. Saponin is a form of phytosterol that can lower blood cholesterol levels by reducing cholesterol absorption and production (Chen et al., 2008). The considerable reduction in serum TC and LDLC levels in the treated groups could be attributed in part to the flavonoid, which lowers blood cholesterol levels by blocking cholesterol production and raising LDL receptor expression (Bolkent et al., 2005).

Harisa et al. (2009) speculate that probiotics' hypotriglyceridemic impact is due to the activation of lipases, a decrease in lipid absorption in the intestine, or an increase in lipid catabolism and/or antioxidant activity. Lipoprotein lipase is in charge of TG metabolism, which results in a normalization of its plasma level. Furthermore, in hypercholesterolemic humans and animals, lowering blood cholesterol and LDL cholesterol lowers the risk of cardiovascular disease. As a result, lowering LDL cholesterol, which is the primary component of serum cholesterol, could be a key contributor in lowering blood total cholesterol. Hepatic absorption regulates blood cholesterol levels via the LDL receptor.

Table 3: Mean values of AChE and MDA hippocampal brain region of experimental obese rat's model exposed to HFD and treated with asparagus extract, *L. plantarum* and the combined mixture.

Treatment Group	Acetylcholinesterase (AChE, µmol SH/g / min)	Malondialdehde (MDA, nmol/g)
Basal diet		
Negative control	2.36 ± 0.07^{e}	18.77 ± 0.73^{d}
Asparagus extract	2.34 ± 0.16^{e}	19.20 ± 0.84^{d}
L. plantarum	2.58 ± 0.16^{de}	18.13 ± 0.72^{d}
Asparagus extract and *L. plantarum*	2.30 ± 0.14^{e}	17.93 ± 0.98^{d}
High fat diet		
Positive control	3.89 ± 0.15^{a}	31.16 ± 1.01^{a}
asparagus extract	3.41 ± 0.05^{b}	28.27 ± 0.68^{b}
L. plantarum	3.20 ± 0.16^{b}	25.73 ± 1.31^{bc}
Asparagus extract and *L. plantarum*	2.80 ± 0.11^{c}	24.96 ± 0.94^{c}

Means having different superscript letters in the same column differ significantly (P<0.05).

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2.3.3.Microbiological analysis

Specific probiotics have been shown to improve human health by modulating the gut microbiota composition by promoting beneficial bacteria and diminishing harmful bacteria. *L. plantarum* DMS 20174, asparagus extract, and synbiotics (combination of asparagus + *L. plantarum*) were given to HF-fed mice to determine whether they affected gut microbiota members differently from mice fed with a standard diet. Plate counting was used to determine the presence of Lactobacillus spp., a beneficial bacterium, and gut pathogens in the caecum content **(Table 5)**. The frequency of Lactobacillus spp. in the HFD group was lower than in the control group. The caecum microbiota of HF-fed mice changed significantly after treatment with probiotic (DMS 20174), asparagus extract, and synbiotics, with greater counts of Lactobacilli and lower counts of total aerobes, coliform bacteria, staphylococci, and fungi.

In general, when compared to the control, the synbiotic diet reduced the population of harmful

microbes. As a result, decreasing or controlling the multiplication of these bacteria is important for preserving health and lowering the risk of disorders such acute gastroenteritis, food allergies, inflammatory bowel disease, and colon cancer. Furthermore, those supplements altered and repaired the gut flora that had been damaged by the HF diet.

Due to nutritional competition, the synthesis of inhibitory compounds, immunological activation, and competition for binding sites, Lactobacillus sp. may be able to inhibit numerous bacterial infections (Georgieva et al., 2015; Inglin et al., 2015). It's also worth noting that mice fed an asparagus extract diet had a higher count of Lactobacilli in their colon. A previous study found that asparagus extract includes prebiotics, which act as a fuel for probiotics (Newgent, 2013). FOS, which is found naturally in that plant and has been demonstrated to promote friendly bacteria in the gut (Xiang et al., 2014), could have contributed to the growth.

A study found that eating more simple carbohydrates than complex carbohydrates causes intestinal dysbiosis and metabolic disruption, which is linked to a shift in bacterial populations from gram-positive to gram-negative species (Arora & Bäckhed, 2016). This enlarged gram-negative bacterial population induces chronic inflammation in people and mice by producing large levels of lipopolysaccharide (LPS) (Moreira et al., 2012). LPS may be involved in the development of sporadic AD (Zhan et al., 2018). Finally, nutrition has been identified as a critical environmental factor that influences the diversity and metabolism of the gut microbiota. Consumption of specific dietary substances, such as mixed, probiotics, and asparagus extract, can also help to modify the microbiota.

Table 4: Mean values of serum lipid profile of experimental obese rat's model exposed to HFD and treated with asparagus extract, *L. plantarum* and the combined mixture.

Treatment Group	Parameters				
	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL
Basal diet					
Negative control	71.83±1.97^{d}	66.03±4.11^{b}	26.43±3.25^{a}	32.17±3.63^{e}	13.23±0.80^{a}
Asparagus extract	76.87±6.47^{c}	68.60±2.88^{a}	24.80±4.60^{ab}	38.37±11.60^{c}	13.73±0.59^{a}
L. plantarum	71.17±2.68^{d}	69.13±1.06^{a}	25.77±3.10^{a}	31.57±4.05^{e}	13.83±0.21^{a}
Asparagus extract and *L. plantarum*	76.13±3.76^{c}	68.83±3.75^{a}	23.03±1.42^{b}	39.30±2.46^{c}	13.77±0.76^{a}
High fat diet					
Positive control	92.00±15.19^{a}	65.47±2.66^{b}	19.47±1.89^{c}	59.43±13.56^{a}	13.10±0.56^{a}
asparagus extract	82.43±5.10^{b}	66.37±1.86^{b}	24.50±5.55^{ab}	44.63±3.16^{b}	13.27±0.35^{a}
L. plantarum	76.03±6.90^{c}	69.23±4.15^{a}	25.23±2.00^{a}	36.97±5.87^{d}	13.83±0.81^{a}
Asparagus extract and *L. plantarum*	80.90±3.59^{b}	66.40±3.72^{b}	23.40±3.18^{b}	40.40±5.16^{c}	13.30±0.75^{a}

Means having different superscript letters in the same column differ significantly (P<0.05).
TC: total cholesterol, TG: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: *very-low-density lipoprotein*

Table 5: Mean values of caecum microbiota of experimental obese rat's model exposed to HFD and treated with asparagus extract, *L. plantarum* and the combined mixture

Treatment Group	Viable count (log cfu/mL)				
	Total aerobes	Lactobacillus sp.	Coliform bacteria	Staphylococci	Fungi
Basal diet					
Negative control	8.66±0.18^{c}	7.81±0.44^{e}	8.15±0.05^{b}	7.78±0.04^{a}	4.15±0.07^{a}
Asparagus extract	8.57±1.06^{d}	8.16±0.96^{d}	7.62±0.11^{c}	7.65±1.32^{b}	3.10±0.37^{c}
L. plantarum	8.39±1.34^{e}	8.76±0.06^{b}	5.06±0.06^{e}	7.21±0.43^{d}	3.06±0.06^{c}
Asparagus extract and *L. plantarum*	8.27±0.05^{g}	9.00±0.09^{a}	4.27±0.23^{f}	6.46±0.08^{f}	2.30±0.84e
High fat diet					
Positive control	9.57±1.09^{a}	7.09±1.17^{g}	8.46±0.48^{a}	7.44±0.58^{c}	4.18±0.54^{a}
asparagus extract	8.99±0.67^{b}	7.28±0.86^{f}	8.37±0.11^{a}	6.83±0.04^{e}	3.24±0.09^{b}
L. plantarum	8.37±0.51^{e}	7.86±0.03^{e}	6.28±0.16^{d}	5.21±0.04^{g}	3.06±0.06^{c}
Asparagus extract and *L. plantarum*	8.33±0.71e^{f}	8.35±0.13^{c}	5.18±0.03^{e}	4.88±0.11^{h}	2.78±1.04^{d}

Conflicts of interest
There are no conflicts to declare.

Conclusions

This study demonstrates how the antimicrobial, antioxidant and anticholinesterase properties of asparagus extract. are enhanced through the fermentation by our probiotic bacteria. The mixed group (*L. plantarum* and asparagus extract) were the best treatment for HFD groups which decrease the phospholipids deterioration. Also, the treated group ameliorate LDL and HDL. Novel applications as functional food dietary supplements or pharmaceutical preparations could be expected.

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