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Research Article
Ameliorative Role of Ashwagandha/Probiotics Fortified Yogurt against $AlCl_3$ Toxicity in Rats

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Abstract

Background and Objective: Yogurt is a distinctive vesicle for active compounds and the widest-spread fermented dairy product. This study aimed to explore the ameliorative role of emulsified yogurt fortified with Ashwagandha Ethanolic Extract (AEE) and probiotic bacteria against Aluminium Chloride ($AlCl_3$)-induced toxicity in rats. **Materials and Methods:** Yogurt was evaluated chemically, microbiologically and sensory as well as biologically using experimental animals. **Results:** Results revealed that fortified yogurt with either AEE, probiotics, or their mixture did not disturb the main chemical composition of yogurt. Yogurt antioxidants and phenolic content increased by adding AEE, alone or in the presence of probiotics; however, it decreased after 15 days of storage but remained higher in the mixture-treated yogurt. Furthermore, all the physicochemical and sensory properties were significantly improved with the addition of AEE, separately or combined with probiotics bacteria. The biological study showed oral administration of rats with the emulsions of yogurt fortified with AEE and probiotic bacteria, alone or in combination, together with Aluminium chloride succeeded to decline $AlCl_3$-induced toxicities; this was evidenced by the significant reduction in serum tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL1β), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), urea, creatinine, cholesterol, triglycerides and low dense lipoprotein-cholesterol (LDL-c) values. Also, reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were markedly increased in liver, kidney and brain tissues coupled with a sharp reduction in the malondialdehyde (MDA) and nitric oxide (NO). The neurochemical markers, dopamine, serotonin and acetylcholinesterase (ACh-ase) were also favorably improved. **Conclusion:** It could conclude that AEE and probiotics succeeded to improve physicochemical, microbiological, sensory qualities as well as health benefits as they restored $AlCl_3$-induced hepato-renal-neuro deteriorations; they are promising-supplement for the protection against toxicities.

Key words: Functional yogurt, ashwagandha ethanolic extract, physicochemical, microbiological, sensory qualities, ameliorative role, toxicity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Yogurt is one of the most common, widely traded and accepted dairy products in all different groups in societies, especially Egyptian society. It provides the body with many important nutrients such as high biological proteins and amino acids important for maintaining good health. Also, it is used to deliver many active biomaterials, beneficial bacteria such as probiotics and other therapeutic compounds by supplementation[1]. Probiotics especially *Lactobacillus* strains show a therapeutic role in cognitive disorders through gut-brain axis communication[2]; most of the fermented foods include probiotics as a natural and dietary intervention for human health. The management of *Lactobacillus* strains was able to rescue the rough eye phenotype seen in AD-induced Drosophila, with a more prominent effect detected upon the administration of *Lactobacillus plantarum* DR7[3].

Aluminium is the third most abundant metal, comprising about (8%) of the earth's crust, is found in combination with oxygen, silicon, fluorine and other elements in soil, rocks, clays and gems and has a significant toxic potential for humans[4]. It gets access to the body via the gastrointestinal and respiratory tracts and accumulates in many tissues, such as kidney, liver, heart, blood, bone and brain[5]. The toxic effect of Aluminium has been suggested to be mediated by reactive oxygen species generation resulting in the oxidative deterioration of cellular lipids, proteins and DNA and also induces changes in the activities of tissue antioxidant enzymes[6], altered gene expression and apoptosis[7]. The induced oxidative stress by Aluminium and its salts is responsible for hepatotoxicity[6], nephrotoxicity[8], cardiac toxicity[9], reproductive toxicity[10] and also neurodegenerative disease and Alzheimer's like neurofibrillary tangle formation[11].

Probiotics are living microorganisms, which applied in sufficient quantities, provide a health benefit to the host and contribute to reducing the risk of disease. They are a subject of increasing interest due to their proven immune-stimulating, antioxidant and anti-cancer efficiencies[12]. Due to their characteristics, dairy products are a good choice for the transfer of many vital and effective nutrients by supplementing them with these bioactive compounds to maximize the health benefit of these compounds, for example, dairy products added to phytosterols, which have confirmed to play a role in reducing cholesterol and blood lipids[13] as well as omega-3-fortified products to reduce the risk of cardiovascular disease in adults[14]. The supplementation is used to compensate for the shortage of nutrients and generally, dairy products fortified with nutrients or bioactive compounds in similar or greater proportions than naturally existing or lack food, these products also include nutrients to compensate for what was lost during various manufacturing processes of food[15].

The diet plays an important role in the development of healthy habits and is referred to as a modifiable risk factor for several non-communicable chronic diseases[16]. Ashwagandha (*Withania somnifera*) or Indian ginseng is cultivated in drier parts of India; it has reported exhibiting anti-inflammatory[17], immunomodulatory[18], anti-arthritic[19] and anti-aging properties[20]. It is found able to modulate the body's' oxidative stress status as its root extract significantly reduces the lipid peroxidation[21] and increases the activity of SOD and CAT, therefore exhibiting free radical scavenging property[22].

In this respect, there is insufficient evidence on the role of functional fortified dairy products in improving health and in preventing risk factors associated with non-communicable chronic diseases; therefore, this study was conducted to prepare a healthy yogurt product that fortified with Ashwagandha extract or/and probiotic bacteria, as well as explores its ameliorating role against toxicities induced by $AlCl_3$ in male rats.

MATERIALS AND METHODS

Materials: This study was carried out in the Department of Dairy at the National Research Centre, Dokki, Egypt, Basic Centre of Science, Misr University for Science and Technology, Giza, Egypt, Zoology department, Faculty of Science, Al-Azhar University, Assuit, Egypt and Medical physiology department, National Research Centre, Giza, Egypt during August, 2019-February, 2020.

Sample collection: Ashwagandha (*Withania somnifera*) roots were obtained from Imtinan Company, Egypt; then identified and found carrying a taxonomic serial number 505824. Fresh skimmed milk was obtained from Animal Production Research Institute, Agriculture Research Center, Giza, Egypt. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* Subsp. *bulgaricus* were obtained from stock cultures of Dairy Microbiology Lab., National Research Centre, Egypt, meanwhile, *Lactobacillus plantarum* was provided by the Northern Regional Research laboratory, Illinois, USA. Due to its higher growth in the presence of AEE (rather than other probiotic bacterial strains), *Lactobacillus plantarum* was chosen for this study.

Methods

Plant extraction: The ethanolic extract of Ashwagandha dry powdered roots was carried out according to the modified method of Filipiak-Szok *et al.*[23]; in brief, 2 g of powder were soaked in 20 mL 70% ethanol at room temperature for 24 h under continuous stirring; then the mixture was filtered through sterile filter paper (Whatman number 42, England). The solvent was evaporated using a rotary evaporator and then the extract was stored at -20°C until further use.

HPLC analysis of phenolic constituents: HPLC analysis was carried out using an Agilent 1260 series (5301 Stevens Creek Blvd. Santa Clara, CA 95051, USA). The separation was carried out using a Kromasil C18 column (4.6×250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 mL/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0-5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (85% A) and 15-16 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µL for each of the sample solutions. The column temperature was maintained at 35°C.

Preparation of fortified yogurt: Ashwagandha roots ethanolic extract (AEE) was mixed with fresh buffalo's skim milk after heat-treated at 90°C/3 min, cooled and adjusted to 42°C; the milk mixture with AEE (at ratios of 0, 200 mg mL^{-1}) was inoculated with 2% yogurt starter (1:1) and 2% probiotic bacteria as follow: C) Control contained yogurt starter only (*Streptococcus thermophilus, Lactobacillus delbrueckii* Subsp. *bulgaricus*); (T_1) contained yogurt starter plus *Lactobacillus plantarum*; (T_2) contained yogurt starter plus AEE (200 mg mL^{-1}); (T_3) contained yogurt starter plus *Lactobacillus plantarum* plus AEE (200 mg mL^{-1}); all treatments were packed in plastic cubs, incubated at 42°C until complete coagulation then cooled and stored at refrigerator till the end of storage[24]. Samples of manufactured yogurt were analyzed for chemical, microbiological, sensory properties at fresh and during the storage period.

Chemical analysis of fortified yogurt: Total solids (TS), fat, total nitrogen (TN), ash content were determined according to AOAC[25]; pH values were measured using a digital laboratory pH-meter (Jenway 3510, UK); Total volatile fatty acids (TVFA) value was determined according to the method described by Koiskowski[26]; diacetyl and acetaldehyde contents were determined according to Less and Jago[27].

Total phenolic compounds and antioxidant activity of fortified yogurt: Total phenolic content and antioxidant activity of yogurt determined according to Salama *et al.*[28].

Enumeration of bacterial strains: Using DeMan-Rogosa-Sharpe broth (MRS broth, Oxoid), the MRS medium was inoculated with 5% active *Lactobacillus* strains with initial count (10^7 cfu mL^{-1}) and incubated at 37°C for 24 h, also, using MRS broth, Oxoid supplemented. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* Subsp. *bulgaricus* were enumerated using modified M 17 and MRS agar, the plates were incubated at 37°C for 48 h according to Harrigan and McCance[29]. Probiotic strains (*Lactobacillus plantarum*) were enumerated on MRS-arabinose agar, that was prepared without dextrose and 10 mL of membrane filtered sterile solution of 10% L-arabinose was added to 20 mL of basal medium (1% final concentration) just before pouring the agar medium. Plates were anaerobically incubated at 37°C for 48h[30].

Microbiological analysis of fortified yogurt: The samples of yogurt were microbiologically examined for each treatment after 0, 7, 15 and 21 days of storage; 25 g of each yogurt sample was added aseptically to 225 mL of a sterile solution (2% w/v) of sterile Buffered Peptone Water (BPW) and homogenized; Total Aerobic Colony Count (TACC) was carried out as the conventional method using plate count agar (Oxoid); enumeration and counts of mold and yeast were carried out in the samples using the media of acidified potato dextrose agar (Mu 96, Himedia, Mumbai) according to the method recommended by FDA[31].

Sensory evaluation: Functional fortified yogurt was sensory evaluated at fresh and 15 days of storage by ten panelists of the staff member of the Dairy Department at Food Industries and Nutrition Division, National Research Centre, using the scores sheet according to Salama *et al.*[24].

Animals and experimental design: Total thirty adult male Wistar rats (150-200 g) were obtained from the Animal Colony, National Research Centre, Egypt; the animals were maintained under temperature (25±1°C) and light-controlled conditions (12/12 h light/dark cycle) on free access to food and water for a week before starting the experiment for acclimatization; they received human care in compliance with the standard institution's criteria for the care and use of experimental animals according to the procedures approved by the Ethics Committee of National Research Centre (FWA 00014747) that follows the recommendations of the National Institutes

of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised in 1985). After acclimatization, the animals were randomly assigned into five groups (6 rats each) as follows: group 1) included rats orally administrated with yogurt starter (2 mL/kg/day) and served as healthy control; group 2) included rats ingested with $AlCl_3$ (300 mg/kg/day); group 3) included rats ingested with AEE-yogurt fortified (2 mL/kg/day) besides to $AlCl_3$; group 4) included rats ingested with probiotic yogurt fortified (2 mL/kg/day) together with $AlCl_3$; group 5) included rats treated with yogurt fortified with the formula (probiotic and AEE) in line with $AlCl_3$ at the same dose.

Blood and tissue sampling: After four weeks of consecutive administrations, the rats were fasted overnight and blood samples were collected from the retro-orbital venous plexus using capillary tubes under diethyl ether anesthesia. Sera were separated using cooling centrifugation and stored at -20°C until further analyses; after blood collection, the animals were sacrificed via cervical decapitation and the brain (two halves), liver and kidney were dissected out, washed in saline, dried and stored at -80°C for biochemical determinations.

Serum biochemical measurements: Serum Aspartate Aminotransferase (ASAT) and Alanine Aminotransferase (ALAT) activities were determined using reagent kits purchased from Human Gesell Schaft fur Biochemical und Diagnostic mbH, Germany; urea and creatinine level were estimated using reagent kits purchased from Vitro Scient Co, Egypt; total cholesterol, triglycerides, LDL- cholesterol and HDL-cholesterol levels were determined using reagent kits purchased from DiaSys Diagnostic System GmbH, Germany; TNF-α and IL-1β were assayed using rat ELISA reagent kits (SG-10179 and SG-10127, respectively) purchased from SinoGeneClon Biotech, China.

Tissue oxidative stress markers: The brain's first half and specimens of each liver and kidney were cool-homogenized separately in a Tris-HCl buffer (pH 7.4) and cool-centrifuged to remove the nuclear and mitochondrial fractions; each supernatant was divided into aliquots and stored at -80°C till biochemical measurements. Levels of GSH and NO and activity of SOD and CAT were determined in brain, liver and kidney homogenates using reagent kits obtained from Biodiagnostic, Egypt. Lipid peroxidation end product (MDA) level was estimated chemically according to the method described by Ruiz-Larrea *et al.*[32].

Brain acetylcholinesterase activity and dopamine and serotonin level: Acetylcholinesterase (AchE) activity was determined in the brain homogenate by the modified method of Ellman *et al.*[33]. The hydrolysis of acetylthiocholine iodide by acetylcholinesterase yields thiocholine, which reacts with 5, 5′-dithiobis-(2-nitrobenzoic acid), reduces it to thionitrobenzoic acid, whose yellow color was read at 412 nm. The brain's second half was homogenized in 0.1 M perchloric acid (containing 3, 4-dihydroxybenzylamine) at a final concentration of 25 ng mL^{-1} then centrifuged 10 min at 36000 rpm g^{-1}. The obtained supernatant was then filtered through 0.25 mm nylon filters, Millipore, USA. Dopamine and serotonin levels were determined using high-performance liquid chromatography (Waters, Milford, USA) by injection of 20 µL sample supernatant into the injector port and using an electrochemical detector following the method of Kim *et al.*[34] with a high-pressure isocratic pump, a sample injector valve, a C-18 reverse phase column (250×4 mm, particle size 5 µm) and an electrochemical detector (464 Pulsed electrochemical detector).

Statistical analysis: Comparisons between means were carried out using one-way analysis of variance (ANOVA) followed by post hock (Duncan's) multiple comparisons test at $p \leq 0.05$, using statistical analysis system (SAS) program software; copyright (c) 1998 by SAS Institute Inc., Cary, NC, USA.

RESULTS AND DISCUSSION

As shown in Table 1 and Fig. 1, mainly 14 phenolic compounds could be identified in AEE, while other constituents more or less couldn't be identified using HPLC analysis. The major identified compound was gallic acid, while the minimal concentration was of Methyl gallate.

Chemical composition, antioxidant activity and total phenolic content of yogurt: The results declared that yogurt was composed mainly of 86.22% moisture, 0.55% fat, 4.54% protein and 0.97% ash for all yogurt samples, as the little addition of either AEE or probiotics did not affect the chemical composition of the resulting yogurt. Regarding Table 2, the obtained results pointed out that addition of AEE and probiotics (either alone or in combination) significantly improved the antioxidant activity and total phenolic content at fresh and maintained high antioxidant activity post-15-day storage in compare to control yogurt; the highest level was noticed in formula-fortified yogurt followed by AEE-fortified

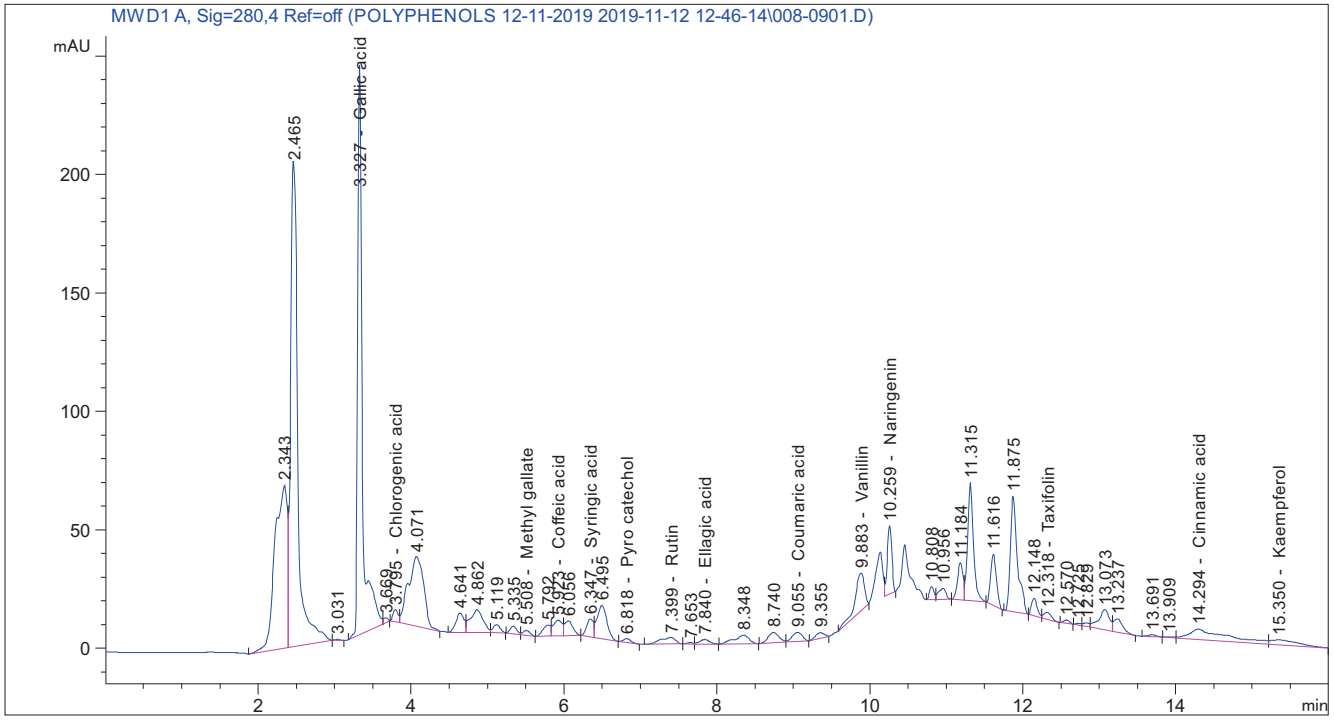

Fig. 1: HPLC analysis of phenolic constituent in ashwagandha ethanolic extract

Table 1: Phenolic constituents of the AEE using HPLC analysis

Phenolic Components	Area	Concentration (μg mL^{-1} = μg/23.3 mg)	Concentration (μg g^{-1})
Gallic acid	1012.74	81.26	3487.40
Chlorogenic acid	23.28	1.81	77.71
Methyl gallate	13.04	0.20	8.43
Caffeic acid	53.89	1.95	83.63
Syringic acid	47.28	1.61	69.12
Pyrocatechol	10.90	1.06	45.28
Rutin	39.60	5.03	215.69
Ellagic acid	16.92	0.97	41.67
Coumaric acid	34.89	0.56	24.17
Vanillin	130.76	2.04	87.66
Naringenin	119.47	7.31	313.66
Taxifolin	19.78	2.25	96.75
Cinnamic acid	150.09	1.58	68.01
Kaempferol	49.72	4.05	173.63

yogurt. One or more reasons could explain this finding; the high phenolic constituents (gallic acid and rutin are the major antioxidant constituents) of AEE exhibit antioxidant activity as achieved by the HPLC analysis (Table 1); Kumar *et al.*[35] suggested that Ashwagandha is a good source of antioxidants and phenolic ingredients; yogurt starter and probiotics were found possess some antioxidant properties[36]; the proteolysis of milk protein[37] and production of organic acids[38] as a metabolic activity throughout fermentation process and cold storage could be other sources of antioxidant activities. During

Table 2: Antioxidant activity and total phenolic content of fortified and control yogurt at fresh and after 15 days' cold storage

		Storage	
Property	Treatment	Fresh	15 day
Antioxidant activity (%)	C	3.88 ± 0.52^{B}	$0.46\pm0.25^{C\#}$
	T_1	5.29 ± 0.34^{A}	$1.33\pm0.28^{B\#}$
	T_2	5.37 ± 0.27^{A}	$1.49\pm0.18^{B\#}$
	T_3	5.42 ± 0.45^{A}	$2.18\pm0.20^{A\#}$
Total phenolic content (mg/100g)	C	4.62 ± 0.03^{B}	$3.69\pm0.20^{D}\#$
	T_1	5.15 ± 0.16^{A}	$4.68\pm0.21^{C\#}$
	T_2	5.37 ± 0.38^{A}	$5.08\pm0.06^{B\#}$
	T_3	5.49 ± 0.30^{C}	$5.31\pm0.32^{A\#}$

All data are expressed as the Mean±SD of three replicates. Data were subjected to one-way analysis of variance (ANOVA) followed by post hoc test (Duncan) at $p\leq0.05$ level. Within the same column, means with superset different letters are significantly different; C (Starter only); T_1 (Starter and probiotics); T_2 (Starter and AEE); T_3 (Starter, AEE and probiotics); # is significant from fresh (same row)

storage, up to 15 days, the antioxidant activity and total phenolic content markedly decreased compared with fresh vales, but still T_3 and T_2 higher than the control; this finding goes in line with many previous reports[28].

For the physicochemical properties of the studied yogurt (Table 3), the results displayed that SN/TN ratio, TVFA, diacetyl and acetaldehyde significantly increased among treatments.

Table 3: Chemical composition of fortified and control yogurt at fresh and after 15 days cold storage

Property	Treatment	Storage		
		Fresh	7 day	15 day
SN/TN (ratio)	C	15.86±0.19^{B}	18.94±0.43^{A}#	22.47±0.33$^{B\#}$
	T_1	15.86±0.26^{B}	18.94±0.17$^{A\#}$	22.69±0.28$^{B\#}$
	T_2	16.30±0.3^{AB}	19.16±0.15$^{A\#}$	23.13±0.04$^{B\#}$
	T_3	16.52±0.52^{A}	19.38±0.24$^{A\#}$	23.79±0.54$^{A\#}$
pH	C	5.12±0.02^{A}	4.98±0.12^{A}	4.76±0.12$^{A\#}$
	T_1	5.10±0.06^{A}	4.95±0.15^{A}	4.69±0.06$^{A\#}$
	T_2	5.07±0.05^{A}	4.91±0.15$^{A\#}$	4.67±0.03$^{A\#}$
	T_3	5.04±0.04^{A}	4.87±0.03$^{A\#}$	4.64±0.01$^{A\#}$
TVFA (mL NaOH 0.1M)/100 g)	C	2.5±0.2^{D}	4.0±0.2$^{D\#}$	5.0±0.0$^{D\#}$
	T_1	4.0±0.0^{C}	5.5±0.2$^{C\#}$	7.0±0.5$^{C\#}$
	T_2	5.0±0.5^{B}	7.5±0.2$^{B\#}$	8.5±0.5$^{B\#}$
	T_3	6.5±0.3^{A}	9.0±0.4$^{A\#}$	10.5±0.5$^{A\#}$
Diacetyl (μm/100 g)	C	9.56±0.14^{Dc}	13.44±0.2$^{C\#}$	16.64±0.21$^{D\#}$
	T_1	10.94±0.17^{C}	13.88±0.34$^{C\#}$	18.58±0.34$^{C\#}$
	T_2	11.48±0.09^{B}	14.56±0.19$^{B\#}$	20.83±0.12$^{B\#}$
	T_3	12.43±0.08^{A}	15.63±0.39$^{A\#}$	22.07±0.07$^{A\#}$
Acetaldehyde (μm/100 g)	C	15.34±0.09^{D}	11.23±0.31$^{D\#}$	3.5±0.36$^{D\#}$
	T_1	18.44±0.17^{C}	14.40±0.12$^{C\#}$	5.84±0.2$^{C\#}$
	T_2	20.77±0.19^{B}	16.58±0.37$^{B\#}$	7.86±0.39$^{B\#}$
	T_3	23.54±0.13^{A}	18.61±0.07$^{A\#}$	11.53±0.21$^{A\#}$

All data are expressed as the Mean±SD of three replicates, Data were subjected to one-way Analysis of Variance (ANOVA) followed by post hoc test (Duncan's) at $p \leq 0.05$ level, Within the same column, means with superset different letters are significantly different, C (Starter only), T_1 (Starter and probiotics), T_2 (Starter and AEE), T_3 (Starter, AEE and probiotics), #: Significant from fresh (same row)

Table 4: Microbiological analysis of fortified and control yogurt at fresh and after 15 days' cold storage

Bacteria type	Treatment	Log cfu mL^{-1} of bacteria/Storage (days)			
		0	7 days	15 days	21 days
L. delbrueckii Subsp. *bulgaricus*	C	8.11±0.04^{Cb}	8.53±0.12^{Ca}	7.64±0.11^{Ac}	7.4±0.05^{Ad}
	T_1	8.21±0.05^{Bb}	8.65±0.07^{Ba}	7.71±0.04^{Ac}	7.32±0.13^{Ad}
	T_2	8.28±0.04^{Bb}	8.71±0.05^{ABa}	7.73±0.02^{Ac}	7.39±0.06^{Ad}
	T_3	8.36±0.01^{Ab}	8.81±0.03^{Aa}	7.74±0.01^{Ac}	7.45±0^{Ad}
S. thermophiles	C	8.4±0.05^{BCb}	8.51±0.04^{Ca}	7.78±0.07^{Ac}	7.16±0.06^{Bd}
	T_1	8.3±0.15^{Ca}	8.40±0.05^{Ca}	7.72±0.01^{Ab}	7.55±0.03^{Ac}
	T_2	8.6±0.15^{Ba}	8.7±0.05^{Ba}	7.77±0.06^{Ab}	7.56±0.04^{Ac}
	T_3	8.85±0.1^{Aa}	8.95±0.1^{Aa}	7.81±0.05^{Ab}	7.58±0.06^{Ac}
L. plantarum	T_1	7.37±0.03^{Bc}	7.86±0.09^{Aa}	7.75±0^{Bb}	7.38±0.02^{Bc}
	T_3	7.62±0.02^{Ac}	7.98±0.01^{Aa}	7.85±0.05^{Ab}	7.8±0.05^{Ab}
Total count of bacteria	C	8.35±0.02^{Ba}	8.41±0.02^{Ca}	7.8±0.05^{Db}	7.68±0.07^{Cc}
	T_1	8.39±0.06^{Ba}	8.40±0.03^{Ca}	8.12±0.04^{Bb}	7.95±0.05^{Bc}
	T_2	8.61±0.02^{Aa}	8.50±0.07^{Bb}	8.00±0.08^{Cc}	7.95±0.01^{Bc}
	T_3	8.69±0.06^{Aa}	8.71±0.02^{Aa}	8.50±0.02^{Ab}	8.25±0.06^{Ac}
Yeast and mold	C	0.0	0.0	0.0	2.3^{A}
	T_1	0.0	0.0	0.0	2.0^{A}
	T_2	0.0	0.0	0.0	0.0
	T_3	0.0	0.0	0.0	0.0

All data are expressed as the Mean±SD of three replicates, Data were subjected to one-way Analysis of variance (ANOVA) followed by post hoc test (Duncan's) at $p \leq 0.05$ level. Within the same column, means with superset different letters are significantly different; C (Starter only), T_1 (Starter and probiotics), T_2 (Starter and AEE), T_3 (Starter, AEE and probiotics), #: Significant from fresh (same row)

Controversially, pH decreased with the addition of AEE and *L. plantarum*, either alone or in combination; however their combination (T_3) recorded the lowest pH value, reflecting the highest acidity; this could be due to one or more reasons; the activity of starter and *L. plantarum* cultures improved as a positive feedback of AEE addition as evidenced from microbiological analyses as summarized in Table 4; also, AEE might play a role as a prebiotic for yogurt starter and probiotic

bacteria[39]; It was described that Ashwagandha contains many active phytochemicals, such as flavonoids and polyphenols[35] that may have an important role in increasing the growth and vitality of yogurt starter and probiotic bacteria. Moreover, this could explain the increase in both SN/TN ratio, TVFA, diacetyl and acetaldehyde values associated with enhanced activity of starter and *L. plantarum* in AEE-enriched yogurt samples. During storage time up to 15 days, all the tested parameters significantly increased except pH and acetaldehyde, which significantly decreased by storage. The pH decreases due to activity of starter and *L. plantarum* that enhanced in presence of the extract, while acetaldehyde decreases due to its conversion to ethanol, as confirmed with many previous reports[24,28,40]; interestingly, it was suggested that the addition of Ashwagandha improves the chemical quality of dairy products, as well as improves the health benefits as confirmed by biological studies[41].

Microbiological examination of yogurt with Ashwagandha and *L. plantarum*: In respect of lactic acid bacteria, a result in Table 4 illustrates the reasonable viability of yogurt starter culture and *L. plantarum* in functional yogurt supplemented with Ashwagandha Ethanolic Extract (AEE). The data indicated that supplementation of yogurt with AEE alone or in combination with probiotic bacteria (T_2, T_3) and progress storage time enhanced the viability of yogurt starter culture that was in agreement with Joung *et al*.,[42]. The viable cell counts of *S. thermophilus* were slightly higher than *L. bulgaricus* even in fresh yogurt or during storage time; this may be attributed to the effect of AEE on *Lactobacillus bulgaricus*[43]. Moreover, the results revealed that the presence of AEE in the yogurt markedly enhanced the growth of *L. plantarum* in the treatment combines yogurt starter with AEE (T_3) more than in yogurt without AEE (T_1), which makes the AEE considered as prebiotic and yogurt with *L. plantarum* as probiotic, this finding is consistent with David *et al*.[44] and Nazia and Rajinder[45]. Additionally, many *in vivo* studies have proven that the presence of *L. plantarum* supports various biological and clinical properties[46,47,2].

Table 5: Sensory evaluation of fortified and control yogurt at fresh and after 15 days cold storage

		Storage	
Property	Treatment	Fresh	15 days
Color and appearance	C	9.0 ± 1.0^{A}	9.0 ± 1.0^{A}
	T_1	9.0 ± 0.5^{A}	9.0 ± 0.0^{A}
	T_2	9.0 ± 0.0^{A}	9.0 ± 1.0^{A}
	T_3	9.0 ± 1.0^{A}	9.0 ± 0.5^{A}
Taste	C	6.0 ± 0.5^{B}	$7.0\pm0.5^{C\#}$
	T_1	6.5 ± 0.4^{AB}	$7.4\pm0.1^{C\#}$
	T_2	7.5 ± 1.5^{AB}	8.3 ± 0.3^{B}
	T_3	8.0 ± 0.5^{A}	$8.9\pm0.1^{A\#}$
Aroma	C	5.2 ± 0.2^{B}	$6.2\pm0.2^{B\#}$
	T_1	6.0 ± 0.5^{B}	6.8 ± 0.3^{B}
	T_2	7.4 ± 0.1^{A}	8.0 ± 0.5^{A}
	T_3	8.0 ± 1.0^{A}	8.5 ± 0.5^{A}
Mouth-feel	C	6.0 ± 1.0^{C}	6.4 ± 0.4^{C}
	T_1	6.5 ± 0.5^{BC}	6.9 ± 0.4^{C}
	T_2	7.5 ± 0.4^{AB}	8.2 ± 0.3^{B}
	T_3	8.2 ± 0.3^{A}	8.9 ± 0.1^{A}
Overall	C	6.1 ± 0.3^{B}	6.5 ± 0.5^{B}
	T_1	6.3 ± 0.3^{B}	6.8 ± 0.3^{B}
	T_2	7.4 ± 0.4^{A}	8.2 ± 0.3^{A}
	T_3	7.5 ± 0.5^{A}	8.4 ± 0.4^{A}

All data are expressed as the Mean±SD of three replicates, Data were subjected to one-way analysis of variance (ANOVA) followed by post hoc test (Duncan's) at $p\leq0.05$ level. Within the same column, means with superset different letters are significantly different, C (Starter only), T_1 (Starter and probiotics), T_2 (Starter and AEE), T_3 (Starter, AEE and probiotics), #: Significant from fresh (same row)

In respect of other microbiological examination, the current study indicated that adding both AEE and *L. plantarum* helped in increasing the total bacterial count. Furthermore, the results in Table 4 also showed that yogurt does not contain mold and yeast in all treatments until the age of 15 days of cold storage, while it appeared after 21 days in the unsupported yogurt as well as in yogurt supported with probiotic bacteria only, indicating the anti-fungal properties of AEE[48].

Sensory evaluation: Yogurts' sensory evaluation results (Table 5) declared that the addition of AEE did not disturb yogurts' color and appearance compared to the control, either at fresh or after15-day storage; while as significantly improved the taste and aroma. More favorable, probiotics greatly improved the taste and aroma by storage. The feeling in the mouth was significantly improved with fortification with AEE, probiotic, or their mixture; the later showed the most favorable feeling. In general, yogurt that fortified with the mixture had the highest overall acceptance; accordingly, yogurt and dairy products are a successful choice to deliver bio-compounds and active ingredients of AEE and probiotics.

Biological study: Aluminium is ubiquitous in the environment; its extensive industrial use provides us the impetus to scope its toxicity. For a long time, it was considered a nontoxic metal, but more attention has been focused on its adverse effects on human and animal health[49]. Despite its presence in drinking water during purification purposes, in

Table 6: Effect of fortified yogurt on serum hepatic and renal functions, lipid profile and inflammatory cytokines of $AlCl_3$-intoxicated rats

Parameters	Control	$AlCl_3$	$AlCl_3$ plus AEE-fortified yogurt	$AlCl_3$ plus probiotics fortified yogurt	$AlCl_3$ plus formula fortified yogurt
ALAT (U L^{-1})	29.7±4.3^{C}	74.0±4.1^{A}	47.0±4.0^{B}	50.2±2.6^{B}	38.7±1.9^{B}
ASAT(U L^{-1})	32.0±4.0^{C}	68.0±3.4^{A}	47.0±6.9^{B}	49.0±3.4^{B}	40.0±1.3^{B}
Urea (mg dL^{-1})	33.7±1.7^{C}	89.5±3.4^{A}	42.0±4.0^{B}	56.0±1.08^{B}	39.0±1.08^{B}
Creatinine (mg dL^{-1})	1.12±0.12^{C}	3.3±0.27^{A}	1.47±0.26^{A}	2.07±0.17^{A}	1.57±0.4^{A}
Total Chol (mg dL^{-1})	65.0±1.2^{D}	127.7±1.2^{C}	80.6±3.1^{B}	90.4±2.7^{B}	69.7±3.5^{B}
Trigly (mg dL^{-1})	59.7±2.2^{D}	103.7±2.6^{C}	79.0±1.8^{B}	80.0±2.7^{B}	75.2±1.6^{B}
LDL-c (mg dL^{-1})	42.0±2.1^{D}	83.0±1.4^{C}	40.0±2.1^{B}	45.0±2.4^{B}	35.7±0.75^{B}
HDL-c (mg dL^{-1})	41.2±1.3^{D}	28.5±1.3^{C}	35.8±1.2^{B}	36.7±1.1^{B}	34.5±0.6^{B}
TNF-α (ng L^{-1})	23.7±2.51^{C}	78.5±4.22^{A}	31.6±3.11^{C}	47.2±5.4^{B}	29.3±3.33^{C}
IL-1β (ng L^{-1})	785±32^{C}	1354±52^{A}	935±44^{C}	1121±56^{B}	887±42^{C}

ALAT: Alanine aminotransferase, ASAT: Aspartate aminotransferase, LDL-c: Low dense lipoprotein-cholesterol, HDL-c: High dense lipoprotein-cholesterol (TNF-α) tumor necrosis factor alpha, (IL-1β) interleukin-1 beta, All data are expressed as the Mean±SE of three replicates, Data were subjected to one-way analysis of variance (ANOVA) followed by post hoc test (Duncan's) at $p \leq 0.05$ level, Within the same row, means with superset different letters are significantly different

many processed foods, medicines and its use in food storage vessels, cans, which may increase its content, particularly in salty, acidic, or alkaline foods[50].

The objective of the present study was to explore the ability of AEE and probiotics alone or in combination to enhance yogurt acceptability and nutri-protective properties against $AlCl_3$-induced toxicity in male rats.

The obtained data (Table 6) revealed that $AlCl_3$-intoxicated animals recorded a significant rise in serum values of ALAT, ASAT, creatinine, urea, total cholesterol, triglycerides, LDL-C, TNFα and IL1β coupled with a notable drop in HDL-c in compare to control group; Favorably, treatment of rats with $AlCl_3$ together with yogurt fortified with either AEE or probiotics alone or in combination markedly restored the values of the above-mentioned measurements in compare to $AlCl_3$-intoxicated group. Animals treated with yogurt fortified with both AEE and probiotics showed the highest degree of improvement; meanwhile, fortification of yogurt with probiotics performed the lowest improving potential.

Fortunately, serum cholesterol and triglycerides were ameliorated by yogurt emulsions containing either AEE or probiotic alone or their combined formula this effect may be attributed to the enhancement of inflammatory status, lowering hepatic lipid content and increase of phosphorylation of 50-AMP-activated protein kinase and acetyl-CoA carboxylase, AEE and probiotic in the liver protects against fatty liver disease due to its anti-inflammatory potential[51].

The previous data indicated that Aluminium has a potential of toxicity in humans and animals[52], which may be mediated by free radical generation and alterations in antioxidant enzymes both *in vivo* and *in vitro*[53] leading to neurotoxicity[54] and hepatotoxicity[55]. Lipid peroxidation of biological membranes results in the loss of membrane fluidity, changes in membrane potential, an increase in membrane permeability and alterations in receptor functions. It is known that Aluminium bounds to transferrin (Fe^{3+}-carrying protein) thereby, rises free intracellular Fe^{2+} that leading to peroxidation of membrane lipids giving rise to membrane damage[11]; another study reported that Aluminium exposure could result in disruptions in mineral balance disturbances in which Aluminium ions/radii replace iron and magnesium ions resulting in the reduction of Fe^{2+} binding to ferritin[56]. Free iron ions released from biological complexes by Aluminium can catalyze hydrogen peroxide decomposition to hydroxyl radical according to Fenton's reaction[56]. Our study pointed out a significant elevation in serum ALAT and ASAT activities matched with a disturbance in the oxidative status of hepatic tissue that monitored from the highly increased hepatic MDA and NO and markedly decreased GSH, SOD and CAT post Aluminium chloride intoxication. These findings are inconsistent with Cheraghi and Roshanaei[57].

Xia *et al.*[58] reported that $AlCl_3$ exposure caused pathological changes in the glomerulus and renal tubule. Mohammed and Kahtani[59] stated that the kidney exerts an active oxidative metabolism, which results in the production of ROS. Abdelhamid[60] mentioned that $AlCl_3$ induces oxidative stress through the formation of ROS that could impair cell function and induce apoptosis. Our results illustrated that $AlCl_3$ caused oxidative stress in the kidney, which likely damaged the structure of the glomerulus and renal tubule; this achieved herein by the increased MDA and NO and reduced GSH, SOD and CAT of renal tissue); additionally, as urea is the end product of protein catabolism, thereby $AlCl_3$-induced liver dysfunction may be a factor of that effect. Aluminium decreases the GSH synthesis probable by decreasing the activity of glutathione synthesis, thus leading to decreased GSH level. On the other hand, it has been demonstrated that Aluminium can inhibit NADPH-generating enzymes such as NADP-isocitrate dehydrogenase and glucose 6-phosphate

Table 7: Effect of fortified yogurt on dopamine and serotonin levels as well as AchE-activity in the brain of $AlCl_3$-intoxicated rats

	Control	$AlCl_3$	$AlCl_3$ plus AEE-fortified yogurt	$AlCl_3$ plus probiotics-fortified yogurt	$AlCl_3$ plus formula fortified yogurt
Dopamine (pg g^{-1})	2309±49^{A}	1433±32^{D}	1892±39^{B}	1585±29^{C}	1625±28^{D}
Serotonin (pg g^{-1})	819±15.9^{A}	507±14.2^{C}	642±12.4^{B}	554±13.8^{C}	572±12.7^{C}
AChE (μmol/min/g)	6473±55^{C}	9606±49^{A}	7606±53^{C}	8568±45^{B}	6635±43^{D}

Ach-ase: acetylcholinesterase, All data are expressed as the Mean±SE of three replicates, Data were subjected to one-way Analysis of Variance (ANOVA) followed by post hoc test (Duncan's) at $p \leq 0.05$ level. Within the same row, means with superset different letters are significantly different

dehydrogenase. Since NADPH is shown to be a main factor for the GSH regeneration[60]; the higher intracellular Aluminium concentration reduced protein synthesis of antioxidant enzymes and subsequently reduced their activities[11]. Our results are in accordance with Newairy *et al.*[61].

Our data revealed that daily administration of rats with $AlCl_3$ led to a significant drop in both dopamine and serotonin level lined with a significant increase in AchE activity away from that of normal control. Interestingly, treatment of rats with yogurt fortified with either AEE or probiotics alone or in combination besides to $AlCl_3$ significantly reduced AchE activity and elevated dopamine and serotonin levels in the brains' homogenate when all were compared with $AlCl_3$-intoxicated group. Mixture-fortified yogurt recorded the highest improvement, but probiotics fortified yogurt achieved minimal restoration (Table 7). Our results showed that $AlCl_3$ caused significant decreases in dopamine and serotonin levels associated with a significant increase in AchE activity; these data are in agreement with those reported previously by Ramachandran *et al.*[62]. The role of oxygen free radicals in neurodegeneration and cognitive decline has been well reviewed[63]. Several findings suggest that Reactive Oxygen Species (ROS) can accumulate excessively in the brain and can severely attenuate the neuronal function[64]. Oxidative stress is therefore implicated as one of the causes of cognitive impairment[65]. Besides, chronic stress is said to promote oxidative stress and demolish antioxidant defense system of the brain[66], which may form the basis for impaired memory. In the present investigation, chronic $AlCl_3$treatment resulted in oxidative damage as indicated by increase lipid peroxidation and depletion of catalase and superoxide dismutase activity, thus strengthening the oxidative theory of cognitive deficits and its complications. In view of the current data, $AlCl_3$ administration provoked disturbance of metabolism indicated by increasing blood cholesterol and triglycerides. In agreement with the present study, Kalaiselvi *et al.*[67] reported disturbance of the lipid metabolism as a result of $AlCl_3$ toxicity in rats. Regarding the disturbed lipid profile in the current study, Wilhelm *et al.*[68] attributed the hypercholesterolemic and hyperlipidemia effect of $AlCl_3$ to the accumulation of $AlCl_3$ in liver, oxidative damage of liver and subsequent lipid metabolism disturbance. In the present study, $AlCl_3$ administration induced a significant increase in serum levels of TNF-α and IL- 1β. These findings are similar to those observed in other previous studies[69].

The oxidative stress status of the liver, kidney and brain showed that $AlCl_3$-intoxication resulted in a significant elevation in the oxidative markers (MDA and NO) level coupled with a marked reduction in the values of antioxidant buttery (GSH, SOD and CAT) in the three organs in comparison with those of normal control. In a promising manner, co-treatment of $AlCl_3$-intoxication rats with yogurt fortified with either AEE or probiotics alone or mixed efficiently reduced the level of oxidation markers (MDA and NO) and strongly restored the values of the anti-oxidative battery (GSH, SOD and CAT) in the studied organs' tissues towards the control group. Similarly, yogurt supplemented with both AEE and probiotics performed the highest alleviative capacity, meanwhile, probiotics fortified yogurt achieved minimal one (Table 8). The present study declared that the ingestion of AEE-fortified yogurt exhibited a protective effect against $AlCl_3$-induced hepato-renal damage, as it markedly modulated their oxidative status and functions. It was reported that AEE contains sitoindoside VIIX and Withaferin A, have antioxidant activity by enhancing the free radical scavenging enzymes such as, dismutase SOD, CAT, GPx and GSH[70]; also, AEE contains Withanolides, which have anti-inflammatory property, thereby may help protect against the liver and kidney damage[71]; all these effects are most likely due to the presence of some active ingredients in ashwagandha root which have antioxidant property. The major constituent of AEE analysis in this study is gallic acid that was suggested inducing the liberation of endogenous antioxidant factors and resistant agents (SOD and CAT enzymes as well as e-NOS and PGE2 non enzymatic); also, it able to reduce and inhibit the initiation of oxidative stress, expression of pro-inflammatory mediators (TNF-α) and expression of apoptotic agents[72]. Moreover, rutin, another constituent found in our AEE, had reduced the oxidative stress in the liver, kidney and brain tissues of rats[73]. Probiotics were found to be beneficial in liberating trophic factors, enzymes and proteins during their intestinal transit so contribute to improving the host immune, defense, digestion and absorption of nutrients[74]. Moreover, a significant improvement in hepatic-renal functions and oxidative status

Table 8: Effect of fortified yogurt on oxidative stress markers of hepatic, kidney and brain tissues of $AlCl_3$-intoxicated rats

Organ	Oxidative stress marker	Control	$AlCl_3$	$AlCl_3$ plus AEE fortified yogurt	$AlCl_3$ plus probiotics fortified yogurt	$AlCl_3$ plus formula fortified yogurt
Liver	MDA (μmol g^{-1})	1.65 ± 0.24^{C}	3.47 ± 0.75^{A}	1.86 ± 0.58^{B}	2.1 ± 0.64^{B}	1.91 ± 0.54^{B}
	NO (nmol g^{-1})	150 ± 35.4^{C}	332 ± 50.9^{A}	197 ± 16.9^{B}	225 ± 15.7^{B}	184 ± 26.5^{B}
	GSH (μmol g^{-1})	350 ± 10^{A}	149 ± 11^{A}	210 ± 13^{C}	186 ± 12^{D}	270 ± 10^{C}
	SOD (U g^{-1})	782 ± 22^{D}	324 ± 34^{A}	615 ± 28^{C}	482 ± 32^{D}	664 ± 21^{B}
	CAT (μmol/min/g)	2240 ± 47^{D}	886 ± 45^{A}	1701 ± 39^{C}	1243 ± 38^{C}	1945 ± 40^{C}
Kidney	MDA (μmol g^{-1})	1.44 ± 0.22^{E}	3.01 ± 0.61^{A}	1.89 ± 0.49^{C}	2.33 ± 0.46^{B}	1.67 ± 0.34^{D}
	NO (nmol g^{-1})	215 ± 21.4^{E}	427 ± 15.6^{A}	292 ± 14.7^{C}	334 ± 19.5^{B}	218 ± 14.7^{D}
	GSH (μmol g^{-1})	410 ± 22.2^{E}	159 ± 39^{A}	295 ± 18.7^{D}	228 ± 14.8^{B}	360 ± 22^{C}
	SOD (U g^{-1})	755 ± 27	342 ± 21^{E}	580 ± 28^{C}	455 ± 29^{D}	677 ± 23^{B}
	CAT(μmol/min/g)	1975 ± 23^{E}	720 ± 19^{A}	1412 ± 23^{C}	1142 ± 19.4^{B}	1632 ± 14.6^{D}
Brain	MDA (μmol g^{-1})	41.1 ± 2.95^{E}	65.7 ± 3.84^{D}	51.8 ± 3.86^{C}	54.2 ± 2.55^{B}	49.7 ± 2.89^{D}
	NO (nmol g^{-1})	38.5 ± 3.6^{E}	109.6 ± 6.1^{A}	63.4 ± 5.9^{C}	71.6 ± 4.1^{B}	52.8 ± 4.9^{D}
	GSH (μmol g^{-1})	302 ± 10.9^{A}	153 ± 8.7^{E}	223 ± 7.7^{C}	194 ± 8.9^{D}	264 ± 10.5^{B}
	SOD (U g^{-1})	28405 ± 298^{E}	12983 ± 274^{A}	22457 ± 28^{C}	19150 ± 267^{B}	26169 ± 341^{D}
	CAT(μmol/min/g)	18781 ± 291^{A}	$10022\pm184^{E}\pm242^{C}$	15242 ± 233^{C}	13350 ± 212^{D}	16740 ± 277^{B}

GSH: Reduced glutathione, SOD: Superoxide dismutase, CAT: Catalase, MDA: Malondialdehyde, NO: Nitric oxide, All data are expressed as the Mean±SE of three replicates. Data were subjected to one-way Analysis of Variance (ANOVA) followed by post hoc test (Duncan's) at $p\leq0.05$ level. Within the same row, means with superset different letters are significantly different

was observed in probiotics-fortified yogurt treated rats; these results come in accordance with Emam *et al*.,[75]. Also, Mahfouz *et al*.[76] found that treatment with probiotics strains of some *Lactobacilli* helps to prevent low-grade inflammation and liver and kidney disorders.

Interestingly, treatment of rats with yogurt emulsions containing either AEE or probiotic alone or their combined formula in combination with $AlCl_3$-intoxication markedly protected rats against $AlCl_3$-induced brain deterioration indicating its radical scavenging activity. The improvement of the levels of hepato-renal-neuro SOD, GSH and MDA showed that AEE and probiotic could effectively decrease oxidative stress reactions. SOD is one of the potent antioxidant enzymes in cells and can catalyze the conversion of superoxide ions into oxygen and hydrogen peroxide[77]. GSH is a major non-enzymatic scavenger that can regulate intracellular redox homeostasis and the mechanistic studies on $AlCl_3$-induced hepato-renal-neuro damage revealed that GSH conjugation played an important role in the clearing of toxic metabolites. Lipid peroxidation is closely related to the pathogenesis of hepatic injury by the free radical derivatives of $AlCl_3$ and mainly leads to cell membrane damage and the consequent release of markers of hepatorenal-neurotoxicity[78]. MDA is the final product of lipid peroxidation, so it is a key marker of lipid peroxidation. After the treatment of rats with yogurt containing either AEE or probiotic alone or their combined formula treatments, the levels of SOD and GSH significantly increased by AEE and probiotic and the level of MDA significantly reduced by AEE and probiotic. Therefore, AEE and probiotic treatment had significant antioxidant properties as hepato-, nephron- and neuroprotective agents.

CONCLUSION

The present study concluded that AEE and probiotics markedly improved yogurts' physicochemical properties and sensory qualities. Also, both possess valuable health benefits as they restored $AlCl_3$-induced hepato-renal-neuro deteriorations; therefore, they serve as very promising-supplements for the protection against different systemic toxicities.

SIGNIFICANCE STATEMENT

This study discovers that the yogurt supplemented by ashwagandha extract, an effective vector of the active compounds contained in the extract, which can be beneficial for the prevention of toxicities and improves public health. This study will help the researcher to uncover the critical areas of $AlCl_3$-induced hepato-renal-neuro deteriorations that many researchers were not able to explore. Thus, a new theory on these beneficial extract and probiotic bacteria combinations in the same product may be arrived at.

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