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Acid curd (Karish) cheese supplemented with ashwagandha and/or probiotics: Modulatory efficiency on induced behavioral and neurochemical changes in rats

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Abstract:
Neurodegenerative disorder leads to a progressive memory loss that has only limited known medications. The use of ashwagandha, probiotics, or their combination may improve cholinergic activity, consequently providing therapeutic potency against amnesia and neuroplasticity disorders. We aimed to explore the modulatory benefits of ashwagandha extract and probiotics against induced behavioral and neurochemical retardations.
Acid curd (Karish) cheese samples were supplemented with ashwagandha extract and/or probiotics and subjected to chemical, microbiological, rheological, sensorial, and biological investigations by standard techniques.
The supplementation of Karish cheese with ashwagandha never deteriorated its chemical composition or rheological parameters. On the contrary, it exerted high antioxidant and phenolic potentials. Also, ashwagandha extract performed antimicrobial action against the tested pathogenic bacteria and showed better prebiotic effects with *Lactobacillus plantarum*. The biological study revealed that treating dementia-modeled rats with Karish cheese supplemented with ashwagandha and/or probiotics resulted in a detectable improvement in the behavioral and neurochemical measurements. However, the cheese supplemented with a formula of ashwagandha and probiotics had the greatest regenerating effect.
The supplementation of Karish cheese with ashwagandha and/or probiotics exhibited a modulatory efficiency against experimentally induced behavioral and neurochemical disorders.

Keywords: Ashwagandha, Karish cheese, *Lactobacillus plantarum*, probiotic, therapeutic effect, dementia, rats

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INTRODUCTION

White soft cheese is a rudimentary product at the breakfast table and a meal, or a basic sandwich, for school children at the beginning of the day [1]. Acid curd, or Karish, cheese is an excellent choice of white soft cheeses mixed with many natural additives such as herbs, spices, or vegetables. Made from defatted milk, acid curd cheese can also be added to edible oils, with olive oil being the most famous. So, this cheese can be used with many additives that increase its health and nutritional value [2]. Several studies have shown that fermented dairy products improve memory functions. In addition, individuals who consume low-fat dairy products, including acid curd (Karish) cheese and yogurt (at least once a week), have a higher level of awareness than others. A close link has also been found between the consumption of fermented dairy products and a lower risk of dementia [3–6].

The physiological effects of fermented dairy products are due to the presence of fatty acids and bioactive peptides. They are naturally released during the fermentation process that occurs due to the presence of lactic acid bacteria in milk and its products [7].

Alzheimer's disease has serious effects on the patient, their family, and society. It is linked with severe cognitive damage and some metabolic defects. Prevention is the best way to fight this disease since its

treatment can neither delay nor stop its progress [8]. Studies indicate links between lower pathogens, lifestyle changes, lower injury rates, and better prevention [9]. Accordingly, following a healthy diet and exercising are some of the most successful ways to avoid many diseases to date [10]. Using herbs and medicinal plants in food for health benefits has been a trend over the past few years. Since milk and dairy products are commonly preferred by different segments of society, they are among the most important carriers of phytochemicals present in herbs (mainly polyphenols) for health benefits [1, 11].

Ashwagandha (*Withania somnifera* L.), Indian ginseng or winter cherry, has many health and medical benefits, both therapeutic and protective [12]. It can efficiently prevent thyroid dysfunction, reduce its complications for the nervous system, and treat hypertension [13, 14]. Ashwagandha is also considered an adaptogen, a memory enhancer, and a cardiovascular protector. It is known for its antioxidant, anxiolytic, antiparkinsonian, antivenom, anti-inflammatory, antitumorous, immunomodulatory, hypolipidemic, and antibacterial properties [15]. Ashwagandha extract was reported to possess antioxidant and anti-inflammatory effects against aluminum neurotoxicity, reduce cholinergic activity by maintaining normal acetylcholine esterase activity, and enhance memory [16]. It is added to many formulations to increase energy, improve overall health and longevity, as well as prevent various diseases. The dried roots of the plant are used to treat nervous and sexual disorders [17].

Probiotics have a positive effect on psychological well-being through enhancing human mood and sleep quality, etc. [18–20]. Different studies on experimental animals indicated a close relation between probiotic consumption and cognitive function. For example, a 12-week probiotic consumption had a positive effect on the cognitive function and some metabolic conditions in Alzheimer's patients [21]. Many previous studies confirmed anti-Alzheimer's properties of *Lactobacillus plantarum* [22]. In particular, this probiotic enhances the production of acetylcholine neurotransmitter, prevents memory deficit, and improves learning ability [23]. Combining probiotics, mainly *L. plantarum*, with ashwagandha extract as a supplement has proven to have a positive effect against aluminum chloride-induced neurotoxicity. Recently, Mustafa *et al.* confirmed the amelioration effect of ashwagandha extract combined with a probiotic strain in bio-yogurt against $AlCl_3$ neurotoxicity in rats [24]. In this regard, we aimed to evaluate the effect of ashwagandha ethanolic extract (AshEE) on the properties of acid curd (Karish) cheese as a milk model system. For this, AshEE and/or a probiotic were added to UF-retentate to deliver health benefits. The supplemented cheese was subjected to chemical, physical, textural, and microbiological determination, as well as a biological evaluation for cognitive or learning difficulties in rats.

STUDY OBJECTS AND METHODS

Our materials were obtained from the following sources:

– skimmed UF-retentate: from the Animal Production Research Institute, the Agriculture Research Center (Giza, Egypt);
– ashwagandha (*Withania somnifera* L., NCBI: txid126910) roots: from Imtenan Co. (Giza, Egypt);
– aluminum chloride ($AlCl_3$, 99%, BN AC196): from Alpha-Chemika (India);
– *Bacillus cereus* (ATCC133018), *Salmonella typhimuum* 9027, and *Staphaurius* (ATCC 25175): from the stock cultures of the Agricultural Research Centre (Giza, Egypt);
– *Escherichia coli* O157:H7 (ATCC 6933), *Listeria monocytogenes* V7, and *Yersenia enterocolitica* (ATCC9610TM): from Liofilchem S.r.l. (Rosetodegli Abruzzi, TE, Italy);
– *Streptococcus thermophiles*, *Lactobacillus delbrueckii* spp. *bulgaricus*, and *Lactobacillus casei*: from the stock cultures of the Dairy Microbiology Lab., the National Research Centre (Giza, Egypt);
– *Lactobacillus rhamnosus* (Tistr 541), *Lactobacillus plantarum* (Dsaz 0174), and *Lactobacillus reuteri* (B-14171): from the Cairo Microbiology Resources Center, the Faculty of Agriculture at Ain Shams University;
– *Bifidobacterium bifidum* (Bb-12) and *Lactobacillus acidophilus* (N4495): from Chr. Hansen's Lab. A/S (Copenhagen, Denmark).

Based on the microbiological results, *L. plantarum* was selected for this study.

Ashwagandha ethanolic extract (AshEE) was prepared as previously described by Mustafa *et al.* [24]. In brief, ashwagandha roots were milled into fine powder, soaked in ethanol (70%), and stirred overnight. The mixture was then filtered (Whatman No. 1) and the solvent was evaporated using a rotary evaporator (under reduced pressure at 40°C), whereas the water residuals were freeze-dried. Finally, the dry extract was stored at –20°C until further *in vitro* and *in vivo* assessments.

The **total phenolic content** of ashwagandha ethanolic extract was analyzed spectrophotometrically by the modified Folin-Ciocalteu colorimetry and expressed as catechin equivalents using the catechin standard curve [24].

Radical scavenging activity. The capacity of ashwagandha antioxidants to quench the DPPH radical was determined as described by Salama *et al.* [25]. The radical scavenging activity (RSA, %) of the extract was calculated according to the following Eq.(1):

$$RSA = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

Preparation of acid curd (Karish) cheese. Karish cheese was made by using skimmed UF-retentate heat-treated at 90°C/5 min and cooled to 42°C. For the experiments, 200 mg of ashwagandha ethanolic extract (AshEE) was added to one litter of UF-retentate, then inoculated with a 2% active starter-mixture and/or a 2% probiotic (*L. plantarum*). The cheese treatments were

divided as follows: 1) the control (C) contained the active starter-mixture (without AshEE); 2) the first treatment (T1) contained the active starter-mixture and the probiotic (*L. plantarum*); 3) the second treatment (T2) contained the active starter-mixture and AshEE; 4) the third treatment (T3) contained the active starter-mixture, the probiotic (*L. plantarum*), and AshEE. The different treatments were packed into plastic cubs (100 mL) and left at 42°C until complete coagulation. After coagulation, the treated cheeses were cooled and stored at 5°C until sensory, chemical, and microbiological analyses before and during storage [2].

Chemical analysis. The cheese samples were analyzed for the content of moisture, fat, total solids, total nitrogen, soluble nitrogen/total nitrogen ratio, and ash according to the Association of Official Analytical Chemists [26]. The pH value was measured using a digital laboratory pH-meter. Diacetyl and acetaldehyde levels were determined spectrophotometrically.

Antioxidant activity and total phenolic compounds. The antioxidant activity and total phenolic content were determined in both the pure ashwagandha ethanolic extract and the supplemented cheese samples according to Salama *et al.* [25].

The texture profile analysis of all the cheese samples was carried out according to the standards of the International Dairy Federation [27].

Screening for anti-microbial activity. The anti-bacterial activity of ashwagandha ethanolic extract (AshEE) against pathogenic strains was evaluated according to Tepe *et al.* [28]. For this, the crude dry extract was considered as level 100%; AshEE was diluted with distilled water to obtain levels 50 and 25%.

Evaluation of prebiotic activity. The prebiotic activity of ashwagandha ethanolic extract was based on the growth of the given probiotic strains according to the methods of Lourence & Viljoen [29].

Microbiological analysis. The total aerobic colony count, as well as mold, yeast, and coliform counts of the cheese samples were microbiologically examined as previously described by El-Shenawy *et al.* during different storage periods [30].

Sensory evaluation. The sensory properties of the cheese samples during different storage periods were evaluated according to Salama [1]. Ten (4 women (35–55 years) and 6 men (25–60 years)) expert panelists from the staff of the Dairy Science Department at the National Research Center (Egypt) were previously trained with commercial samples of cheese according to Cost-95. They evaluated appearance (10), body & texture (40), and flavor (50) of the cheese samples. The cheese samples were cut into cubes (1.5×1.5×1.5 cm), covered with plastic wrap to prevent dehydration, coded with three-digit random numbers, and held for at least 1 h at 20°C to equilibrate. Each panelist was given three cheese cubes to score each sample on the hedonic scale. The evaluation took place in a tasting room equipped as specified in Standard IS0 8589 (1988).

Animals and experimental study. Thirty adult female Wistar albino rats (150–200 g) were obtained from the Animal Colony of the Research Institute of Ophthalmology, Egypt. They were housed in suitable plastic cages and maintained under controlled conditions (temperature 25 ± 2°C, humidity 50 ± 5%, and 12 h light-dark cycles). The rats had free access to a commercial ration used as a basal diet and water ad libitum. After seven days (acclimatization period), they were randomly assigned into five groups (6 animals each) as follows. Group I included rats that orally received 2 mL/kg/day of starter-emulsified cheese for one month and served as the control. Group II included rats orally intoxicated with $AlCl_3$ (300 mg/kg/day) for one month to induce dementia [31]. Group III included rats that orally received 2 mL/kg/day (equivalent to 10^9 CFU/kg/day) of probiotic/starter-emulsified cheese in addition to intoxication with $AlCl_3$ for one month [32]. Group IV included rats that orally received 200 mg/kg/day of ashwagandha ethanolic extract (AshEE)-emulsified cheese alongside their intoxication with $AlCl_3$ for a similar period [24]. Group V included rats that orally received 2 mL/kg/day of starter/probiotic/AshEE-emulsified cheese, as well as being $AlCl_3$-intoxicated for a month. All the animals were gavaged once a day, with the doses adjusted on a weekly basis according to their body weights. At the end of the experiment, behavioral observations and biochemical measurements were conducted.

Behavioral profile. ***Open field test.*** In this test, the rats were gently placed into a corner of a cleaned and sterilized planed arena and observed for 3 min. Both exploratory behaviors (ambulation and crossing of squares as well as rearing) and non-exploratory measures (grooming) were scored as absolute counts [33].

Modified elevated plus maze test. Depending on the aversion of rats to the open space, their spatial long-term memory was measured as described by Hliňák & Krejčí [34].

The ***novel object recognition test*** (hippocampus-dependent memory impairment) was performed via an automated tracking of rats with a video tracking system (Anymaze 4.20, Stoelting, USA). All exploratory actions were measured automatically and manually as explained by Gümüş [35].

Y-maze test. The short-term memory and locomotor activity were measured according to Wright [36]. The rats were video-tracked for 5 min using a video tracking system (Anymaze 4.20, Stoelting, USA) that recorded the number of arm entries and the distance travelled by each animal. The alternation percent was calculated according to the Eq. (2):

$$\text{Alternation persent} = \frac{\text{Total alternations}}{\text{Entries number} - 2} \times 100 \qquad (2)$$

Brain tissue sampling. After the last administration and behavioral tests, the animals were fasted overnight and euthanized by sudden decapitation. The brains of some animals (in each group) were dissected out and anatomized into two similar halves. The first half was

homogenized in phosphate buffer (0.1 M, pH 7.4) to determine oxidative stress markers. The second half was homogenized in 0.1 M perchloric acid containing 3, 4-dihydroxybenzylamine at a final concentration of 25 ng/mL to assess acetylcholinesterase activity and the level of biogenic amines. The brains of the rest of the animals (in each group) were dissected out and immersed in formaldehyde-saline (10 % v/v) buffer for histopathological examination.

Acetylcholinesterase activity, dopamine, and serotonin. Acetylcholinesterase activity was determined according to the modified method of Ellman [37]. Dopamine and serotonin levels were measured by high-performance liquid chromatography (Waters, Milford, USA) following the method of Kim [38].

Oxidative stress status. Oxidative stress markers (glutathione, nitric oxide, malondialdehyde, and superoxide dismutase) were determined in brain phosphate buffer homogenates using reagent kits obtained from Biodiagnostic (Giza, Egypt).

Histopathological examination. Formaldehyde-saline (10 % v/v) buffer-immersed brains were hydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin. Then, 5-µm-thick sections were cut and stained with hematoxylin and eosin for pathohistological examination under light microscopy.

Statistical analysis. Comparisons between means were carried out using one-way ANOVA, followed by a post-hock (Duncan) multiple comparisons test at $p \leq 0.05$, using the statistical analysis system (SAS) program software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The total antioxidant capacity and total phenolic content of ashwagandha ethanolic extract (AshEE) are presented in Fig. 1. As can be seen, AshEE can be considered an excellent source of antioxidants and phenolic compounds, as previously reported by Munir *et al.* [39]. In addition, the probiotic (*Lactobacillus plantarum*) and starter used in our study performed a detectable antioxidant activity resulting from metabolic and milk protein hydrolyses via the spontaneous action of the starter and beneficial bacterial strains, as previously reported by Ali *et al.* and Wang *et al.* [40, 41].

Figure 2 illustrates the main chemical analysis of different acid curd (Karish) cheese samples supplemented with AshEE and/or probiotic bacteria. As we can see, neither AshEE nor the probiotic bacteria made any changes in the chemical composition of manufactured Karish cheese, as previously reported [1, 22].

Antimicrobial activity. Table 1 illustrates the antimicrobial activity of ashwagandha ethanolic extract (AshEE) at different concentrations (25, 50, and 100%) evaluated against the selected foodborne microorganisms. The AshEE showed a varying ability to inhibit the growth of the pathogenic strains, depending on its concentration. Particularly, the highest concentration (100%) exhibited high antimicrobial activity against all the selected micro-

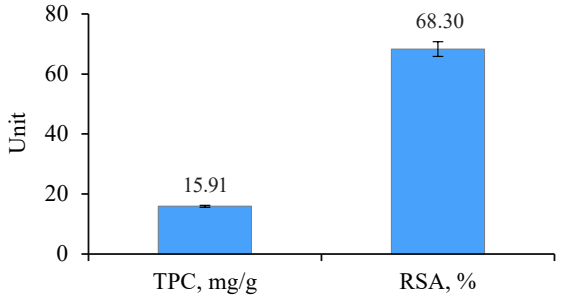


Figure 1 Total pnolic content (TPC) and radical scavenging activity (RSA) of ashwagandha ethanolic extract

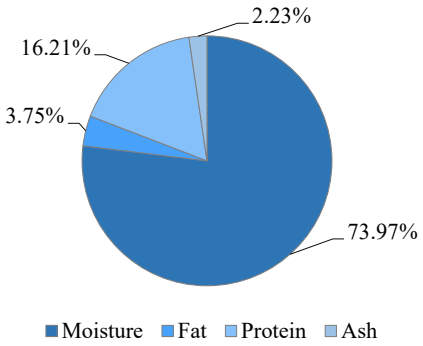


Figure 2 Chemical analyses of acid curd (Karish) cheeses supplemented with ashwagandha ethanolic extract, probiotic bacteria, or their combination

Table 1 Antimicrobial activity of ashwagandha ethanolic extract (AshEE), mm

Bacterial strains	Inhibition zone, mm		
	Pure AshEE	Diluted AshEE 50%	Diluted AshEE 25%
Escherichia coli O157:H7	$7.0^{Af} \pm 0.3$	$2.0^{Bf} \pm 0.4$	$2.0^{Bd} \pm 0.4$
Yersenia enterocolitica ATCC 9610	$15.0^{Ad} \pm 0.2$	$12.0^{Bc} \pm 0.6$	$6.0^{Cb} \pm 0.4$
Salmonella typhimurium ATCC 9027	$12.0^{Ae} \pm 0.8$	$6.0^{Be} \pm 0.3$	$3.0^{Cc} \pm 0.2$
*Listeria monocytogenes*V7	$20.0^{Ac} \pm 0.1$	$8.0^{Bd} \pm 0.4$	$3.0^{Cc} \pm 0.7$
Staphylococcus aureus ATCC 25175	$22.0^{Ab} \pm 0.7$	$15.0^{Bb} \pm 0.1$	$3.0^{Cc} \pm 0.1$
Bacillus cereus ATCC 33018	$25.0^{Aa} \pm 0.3$	$20.0^{Ba} \pm 0.7$	$12.0^{Ca} \pm 0.3$

The data are represented as mean ± SD. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within a row, the means with different capital (A, B…) superscript letters are significantly different. Within a column, the means with different small (a, b, c) superscript letters are significantly different

organisms, especially *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes* (gram-positive), with inhibition zones of 25, 22, and 20 mm, respectively. The minimum inhibition zones of 7 and 12 mm, however, were noted with *Escherichia coli* and *Salmonella* (gram-negative), respectively. These findings confirmed the previous results demonstrated in [42, 43]. According to Khanchandani *et al*., AshEE is more effective against gram-positive than gram-negative bacteria [43]. This might be due to bioactive compounds in AshEE that confer resistance against microbial pathogens. Alternatively, AshEE contains phenolic compounds associated with antimicrobial efficiency through their hyper-acidification effect at the plasma membrane interface of the pathogen, consequently disrupting the cell wall synthesis [42]. More than 80 chemical compounds have been found in AshEE, mainly alkaloids, flavone glycosides, steroidal lactones, and polyphenols [44–46]. These compounds play an active role in antibiotic, anti-inflammatory, and cytotoxic activities [14]. A recent study has demonstrated that AshEE showed antagonistic potential against pathogenic bacteria due to the presence of endophytes, which have bio-control potential against pathogens [44].

Antioxidant activity and total phenolic content. Interestingly, our study evidenced that adding ashwagandha ethanolic extract (AshEE) and probiotic bacteria to Karish cheese improves its antioxidant activity and total phenolic content (Table 2). Among the fresh cheeses, the highest antioxidant activity and total phenolic content (6.49% and 6.24 mg/100 g, respectively) were found in the AshEE/probiotic-supplemented sample (T3), followed by the AshEE-treated cheese without probiotic bacteria (T2) and the probiotic-supplemented sample without AshEE (T1). The lowest values were found in the control. However, 15 days of cold storage significantly decreased the antioxidant activity and total phenolic content of all the cheese treatments. The values were still quite high though, with the lowest found in the control (1.94% and 4.89 mg/100 g, respectively). The decrease in antioxidants and total phenols during storage agrees with the previous studies [24, 25].

Physicochemical properties of acid curd (Karish) cheese. Table 3 shows the physicochemical analysis of different treatments of Karish cheeses before and after 15 days of cold storage. In the fresh samples, the soluble nitrogen/total nitrogen ratio significantly increased in the cheese treated with ashwagandha ethanolic extract (AshEE) and probiotic bacteria, while being equal in the control and the probiotic-supplemented cheese (T1). During cold storage (7 and 15 days), the soluble nitrogen/total nitrogen ratio also significantly increased. This is attributed to the addition of AshEE, which markedly enhanced the effect of starter and probiotic bacteria as a prebiotic improving the growth and fermentation. This finding clarifies the upsurge of the soluble nitrogen/total nitrogen ratio in the fresh samples or during storage [47, 48].

The pH values decreased slightly between the fresh samples as AshEE was added alone or in combination with probiotic bacteria. However, they decreased significantly throughout storage up to 15 days. As mentioned before, the decrease in pH spontaneously resulted from the action of the starter and probiotic bacteria, which was markedly enhanced by the presence of AshEE.

Total volatile fatty acids and diacetyl values increased significantly among the treatments, as well as during storage, with the highest values noted in the cheeses treated with AshEE and/or probiotics.

Acetaldehyde values significantly elevated in the fresh treatments and markedly decreased during storage, with the lowest values recorded at the end of storage. The samples supplemented with AshEE and/or probiotics had higher acetaldehyde levels compared to the control.

These physicochemical changes were consistent with several previous studies [25, 49, 50].

Texture analysis is an important test that reflects the product's acceptability, varying with the type of cheese. Figure 3 presents the texture profile of acid curd (Karish) cheeses supplemented with ashwagandha ethanolic extract (AshEE) and/or probiotic bacteria. While no significant differences were observed in the examined texture profile parameters among the treatments, there was a slight difference when comparing them with the control. This difference might be due to the addition of AshEE, probiotics, and/or starter culture. Alternatively, it could result from the changes in pH and soluble nitrogen/total nitrogen ratios that affected the texture profile during storage. It was previously reported that the texture profile correlated with the physicochemical changes and the chemical composition of Karish cheese [51]. Generally, the addition of AshEE and probiotic bacteria does not have a significant effect on the texture properties compared with the control.

Sensory evaluation is one of the most important tests that determines the extent of acceptance of the

Table 2 Antioxidant activity and total phenolic content of acid curd (Karish) cheese supplemented with ashwagandha ethanolic extract and/or probiotic bacteria before and after 15 days of cold storage

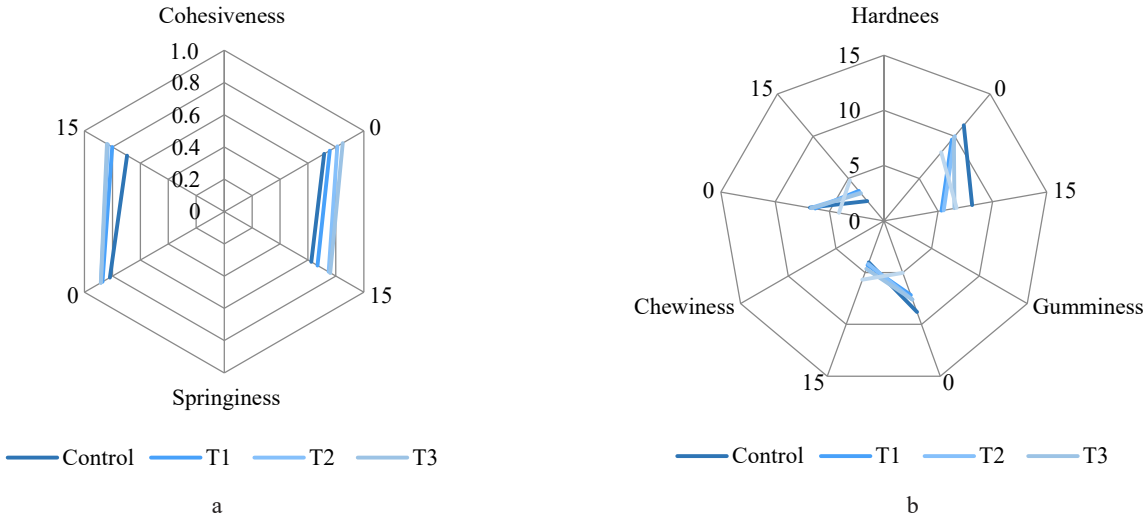
Property	Treatment	Storage	
		Fresh	15 days
Antioxidant activity, %	Control	4.97 ± 0.13^{B}	1.94 ± 0.03^{D}#
	T1	6.34 ± 0.09^{A}	2.25 ± 0.10^{C}#
	T2	6.39 ± 0.07^{A}	2.57 ± 0.13^{B}#
	T3	6.49 ± 0.06^{A}	3.51 ± 0.12^{A}#
Total phenolic content, mg/100 g	Control	5.80 ± 0.13^{B}	4.89 ± 0.04^{C}#
	T1	5.94 ± 0.13^{B}	5.28 ± 0.03^{B}#
	T2	5.96 ± 0.04^{B}	5.87 ± 0.09^{A}
	T3	6.24 ± 0.05^{A}	5.89 ± 0.10^{A}#

The data are expressed as mean ± SD of three replicates. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same column, the means with different superscript letters are significantly different; Control (starter only); T1 (starter and probiotics); T2 (starter and AshEE); T3 (starter, AshEE, and probiotics). Within the same row, # is significantly different from the fresh sample

Table 3 Physicochemical properties of acid curd (Karish) cheese supplemented with ashwagandha ethanolic extract (AshEE), probiotic bacteria, or their combination before and during cold storage

Property	Treatment	Cold storage		
		Fresh	7 days	15 days
Soluble nitrogen/total nitrogen ratio	Control	7.47 ± 0.02^{C}	8.07 ± 0.05^{D}#	9.41 ± 0.08^{C}#
	T1	7.47 ± 0.02^{C}	8.29 ± 0.08^{C}#	9.58 ± 0.13^{C}#
	T2	7.83 ± 0.01^{B}	9.19 ± 0.01^{B} #	13.29 ± 0.01^{B} #
	T3	7.91 ± 0.06^{A}	9.30 ± 0.02^{A}#	14.44 ± 0.01^{A}#
pH	Control	5.27 ± 0.01^{A}	$5.13 \pm 0.03^{A\#}$	4.76 ± 0.04^{A} #
	T1	5.25 ± 0.05^{A}	$5.03 \pm 0.02^{B\#}$	4.69 ± 0.01^{B} #
	T2	5.15 ± 0.04^{B}	4.95 ± 0.02^{C}#	4.58 ± 0.00^{C}#
	T3	5.18 ± 0.03^{B}	4.88 ± 0.01^{D}#	4.49 ± 0.01^{D}#
Total volatile fatty acids, mL/100 g	Control	5.00 ± 0.20^{D}	8.00 ± 0.10^{D}#	10.00 ± 0.20^{D}#
	T1	8.00 ± 0.10^{C}	11.00 ± 0.02^{C}#	14.00 ± 0.40^{C}#
	T2	10.00 ± 0.40^{B}	15.00 ± 0.30^{B}#	17.50 ± 0.85^{B}#
	T3	13.00 ± 0.20^{A}	18.00 ± 0.20^{A}#	21.00 ± 0.20^{A}#
Diacetyl, µm/100g	Control	25.20 ± 0.20^{D}	34.18 ± 0.32^{C}#	39.20 ± 0.30^{D}#
	T1	28.00 ± 0.50^{C}	35.67 ± 0.22^{B}#	42.40 ± 0.20^{C}#
	T2	29.60 ± 0.20^{B}	36.97 ± 0.08^{A}#	46.40 ± 0.40^{B}#
	T3	31.60 ± 0.10^{A}	37.33 ± 0.21^{A}#	48.40 ± 0.20^{A}#
Acetaldehyde, µm/100 g	Control	20.84 ± 0.11^{D}	16.56 ± 0.24^{D}#	5.06 ± 0.03^{D}#
	T1	23.80 ± 0.16^{C}	19.75 ± 0.10^{C}#	7.70 ± 0.01^{C}#
	T2	25.60 ± 0.60^{B}	21.48 ± 0.16^{B}#	9.06 ± 0.04^{B}#
	T3	28.62 ± 0.02^{A}	24.23 ± 0.28^{A}#	12.82 ± 0.28^{A}#

The data are expressed as mean ± SD of three replicates. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same column, the means with different superscript letters are significantly different; within the same row, # is significantly different from the fresh samples. C (starter only), T1 (starter and probiotics), T2 (starter and AshEE), T3 (starter, AshEE, and probiotics)



Control – starter only; T1: starter and probiotics; T2: starter and AshEE; T3: starter, AshEE, and probiotics

Figure 3 Texture profile of Karish cheeses supplemented with ashwagandha ethanolic extract (AshEE) and/or probiotic bacteria: (a) cohesiveness & springiness; (b) hardness, chewiness, & gumminess

product by the consumer. Table 4 shows different sensory properties of the Karish cheeses fortified with ashwagandha ethanolic extract (AshEE) and/or probiotics before and during refrigerated storage up to 15 days. As can be seen, the addition of AshEE and/or probiotic bacteria had no effect on the appearance of the fresh or cold-stored samples compared to the control. Neither did we find any significant changes in the body, texture, or taste of the treated samples. The total acceptance of the cheeses showed significant differences, with T3 given the highest acceptance, followed by T2, T1, and the control. Generally, all the samples were acceptable. This may be because we added a minimal amount of AshEE that did not affect the taste, appearance, or any of the

Table 4 Sensory properties of acid curd (Karish) cheeses supplemented with ashwagandha ethanolic extract (AshEE) and/or probiotic bacteria before and during cold storage

Sensory properties	Storage period	Cheese treatments			
		Control	T1	T2	T3
Appearance (10)	Fresh	$8.50^{A} \pm 0.89$	$8.50^{A} \pm 1.50$	$8.50^{A} \pm 0.50$	$8.50^{A} \pm 1.50$
	7 days	$8.50^{A} \pm 0.89$	$8.50^{A} \pm 1.50$	$8.50^{A} \pm 0.50$	$8.50^{A} \pm 1.50$
	15 days	$8.50^{A} \pm 0.89$	$8.50^{A} \pm 1.50$	$8.50^{A} \pm 0.50$	$8.50^{A} \pm 1.50$
Body and texture (40)	Fresh	$34.00^{A} \pm 3.00$	$35.00^{A} \pm 5.00$	$36.00^{A} \pm 2.00$	$36.50^{A} \pm 2.50$
	7 days	$34.80^{A} \pm 2.20$	$36.50^{A} \pm 1.50$	$36.80^{A} \pm 2.20$	$37.00^{A} \pm 2.00$
	15 days	$35.40^{A} \pm 3.40$	$37.60^{A} \pm 1.40$	$37.45^{A} \pm 1.55$	$37.80^{A} \pm 1.20$
Flavor (50)	Fresh	$34.00^{A} \pm 1.00$	$34.37^{A} \pm 2.63$	$35.00^{A} \pm 3.00$	$36.50^{A} \pm 1.50$
	7 days	$34.00^{A} \pm 2.00$	$34.50^{A} \pm 2.50$	$36.43^{A} \pm 1.57$	$37.00^{A} \pm 2.00$
	15 days	$34.53^{C} \pm 1.47$	$35.23^{BC} \pm 0.77$	$36.73^{AB} \pm 1.27$	$37.65^{A} \pm 0.75$
Total (100)	Fresh	$76.50^{C} \pm 1.00$	$77.87^{BC} \pm 2.13$	$79.50^{AB} \pm 1.00$	$81.50^{A} \pm 1.50$
	7 days	$77.30^{B} \pm 1.30$	$79.50^{AB} \pm 0.50$	$81.73^{A} \pm 0.73$	$82.50^{A} \pm 3.50$
	15 days	$78.43^{B} \pm 0.57$	$81.33^{A} \pm 2.33$	$82.68^{A} \pm 0.95$	$83.95^{A} \pm 1.45$

The data are presented as mean ± SD. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same row, the means with dissimilar superscript letters are significantly different. Control (starter only), T1 (starter and probiotics), T2 (starter and AshEE), T3 (starter, AshEE, and probiotics)

Table 5 Prebiotic activity of ashwagandha ethanolic extract on bacterial counts at different incubation times (log CFU/mL)

Strains	12 h		24 h		48 h	
	Control	Treatment	Control	Treatment	Control	Treatment
Lactobacillus reuteri	8.07 ± 0.06^{Ba}	8.63 ± 1.00^{A}	8.13 ± 0.11^{Ba}	$8.74 \pm 0.05^{A\#}$	8.19 ± 0.00^{Ba}	$8.79 \pm 0.07^{A\#}$
Lactobacillus plantarum	8.29 ± 0.07^{Aa}	8.54 ± 1.00^{A}	8.38 ± 0.09^{Ba}	$9.35 \pm 0.03^{A\#}$	8.39 ± 0.19^{Ba}	$9.40 \pm 1.00^{A\#}$
Lactobacillus acidophilus	7.89 ± 0.10^{Bb}	8.39 ± 0.09^{A}	8.10 ± 0.10^{Bab}	$9.80 \pm 0.03^{A\#}$	8.60 ± 0.53^{Ba}	9.41 ± 0.11^{A}
Lactobacillus rhamnosus	8.88 ± 0.12^{Ac}	8.92 ± 0.12^{A}	9.762 ± 0.200^{Aa}	$9.47 \pm 0.03^{A\#}$	9.200 ± 0.006^{Ab}	$9.23 \pm 0.20^{A\#}$
Bifidobacterium bifidum	8.13 ± 0.12^{Bb}	8.80 ± 0.10^{A}	8.99 ± 0.17^{Ba}	9.40 ± 0.62^{A}	9.26 ± 0.30^{Ab}	$9.55 \pm 0.23^{A\#}$
Enterococcus facium	8.30 ± 0.11^{Ab}	8.20 ± 0.10^{A}	8.90 ± 0.02^{Aa}	$8.80 \pm 0.11^{A\#}$	8.30 ± 0.06^{Ab}	$8.11 \pm 0.01^{B\#}$
Lactobacillus casei	7.95 ± 0.05^{Ac}	7.54 ± 0.05^{B}	8.18 ± 0.01^{Bb}	$8.20 \pm 0.01^{A\#}$	8.28 ± 0.01^{Aa}	$8.27 \pm 0.25^{A\#}$

The data are presented as mean ± standard deviation. The data were subjected to one-way ANOVA followed by a post-hoc (Duncan) test at $p \leq 0.05$. Within the row of the same incubation period, the means with different superscript capital letters (A, B) are significantly different. Within the same row, the means with different superscript small letters (a, b, c) are significantly different from the control at different incubation times.

studied sensory parameters. Therefore, Karish cheese supplemented with AshEE and/or probiotic bacteria can be consumed by different age groups, especially children, to gain benefit from its nutritional and health properties. The same observation was made by Mustafa *et al.* who used ashwagandha extract with probiotic bacteria to manufacture a healthy functional yogurt [24]. Ashwagandha has been reported to improve the chemical quality of fermented dairy products containing probiotic bacteria, which in turn is reflected in its sensory quality [52]. Many researchers recommend the use of ashwagandha in dairy products, including yogurt, ice cream, and fermented milk, as well as in other food products such as probiotic juice [24, 47, 53, 54].

Prebiotic activity. Ashwagandha ethanolic extract (AshEE) exhibits excellent prebiotic properties due to its high contents of phytochemicals, polyphenols, and other bioactive compounds. Therefore, we evaluated the prebiotic activity of various concentrations of AshEE against foodborne microorganisms to choose the best strain with high prebiotic activity (best growth) for the manufacture of acid curd (Karish) cheese. Table 5 presents the selected probiotic strains in culture media containing AshEE in comparison with the control. We found no significant changes in bacterial counts during the first 12 h. After that period, particularly after 24 h, the growth significantly increased. When the bacterial population reached its maximal growth, it remained constant during 48 h. *L. plantarum* showed significant growth after 24 h to reach a count of 9.35 CFU/mL, 1.0 log higher than that of the control group (8.38 log CFU/mL). As a result, we selected *L. plantarum* as a good carrier of AshEE and probiotic bacteria to use in manufacturing Karish cheese. We also found increasing growth rates of the other probiotic strains at all the interval times. This is because AshEE is a rich source of carbohydrates with high prebiotic potential [55]. Generally, herbs contain a good amount of carbohydrates, protein, minerals, and some vitamins. They serve as prebiotics and are a source of carbon and nitrogen enhancing the growth of different probiotic strains [56]. On the other hand, ashwagandha contains steroidal lactones and flavonoids, while its roots have suitable amounts of sominie, somniferin, somniferinine, withanine, and withanonine that supply the pH and titratable acidity [57].

Viability of *L. plantarum* and starter culture in Karish cheese. The viability of probiotics, or the number of viable counts, in the final product until the end of its storage is an important quality indicator. A functional product should have a viable count of at least 10^{-9}–10^{-10} CFU/mL. Therefore, all fermented products are tested for viable counts until consumption. Table 6 shows the viability of *L. plantarum* and starter culture in Karish cheeses supplemented with ashwagandha ethanolic extract (AshEE). As can be seen, the presence of AshEE enhanced the growth of *L. plantarum* in fresh Karish cheese and during storage at 4°C, compared to the control, increasing the production of lactic acid. The viable counts of *L. plantarum* reached 7.51 log CFU/mL after 15 days of cold storage.

According to the Codex Alimentarius, a commercial probiotic beverage should possess a minimum viable count of 10^6 CFU/mL at the time of consumption [58]. The growth rates recorded in our study showed that ashwagandha is a good medium for probiotic growth and it can be considered a prebiotic. Also, the starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*) showed greater growth in the AshEE-treated cheese (T2) compared to the sample free from the extract, although the growth of *S. thermophiles* was higher in the control. However, the starter cultures were still above the minimum viable counts recommended by the Codex Alimentarius. These results are in contrast with those reported by Momin and Prajapati, who indicated that the increase in the log viable count was lower in the fermented milk supplemented with ashwagandha [47]. Nevertheless, our results are consistent with those of Khatoon and Gupta, who reported a suitable growth of starter culture in various combinations with AshEE [54]. At the end of the storage period (15 days), the total bacterial count in all the Karish cheese treatments ranged from 7.8 (Control) to 8.37 (T3) log CFU/g. Molds, yeasts, or coliform bacteria were not detected in any of the samples throughout cold storage. This indicates the microbial quality of the final product until the end of storage, as well as good hygiene during preparations, manufacture, and storage.

Biological study. Regarding behavioral measurements (Figs. 4–7), we found that the co-administration of rats with cheese supplemented with ashwagandha ethanolic extract (AshEE), probiotics, or their combination significantly restored the behavioral deteriorations resulting from $AlCl_3$ intoxication. This was evidenced by the marked improvement in locomotion deficits (vertical and horizontal activities) in the open field test. In particular, AshEE significantly increased the number of arms in the maze, while the formula (AshEE plus probiotics) increased the spontaneous alternation percentage, compared to the $AlCl_3$-intoxicated rats' group. In the modified elevated plus maze, the probiotics, AshEE, and their formula significantly decreased the transfer latency. Further, the AshEE-treated rats spent significantly more time exploring in the novel object recognition test, compared to the probiotic-treated rats, whereas the formula-treated rats showed a high discrimination ratio, as well a high recognition index, compared to the other groups.

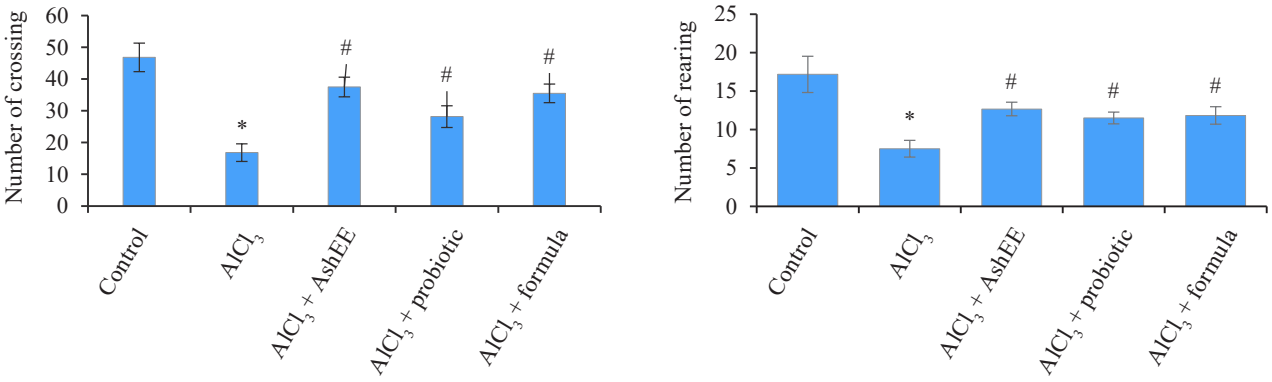
The open field test was used to assess the rats' locomotion and emotionality [59]. We found that AshEE was able to alleviate some motor deficits and anxiety caused by $AlCl_3$, which might be due to its chemical constituents with antioxidant properties. Thus, AshEE can reverse the $AlCl_3$-induced cognitive defects. These findings are consistent with some previous experiments [60]. The effect of AshEE on the rats' motor activity was confirmed by the number of arms entered in the Y-maze, which also increased significantly in the AshEE-treated group when compared with the other groups. In the Y-maze test, the formula was found better able to treat short-term memory deficits in the rats, compared to AshEE or probiotics.

The modified elevated plus maze test is used to measure the long-term spatial memory of rats [61]. In our study, both AshEE and probiotics, as well as their formula, markedly decreased the time to transfer to both

Table (6) Viability of starter culture and *Lactobacillus plantarum* in Karish cheeses supplemented with ashwagandha ethanolic extract (AshEE) during storage at 4°C (log CFU/g)

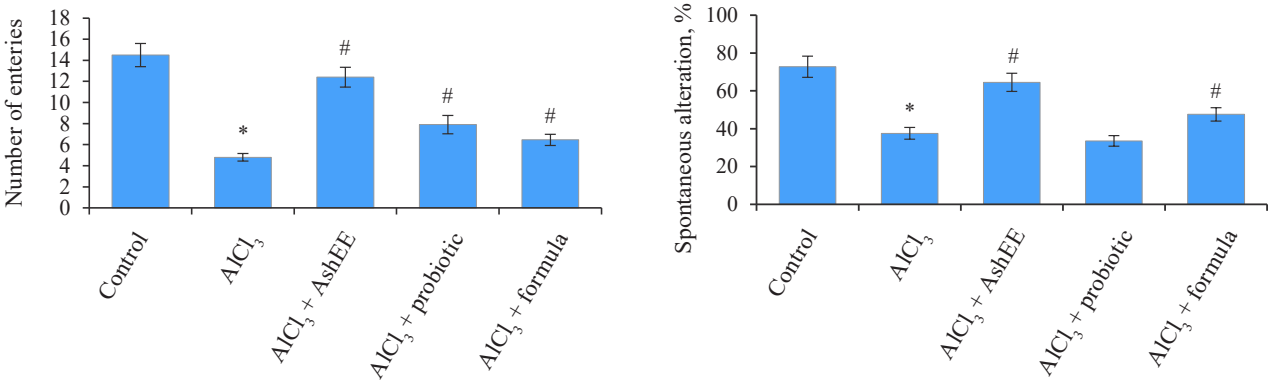
	Storage time	Control	T1	T2	T3
Total bacterial count	Fresh	$7.80^{A} \pm 0.11$	$8.10^{A} \pm 0.09$	$7.20^{A} \pm 0.08$	$8.20^{A} \pm 0.10$
	7 days	$7.90^{B} \pm 0.18$	$8.20^{A} \pm 1.01$	$7.18^{C} \pm 0.23$	$8.21^{A} \pm 0.21$
	15 days	$7.90^{B} \pm 0.10$	$8.32^{A} \pm 0.02$	$7.30^{C} \pm 0.03$	$8.30^{A} \pm 0.02$
Streptococcus thermophilus	Fresh	$7.90^{A} \pm 0.10$	$7.53^{B} \pm 0.41$	$7.65^{B} \pm 0.05$	$7.69^{B} \pm 0.51$
	7 days	$8.00^{A} \pm 0.10$	$7.14^{C} \pm 0.02$	$7.70^{B} \pm 0.01$	$7.73^{B} \pm 0.08$
	15 days	$8.14^{B} \pm 0.11$	$7.25^{D} \pm 0.22$	$7.73^{C} \pm 0.21$	$7.82^{A} \pm 0.07$
Lactobacillus delbrueckii spp. *bulgaricus*	Fresh	$7.10^{B} \pm 0.10$	$7.50^{A} \pm 0.05$	$7.50^{A} \pm 0.10$	$7.60^{A} \pm 0.02$
	7 days	$7.11^{C} \pm 0.01$	$7.67^{B} \pm 0.13$	$8.10^{A} \pm 0.10$	$8.16^{A} \pm 0.04$
	15 days	$7.30^{B} \pm 0.10$	$8.12^{A} \pm 0.01$	$8.13^{A} \pm 0.03$	$8.14^{A} \pm 0.12$
Lactobacillus plantarum	Fresh	–	$7.30^{B} \pm 0.06$	–	$7.80^{A} \pm 0.10$
	7 days	–	$7.51^{B} \pm 0.01$	–	$7.69^{A} \pm 0.01$
	15 days	–	$7.495^{A} \pm 0.100$	–	$7.51^{A} \pm 0.05$

The data are expressed as mean ± SD of three replicates. Within the same row, the means with different capital letters are significantly different at $p \leq 0.05$. Control (starter only); T1 (starter and probiotics); T2 (starter and AshEE); T3 (starter, AshEE, and probiotics)



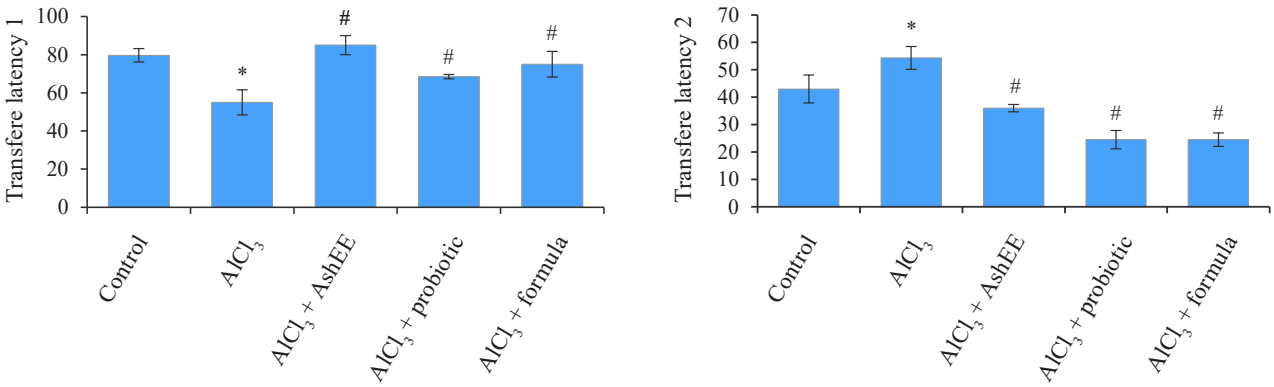
The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AshEE – ashwagandha ethanolic extract

Figure 4 Effect of AshEE- and/or probiotic-supplemented Karish cheese on the number of crossings and rearings in $AlCl_3$-intoxicated rats in the open field test



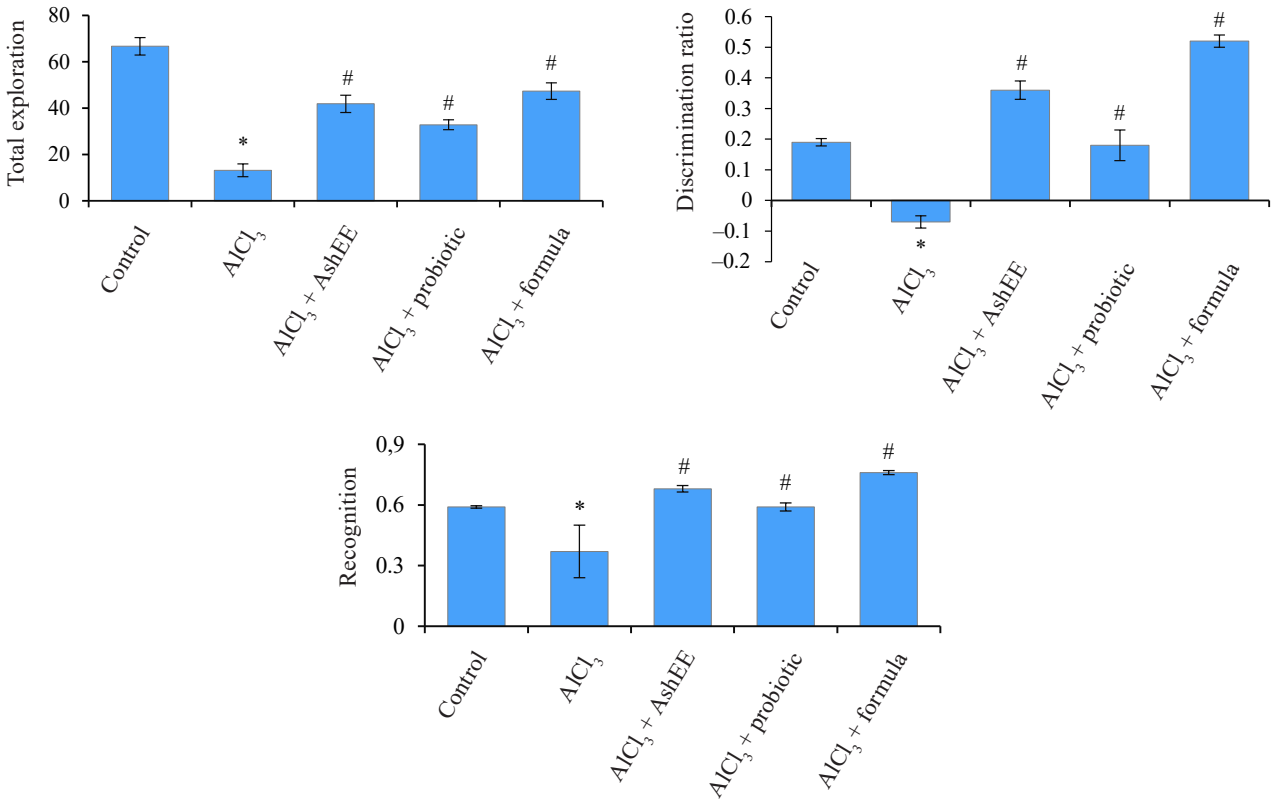
The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AshEE – ashwagandha ethanolic extract

Figure 5 Effect of AshEE- and/or probiotic-supplemented Karish cheese on the number of arm entries and spontaneous alternation percentage in $AlCl_3$-intoxicated rats in the Y-maze test



The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AshEE – ashwagandha ethanolic extract

Figure 6 Effect of AshEE- and/or probiotic-supplemented Karish cheese on transfer latency 1 and 2 in $AlCl_3$-intoxicated rats in the modified elevated plus maze test



The data are expressed as mean ± standard error. The data were subjected to one-way ANOVA followed by a post-hoc (Bonferroni) test at $p \leq 0.05$. * Significantly different from the control starter group; # significantly different from the $AlCl_3$-intoxicated rats' group; AshEE – ashwagandha ethanolic extract

Figure 7 Effect of AshEE- and/or probiotic-supplemented Karish cheese on total exploration time, discrimination ratio, and recognition index in $AlCl_3$-intoxicated rats in the novel object recognition test

closed arms. This indicated their ability to restore the long-term memory in the treated rats compared to the untreated-$AlCl_3$ rats.

The novel object recognition test is used to assess the ability of rats to recognize an object or stimulus seen in the previous 24 h [62]. The test requires intact dorsal hippocampus and cortex [63]. Our data revealed that the administration of AshEE- or formula-supplemented cheese remarkably restored the cognitive deficits and recognitive memory disruptions that accompanied $AlCl_3$-intoxication. This was indicated by the time spent exploring a novel object, a recognition index, and a discrimination ratio. In animal model investigations, strong associations have been reported between gastrointestinal microbiota and stress behavior. Particularly, the disruption or absence of gut bacteria was shown to increase the neuroendocrine stress response and behaviors associated with anxiety and stress-induced memory dysfunction [64, 65]. It was reported that probiotics reduced anxiety behavior in rodents, effectively reversing the effects of stress and improving memory and learning performance in terms of object recognition [66–68].

$AlCl_3$ is known to induce behavioral, biochemical, and histopathological effects that are linked with cognitive impairments [69–72]. In our study, the supplementation of Karish cheese with AshEE and/or probiotics appreciably ameliorated the neuronal, biochemical, and behavioral aberrations in the $AlCl_3$-challenged demented rats. This indicated their curative and neuroprotective actions against dementia complications.

With respect to the neuro-and-biochemical investigations, we found that $AlCl_3$ significantly decreased brain monoamines (dopamine and serotonin) and raised acetylcholinesterase activity, as previously observed [72–74]. The levels of monoamines were similar to those in patients with Alzheimer's disease, suggesting a prominent role of $AlCl_3$ in aging and development of neurodegenerative diseases [75]. Interestingly, the supplementation with AshEE and/or probiotics was found to significantly alleviate the effect of $AlCl_3$ on the biogenic monoamines, producing a therapeutic effect against neurodegenerative disorders. In addition, AshEE and probiotics possessed significant antioxidant properties, which may be related to their contents of polyphenols, flavonoids, and other compounds. Compared with the control group, $AlCl_3$-intoxication resulted in a significant decrease in dopamine and serotonin levels (–37.9 and –49.7%, respectively). However, a significant elevation (63.1%) was shown in acetylcholinesterase activity. Interestingly, the treatment of rats with Karish cheese supplemented with AshEE, probiotics, or their combination (formula),

in line with $AlCl_3$-intoxication, markedly restored (at different degrees) the $AlCl_3$-deteriorated neurochemical measurements. This was evidenced by a significant reduction in acetylcholinesterase activity (–28.4, –18.8, and –24.1%, respectively), as well as a notable increase in dopamine (45.3, 17.2, and 26.6%, respectively) and serotonin (69.6, 39.1, and 57.4%, respectively), compared with the $AlCl_3$-intoxicated group. The highest improvement was performed by the AshEE-supplemented cheese (Table 7).

Our data showed a significant increase in the levels of malondialdehyde (59.6%) and nitric oxide (183.6%) in the brains of $AlCl_3$-intoxicated rats. We also found a significant drop in the anti-oxidative battery resulting from a marked decrease in glutathione (–49.4%) and superoxide dismutase activity (–36.6%), compared to the control group. Noteworthily, the treatment of $AlCl_3$-intoxicated rats with the Karish cheese supplemented with AshEE, probiotics, or their combination (formula) alleviated some $AlCl_3$-induced oxidative deteriorations. This was evidenced by a marked decrease in the levels of malondialdehyde (–28.6, –12.8, and –16.3%, respectively) and nitric oxide (–42.1, –21.2, and –30.2%, respectively), as well as a remarkable rise in glutathione (39.8, 13.9, and 23.2%, respectively) and superoxide dismutase activity (39.8, 14.4, and 28.5%, respectively), compared to the $AlCl_3$-intoxicated rats (Table 8).

Regarding the histopathological examination (Fig. 8), the brains of the control animals had a normal histological structure in different brain regions, with only the cerebral cortex exhibiting some congested blood vessels (Fig. 8, plate 1). In particular, the striatum was histologically normal (Fig. 8, plate 2), and both the hippocampus and the cerebellum were also normal (Fig. 8, plates 3 & 4). However, the intoxication of rats with $AlCl_3$ resulted in serious histological alterations in brain tissue, with many dark degenerated neurons observed in the cerebral cortex (Fig. 8, plate 5) with neuronophagia. The hippocampus showed changes in blood vessels accompanied by mild glial infiltrations (Fig. 8, plate 6), as well as marked neuronal damage resulting in an obvious loss of cell density. Pink fibrillar material was seen between the degenerating neurons of the hippocampus with glial cells infiltration (Fig. 8, plate 7). Purkinje cell necrosis was commonly seen in almost all the examined cerebellar sections (Fig. 8, plate 8).

According to our results, the treatment of $AlCl_3$-intoxicated rats with AshEE-supplemented Karish cheese resulted in a moderate protection against brain injury. In particular, the cerebral cortex appeared normal (Fig. 8, plate 9) and free from angiopathies; the hippocampus also appeared histologically normal (Fig. 8, plate 10). Similarly, the treatment of the $AlCl_3$-intoxicated animals with probiotic-supplemented Karish cheese resulted in a mild alleviative action against $AlCl_3$-induced brain damage. However, the cerebral cortex appeared normal, except for a few degenerating neurons with focal glial infiltrations (Fig. 8, plate 11). Cerebral angiopathy was also noticed with mild perivascular lymphocytic cuffing (Fig. 8, plate 12). A few degenerating neurons were observed in the hippocampus and the cerebellum (Fig. 8,

Table 7 Effect of Karish cheese supplemented with ashwagandha ethanolic extract (AshEE) and probiotics on $AlCl_3$-induced neurochemical changes

Parameter	Dopamine		Serotonin		Acetylcholinesterase	
	pg/g	%	pg/g	%	µmol/min/g	%
Control	2431 ± 54^{A}		863.0 ± 17.2^{A}		6814 ± 65^{E}	
$AlCl_3$	1509 ± 38^{E}	–37.9*	434.0 ± 15.9^{E}	–49.7*	$11\ 112 \pm 57^{A}$	63.1*
$AlCl_3$ + AshEE	2192 ± 42^{B}	45.3#	736.0 ± 13.8^{B}	69.6#	7054 ± 64^{D}	–36.5#
$AlCl_3$ + probiotic	1769 ± 34^{D}	17.2#	604.0 ± 15.4^{D}	39.1#	9019 ± 55^{B}	–18.8#
$AlCl_3$ + formula	1911 ± 33^{C}	26.6#	683.0 ± 13.5^{C}	57.4#	8437 ± 49^{C}	–24.1#

The data are presented as mean ± standard error, subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. The means with dissimilar superscript letters are statistically significant. AshEE (ashwagandha ethanolic extract); * is percentage of change calculated for the control group; # is percentage of change calculated for the $AlCl_3$ group. Formula (cheese supplemented with starter, AshEE)

Table 8 Effect of Karish cheese supplemented with ashwagandha ethanolic extract (AshEE) and/or probiotics on $AlCl_3$-induced oxidative stress status of the animals' brain

Parameter	Malondialdehyde		Nitric oxide		Glutathione		Superoxide dismutase	
	µmol/g	%	µmol/g	%	µmol/g	%	U/g	%
Control	43.26 ± 3.65^{C}		4.03 ± 0.52^{D}		318.4 ± 12.5^{A}		299.0 ± 32.5^{A}	
$AlCl_3$	69.15 ± 4.57^{A}	59.8*	11.43 ± 0.75^{A}	183.6*	160.9 ± 9.3^{D}	–49.4*	189.30 ± 28.7^{D}	–36.7*
$AlCl_3$ + AshEE	49.34 ± 3.98^{C}	–28.6#	6.62 ± 0.25^{C}	–42.1#	224.9 ± 11.7^{B}	39.8#	264.9 ± 38.7^{B}	39.8#
$AlCl_3$ + probiotic	60.29 ± 2.78^{B}	–12.8#	9.01 ± 1.08^{B}	–21.2#	183.3 ± 8.9^{C}	13.9#	216.5 ± 26.8^{C}	14.4#
$AlCl_3$ + formula	57.82 ± 4.57^{B}	–16.3#	7.98 ± 0.98^{C}	–30.2#	198.2 ± 7.7^{C}	23.2#	243.2 ± 21.7^{C}	28.5#

The data are presented as mean ± standard error. The data were subjected to one-way ANOVA followed by a *post-hoc* (Duncan) test at $p \leq 0.05$. Within the same column, the means with dissimilar superscript letters are statistically significant. AshEE (ashwagandha ethanolic extract); Formula (AshEE + probiotic); * is percentage of change calculated for the control group; # is percentage of change calculated for the $AlCl_3$ group

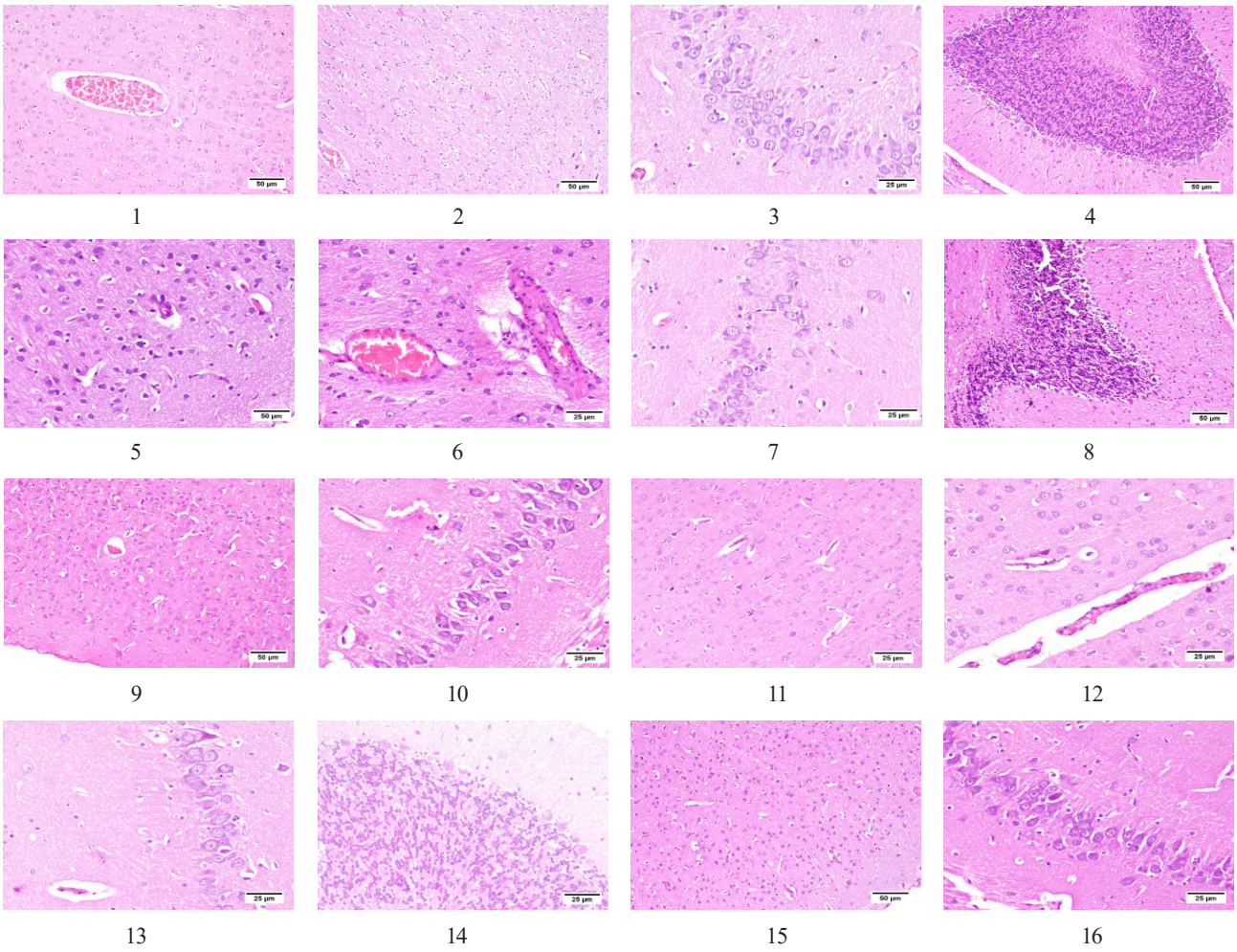


Figure 8 Photomicrograph of rat brain, H&E-stained: 1 – control group, showing normal cerebral cortex with congested cerebral blood vessels; 2 – control group showing normal striatum; 3 – control group, with normal architecture of hippocampus; 4 – control group, showing cerebellar cortex, normally appearing molecular layer, Purkinje cell layer, and granule cell layer; 5 – $AlCl_3$ group, showing degenerating (dark) neurons with neuronophagia, noticeable angiopathy; 6 – $AlCl_3$ group, showing a congested blood vessel with thickening in its wall, as well as focal perivascular lymphocytic aggregation; 7 – $AlCl_3$ group, showing loss in hippocampus cells with presence of pink fibrillar material and glial cells infiltrations; 8 – $AlCl_3$ group, showing necrosis of Purkinje cells; 9 – ashwagandha group, showing apparently normal cerebral cortex; 10 – ashwagandha group, showing apparently normal hippocampus; 11 – probiotic group, showing a few degenerating neurons in cerebral cortex with focal gliosis; 12 – probiotic group, showing cerebral vessels angiopathy with perivascular lymphocytic infiltration; 13 – probiotic group, showing a few degenerating neurons in the hippocampus; 14 – probiotic group, showing a few degenerating Purkinje cells; 15 – formula group, showing apparently normal cerebral cortex; 16 – formula group, showing apparently normal hippocampus

plates 13 & 14). The treatment of the animals with the formula (AshEE and probiotics)-supplemented Karish cheese exerted the best neuroprotective action. The cerebral cortex appeared normal (Fig. 8, plate 15), although both the hippocampus and the cerebellum showed a few necrotic cells (Fig. 8, plate 16).

The $AlCl_3$-induced significant elevation in malondialdehyde and reduction in enzymatic (superoxide dismutase and CAT) and non-enzymatic (glutathione) antioxidant efficiency, as well as the extensive neuronal damage in the hippocampus were not consistent with the reports of Igbokwe *et al.* and Makhdoomi *et al.* [76, 77]. Since Al^{3+} and Fe^{3+} have similar ionic radii, Al^{3+} can bind to the Fe^{3+}-binding protein transferrin and pass Al^{3+} by transferrin receptors to deliver Al^{3+} to the brain and initiate oxidative damage [78]. Moreover, Al^{3+} alters Ca^{2+} flux and causes abnormal augmentation of intracellular Ca^{2+}, which can increase the production of reactive oxygen species via mitochondrial dysfunction [79]. This can result in oxidative damage, neuronal degeneration, neurochemical changes, and cognitive impairments. Since both inflammation and oxidative stress are interrelated, the latter induces inflammatory cytokine genes [80]. Thus, exposure to metals such as aluminum can increase the levels of proinflammatory cytokines in the brain [81]. Moreover, elevated nitric oxide inhibits microglia proliferation and stimulates glutamate release from astrocytes, leading to excitotoxicity of neurons and glia. Our study revealed that AshEE- and/or probiotic-fortified cheese efficiently alleviated the $AlCl_3$-

associated pathophysiological deteriorations. It has been reported that AshEE exhibited neuroprotection against oxidative stress by activating the Nrf2 pathway and up-regulating cytoprotective genes, as well as Keap-Nrf2-ARE signaling [82, 83]. Ashwagandha appears to increase the expression and translocation of Nrf2 that binds on ARE and causes the induction of several phase I and phase II metabolizing enzymes, phase III detoxifying proteins, and antioxidant proteins. The up-regulation of detoxification enzymes enhances cell survival and protection due to an improved redox state which prevents glutathionylated protein accumulation. Also, AshEE-mediated neuroprotection was reported showing that Withanolide-A (the main constituent of AshEE) increased glutathione synthesis in neuronal cells [84].

CONCLUSION

Acid curd (Karish) cheese as a dairy product model can consider an excellent delivery system for ashwagandha and probiotics, mainly *Lactobacillus plantarum*, to provide maximum health benefits. The addition of ashwagandha extract and/or *L. plantarum* enhances the nutri-pharmaceutical value of Karish cheese without affecting its properties. The combination of ashwagandha extract and *L. plantarum* performed a modulatory effectiveness against induced neurotoxicity, especially related Alzheimer's disease and learning difficulties. This effect was achieved through a remarkable improvement in the behavioral, neurochemical, and oxidative status.

CONTRIBUTION

L.K. Hassan and H.H. Salama were involved in the original research and manuscript writing. K.G. Abdel-Wahhab and H.M.A. Khalil conducted the biological study. S.M. Abdelhamid performed the microbiological assessment. All the authors were involved in editing, reviewing, and proofreading the manuscript. All the authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest which could hinder the publication of this article.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATION

The sensory test was carried out in compliance with the standard institutional criteria established by the Ethical Committee of the National Research Centre. After the participants had been given a full explanation of the study, they had to provide their signed written informed consent to participate in the research. The experimental study was approved by the Veterinary Institutional Animal Care and Use Committee (Approval No. Vet CU23012020113) in accordance with the American Guidelines for Animal Care and Use.

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