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Boosting the nutritional value of stirred yogurt by adding nano-sized avocado seed powder and Lactobacillus acidophilus

Samah M. El-Sayed* and Hoda S. El-Sayed
Dairy Department, National Research Centre, 33 El Bohouth St., Dokki, Giza, P.O. 12622, Egypt.

In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract
The nano-sized avocado seed powder (nano-ASP) was prepared to integrate in stirred yogurt as highly nutritional agriculture's removal discarded. The particle size, nutritional composition and phenolic contents of nano-ASP were determined. Also, their effect as antimicrobial agents plus activation supplements for probiotics was evaluated. The stirred yogurt was prepared as carrier to supply vital nano-ASP to human using concentration of (0.0, 1.0, 2.0, and 3.0%). All stirred yogurt treatments were evaluated for physicochemical, color, microbiological loaded and sensory properties during 30 days. The contaminated stirred yogurt with A. niger was designated to confirm their antimicrobial influence. The data indicated that nano-ASP was located at the nanoscale with seven phenol components mainly methyl gallate, catechin, and ellagic acid. The strongest inhibitory zones were obtained at (17, 16, and 19 mm) for S. aureus, A. niger, and A. flavus, respectively at the concentration 1% and capable for increasing the probiotic strains' proliferation. Moreover, the nano-ASP was considered the vigorous source for protein, carbohydrates, minerals, and fiber with highly antioxidant activity that added nutrition and healthy components to stirred yogurt. The viable counts of L. acidophilus loaded in stirred yogurt were improved during storage, especially at 2% nano-ASP that adequate to progress L. acidophilusfor two weeks and as well amplified the starter cultures counts. Stirred yogurt with 1.0% and 2.0% nano-ASP received higher overall acceptability scores. In the contaminated part, the levels of A. niger increased more with storage under control than with other treatments. From results, the appropriate stirred yogurt shelf life was 21 days.
Key words: Avocado seed powder; nanoscale; antimicrobial activity; stirred yogurt; probiotics; physiochemical evaluation

1. Introduction

Yogurt is a popular dairy product, and it's considered the most widely consumed fermented milk product in the world, with an excellent vehicle for the delivery of several ingredients with functional properties [1]. Yogurt is considered to be the main functional dairy product owing to its nutritional, beneficial, and probiotic effects [2]. Furthermore, probiotics are being added to yogurt to increase its health benefits; the end result is known as "bio-yogurt" [3]. Probiotics are a crucial group of functional foods when ingested in sufficient amounts since they help the host's health [4,5].

Probiotic yogurt has undergone a number of measures to boost its biological activity, including supplementation with fruits, vegetables, minerals, or herbs. For instance, this addition enhanced the yogurt's anticancer, antibacterial, and probiotic culture development as well as its flavor and antioxidant activity [6-11].

The avocado fruit (Percea Americana Mill.) is a member of the Lauraceae genus of flowering plants. It is indigenous to Mexico, Central America, and South America. Due to its great nutritional content and medicinal qualities, it has been a well-liked food and is frequently advertised as a "superfood" [12,13]. Avocados are often consumed because of their tasty and pleasant texture. However, despite having more beneficial components than the edible parts, avocado seeds (which make up between 13 and 40% of the fruit's weight) are typically thrown away as waste [14,15]. The seed part of the avocado is usually not consumed due to its weak taste. Consequently, different people never prefer avocado seeds, mainly because they have little information on their nutritional value and are not informed of their medicinal and photochemical compositions [16].

Avocado seeds have 70% of the antioxidants found in the whole avocado fruit. Moreover, it has more soluble fiber and more antioxidants than most fruits [17]. Also, the avocado seed contains significant amounts of phenolic compounds (64% of the seed's weight), phytochemicals, and minerals, many of which have positive therapeutic qualities. Locally, dry avocado seeds are used to treat diarrhea

* Corresponding author: samah_mosbah80@yahoo.com; (Samah M. El-Sayed).
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or dysentery, as a relief drink, skin diseases, and toothaches [16].

Avocado seeds contain high biological activities for instance antibacterial activity against numerous pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli*[18], antifungal activity against *Aspergillus niger*[19], antiviral and wound healing properties [20] antioxidant properties[21], anti-inflammatory and analgesic activities [22], prevention and treatment of many health conditions, including heart disease, cancer, high blood pressure and diabetes [14, 23-27] biotechnological, pharmaceutical, chemical, and food industries [28,29].

Due to the high nutritional value, antioxidants, and antimicrobial properties of avocado seeds, as well as the paucity of information regarding their use in functional dairy products, the goal of this study was to investigate the addition of avocado seed powder in nanoscale to stirred yogurt and assess its physicochemical, nutritional, sensory, and microbiological properties during the period of 30 days of storage.

2. Materials and methods

2.1. Materials and chemicals

The local Egyptian fruit market provided the fresh avocado fruit. From the dairy farm at Cairo University's Faculty of Agriculture, Egypt's skim milk was obtained. Analytical-grade substances were also used in the investigation.

2.2. Preparation of nano-sized avocado seed powder (ASP)

Fresh avocados were cleaned with water, dried, and then the seeds were manually removed with a stainless-steel knife. Avocado seeds were thinly sliced using a vegetable slicer and submerged in water containing 150 mmol/liter citric acid for 10 min. The little pieces were dried in an oven set to 50 °C for around 48 hours. For the application study, dried seeds were ground in a blender and sieved through BS 72 (120 m) mesh to produce a fine powder, which was then kept in glass bottles at 4°C with light protection (Figure1).

2.3. Particle size distribution of nano-ASP

The particle size distribution of avocado seed powder was examined using the Dynamic light scattering (DLS) instrument, NICOMP 380 ZLS, from PSS in Santa Barbara, California, USA. The incident light was the 632 nm line of a HeNe laser at a Zeta potential external angle of 18.90.

Figure 1: Flow sheet diagram for preparation of nano-sized avocado seed powder

2.4. Chemical analysis of nano-ASP

According to AOAC [30], the composition proximate of nano-ASP was analyzed for protein content using the semi-micro-Kjeldahl process, total fat content using the Soxhelt extraction technique, moisture, crude fiber, and ash. The carbohydrate values were obtained by calculation. Carbohydrates expressed as a percentage = [100-(Moisture + Ash + Protein + Fiber + Fat)].

2.5. HPLC conditions of phenolic compounds of nano-ASP

Using a Kromasil C18 column (4.6 mm x 250 mm i.d., 5 µm), the phenolic compounds were separated using an Agilent 1260 HPLC system. Water (Solvent A) and 0.05% trifluoroacetic acid in acetonitrile (Solvent B), both at a flow rate of 1 ml/min, were used to carry out the gradient elution. The mobile phase's programming followed the format shown in Table (1). Ten µl of injection volume were given to each sample solution. At 280 nm, the multi-wavelength detector was observed. The column was kept at a 35 °C temperature [31].

Table 1: The gradient of elution solvents

No.	Time (min)	Solvent A,%	Solvent B,%
1	0 min	82	18
2	0-5 min	80	20
3	5-8 min	60	40
4	8-12min	60	40
5	12-15min	85	15
6	15-16 min	82	18

2.6. Mineral elements analysis

Mineral contents (K, Ca, Mg, Fe, Cu, Mn, and Zn) were determined by an atomic absorption spectrometer (Chemtech CTA-2000, England). Samples were digested by dry ashing and dissolved in 1 M HCl, as described by El-Sayed[32].

2.7. Antimicrobial action of nano-ASP against pathogens

The antimicrobial activity of nano-ASP was achieved according to BSAC [33]. The antimicrobial effect was examined against Salmonella Typhimirum ATCC 14028, Listeria monocytogenes ATCC 5980, Staphyloco ccusaureus ATCC 6538, Escherichia coli ATCC 8739, Psedomonas aeruginosa ATCC 27853, Aspergillus niger ATCC 10404 and Aspergillus flavus ATCC 9643. Identical colonies were chosen after the microbial strains were cultured on nutrient agar for 24 h for bacteria and 48 h for fungi. The identical colonies were put into tryptic soya broth (about 5 ml per tube) and incubated at suitable temperatures (35ºC for bacteria and 25ºC for fungi) up until obvious turbidity at 0.5 ml of the "McFarland" standard solution. After applying each strain's inoculum to Mueller-Hinton agar plates with a sterile cotton swab (using approximately 25 ml of agar media per plate), the plates should rest for 15 minutes. By using a 6 mm cropper, the various wells in the agar media were created. Each well was filled with 100 µL of 0.1, 0.5, and 1.0% nano-ASP that had been dissolved in DMSO (w/v). For bacteria, all test plates were incubated for 24 h at 35ºC and for fungi, for 48 h at 25ºC. The zone of inhibition diameter (mm) was measured after the incubation period.

2.8. Evaluation the viability of probiotic strain in presence of nano-ASP

The different probiotic strains are: Lactobacillus acidophilus CH-2, Lacticasei bacillus rhamnosus NRRL B-442, Ligilacto bacillus salivarius NBIMCC 1589, Lacticasei bacilluscasei NRRL B-1922, Limosilacto bacillusreteri NRRL B-14171, Lactiplanti bacillus (L. paraplantarum. The novel taxonomy developed by Zheng et al. [34] was exploited to classify different Lactobacillus strains and to evaluate the nano-ASP powder's potential as a probiotic strain growth agent. 10 ml of MRS broth were mixed with various amounts (0.1, 0.2, and 0.3 g, w/v) of nano-ASP powder before being inoculated with 0.5 ml of each strain. All inoculation test tubes underwent a 48 h incubation period at 37°C. As a control, other inoculation test tubes without the nano-ASP were used. On MRS agar medium, probiotic strains were counted using the pour-plate method after each strain was given an appropriate serial dilution in the plates. All MRS agar plates underwent a 48 h incubation period at 37°C, during which the growth colonies were noted.

2.9. Production of stirred yogurt fortified with nano-ASP

Fresh skimmed cow's milk was heated for 15 minutes at 82 °C and then quickly cooled to 42 °C to make stirred yogurt [1]. The yogurt cultures L. bulgaricus and S. thermophilus at 2% (w/w) and L. acidophilus as a probiotic model strain at 2% were used to inoculate the milk. The inoculated milk was then divided into four equal parts; the first part served as the control, and the other three were given treatments T1, T2, and T3, respectively, by adding 1.0, 2.0, and 3.0% (w/w) nano-ASP. At a temperature of 42 °C, the treatments were incubated for around 3 h until yogurt curd formed. The yogurt treatments that were produced were then cooled and stirred with a glass rod. All treatments were placed in separate, sterile plastic cups (50 mL) and kept in cold storage for 30 days at 5±1°C. At intervals, the stirred yogurt treatments were assessed for each seven days in terms of microbiology, chemically, and sensory properties.

2.9.1. Physicochemical analysis of yogurt fortified with nano-ASP

The ash, fat, total protein, and total solids contents of probiotic yogurt fortified with nano-ASP and control probiotic yogurt were measured using AOAC, [35]. The pH measurements of yogurt samples were carried out using a digital pH meter (Hanna Instruments model, Germany). All of the analyses were run in triplicate.

2.9.2. Color measurements

The stirred yogurt and nano-ASP samples' color properties were evaluated using a Hunter colorimeter model D2s A-2 (Hunter Assoc. Lab Inc., VA, USA). The instrument's top and bottom standards were made out of white tiles (bottom of the scale). A portion of stirred yogurt was placed at the specimen port (in a flat sheet). The color values (l, a, and b) were calculated using the appropriate colorimeter button. Where (a) represents the range of colors from red (+) to green (-); (b) represents the range of colors from yellow (+) to blue; and (l) represents the value of the range of darkness from black (0) to white (100); (-).

2.9.3. Determination of antioxidant activity

According to Matthus [36], the antioxidant activity of nano-ASP and stirred yogurt treatments was evaluated using the stable 2, 2-diphenyl-1-picryl-hydrazyl (DPPH, Sigma Aldrich, Germany) radical scavenging method. The following formula was used to determine the percentage inhibition of DPPH, which was used to express the radical-scavenging capacity of the samples under study:

Inhibition, % = (($A_{control} - A_{0sample}$) / $A_{control}$) × 100

Where: A_0, the final absorbance of the test sample at 515 nm; A, the absorbance of the control sample at 515 nm.

2.9.4. The microbiological evaluation

The stirred yogurt treatments were tested for the yogurt strains during storage as follows after a 10-fold serial dilution with saline (0.9% NaCl): *S. thermophilus* was detected using M17 agar medium after the plates were incubated aerobically for 48 h at 37°C IDF [37]. MRS agar supplemented with 10% sorbitol and anaerobically incubated at 37°C for 48 h was used to recognize *L. bulgaricus* [38]. The probiotic *L. acidophilus* count was evaluated using MRS agar that had been completed with 0.02% bile salts and anaerobically incubated at 37°C for 48 h [39]. Additionally, nutrient agar medium was used to detect the total bacterial counts, and it was cultured aerobically for 48 h at 30 ºC IDF [37]. Chloramphenicol rose Bengal medium was used to detect the mold and yeast counts, and it was cultured aerobically for 4 days at 25 ºC [40].

2.9.5. Sensory properties

Twenty dairy department staff members from the National Research Centre evaluated sensory properties. Fresh samples and samples fortified with nano-ASP that had been stored for a week both were evaluated. The parameters of evaluation included overall acceptability, body and texture, color, appearance, and flavor. The range of the evaluation degree was 1 to 5, with 5 being the highest favorable degree [41].

2.10. Evaluated the nano-ASP integrated in stirred yogurt as antimicrobial agent

To evaluate the behavior of nano-ASP as an antimicrobial agent, four more parts of stirred yogurt treatments were prepared independently as in the preceding section (2.5), but these treatments were contaminated with *A. niger* as the strain model. *A. niger* counts were tracked throughout storage at intervals of seven days using a 10-fold serial dilution and spread-plate technique. The counts of *A. niger* were enumerated after incubating the plates of chloramphenicol-rose Bengal medium spread with the appropriate dilution and aerobically incubated them for 4 days at 25 ºC [40].

2.11. Statistical analysis

According to SAS Institute, Inc.'s (USA) Statistical Analysis System Users Guide, the results for stirred yogurt treatments were examined SAS [42]. The Duncan's multiple range tests were performed to evaluate whether there was a significant difference between the means ($p > 0.05$).The findings were reported as mean ± standard deviation.

3. Results and discussion

3.1. Particle size distribution

Using a dynamic light scattering approach, Figure (2) shows the avocado seed powder's particle size distribution. The average diameter of avocado seed powder is 119.9 nm, indicating that the powder's particles are nanoscale. According to a previous study Otoni et al. [43], polydispersity index values less than 1.0 indicate good polydispersion, and the polydispersity is approximately 0.224, corresponding to an excellent homogeneous particle size distribution. This suggested that avocado seed powder could improve the consistency and caliber of yogurt.

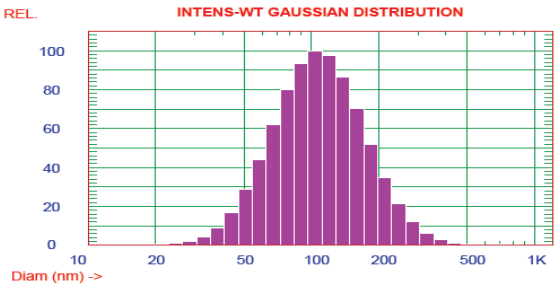


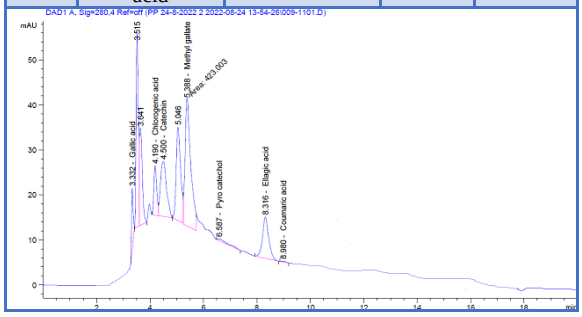
Figure 2: Mean diameter and comparative particle size indices for nano-ASP.

3.2. Total phenolic content of the nano-ASP

Phenolic compounds are a large class of plant phytonutrients that are used in products as antioxidants or protective ingredients. Table (2) shows the total phenolic content of the nano-ASP (µg /g seed powder) as well as the HPLC chromatogram. According to the data, nano-ASP contains seven phenol components. Methyl gallate, catechin, and ellagic acid were the most abundant phenolic compounds found. Chlorogenic acid, gallic acid, pyrocatechol, and coumaric acid are negligible phenolic compounds lower than 100 µg /g seed powder. Most of these phenolic compounds are well-known for their beneficial health effects, which include antioxidative, anticarcinogenic, antiangiogenic, tonic effects on the nervous system, and cardiopreostatic effects [44].

Table 2: Quantification and identification and of phenol compounds by the HPLC profile for nano-ASP.

Peak	Phenol compounds	Retention time (min)	Peak area	Conc. (µg/g)
1	Gallic acid	3.33	53.90	4.37
2	Chlorogenic acid	4.19	82.40	11.40
3	Catechin	4.50	193.40	46.23
4	Methyl gallate	5.38	423.00	29.29
5	Pyro catechol	6.58	11.66	1.61
6	Ellagic acid	8.32	147.27	30.30
7	Coumaric acid	8.98	2.09	0.05



3.3. Antimicrobial action of nano-ASP against pathogens

The antimicrobial efficacy of nano-ASP prepared with DMSO at varying concentrations can be seen in Table (3). Based on the results from the well diffusion method, it was revealed that amplification the nano-ASP concentration also boosted the microbial inhibition zones. With the highest concentration of nano-ASP, the strongest inhibitory zones were obtained (17, 16, and 19 mm for *S. aureus, A. niger*, and *A. flavus*, respectively). With *S. Typhimurium* (7.0 mm at 1% concentration), the minimal microbial reduction inhibitory zone was obtained. Also, *P. aergenosia* did not show any inhibitory activity against concentrations of nano-ASP, which may be explained by the structure and composition of its cell wall. The cell wall of Gram-negative bacteria typically contains a lipid bilayer, which provides additional protection against antimicrobial agent's substances [45]. Moreover, based on the structure of their membranes, Melgar et al. [46] and Rodríguez-Carpena et al. [26] showed that the same concentration of avocado seeds was necessary to inhibit both Gram-positive and Gram-negative bacteria growth. As indicated in section (3.2) the phenolic chemicals gallic acid, chlorogenic acid, catechin, ellagic acid, coumaric acid, and methyl gallate contained in the nano-ASP are primarily responsible for the avocado seed's bactericidal effect. According to Soledad et al.[12], high *S. aureus* and *S.Typhimurium* log reductions were 4.0 0.3 and 1.8 0.3 log CFU at 2000 mg/L of avocado seeds extract, respectively, with no significant effect of the solvents.

Table 3: Antimicrobial action of nano-ASPagainst pathogens

Strains	nano-ASPconcentrations (%)		
	0.1	0.5	1.0
S. Typhimurium	N.D	N.D	7.00^{Ae} ±0.038
L. monocytogenes	N.D	N.D	14.00^{Ad} ± 0.017
S. aureus	3.00^{Cb} ± 0.033	10.00^{Bc} ± 0.039	17.0^{Ac} ±0.029
E. coli	N.D	6.00^{Bd} ± 0.028	14.00^{Ad} ± 0.035
P. aergenosia	N.D	N.D	N.D
A. niger	5.00^{Ca} ± 0.020	11.00^{Bb} ± 0.045	16.00^{Ab} ± 0.022
A. flavus	6.00^{Ca} ± 0.033	14.00^{Ba} ± 0.022	19.00^{Aa} ± 0.020

N.D: not detectable. Data are represented as mean of three replicates ± SD. Means in the same row and capital superscript letters (effect between concentrations) are not significantly different ($p > 0.05$). Means in the same column and small superscript letters (effect between strains) are not significantly different ($p > 0.05$).

3.4. Evaluation the viability of probiotic strain in presence of avocado seeds nano-powder

The viability of probiotic bacteria when incubated with various doses of nano-ASPis shown in Figures (3 a, b). Overall, compared to the control, all concentrations of nano-ASP considerably increased the probiotic strains' proliferation. On the other hand, it was evident that when probiotic strains were incubated with 3% nano-ASP, the growth was significantly higher and varied between 10.0 and 10.83 log CFU/g (Figure 3a), with enhancement between 1.20 and 2.05 log cycles (Figure 3b) depending on the strain. Additionally, at 2% nano-ASP, the probiotic counts increased by between 9.70 and 10.40 log CFU/g, with increases of between 0.95 and 1.55 log cycles. Additionally, the probiotic strains could be grown with 1% nano-ASP better than the control with lower counts between 8.95 and 9.49 log CFU/g (about 0.18 to 0.92 log cycles). According to the statistical analysis, the probiotic *L. acidophilus* was the highest strain raised by nano-ASP, but there was little significant difference between the other tested strains. Generally, the progress of the probiotic strains was indicated by the nutrient content of nano-ASP, especially the content of phenols and minerals. Phenolic compounds contained in different seeds and peels of fruits are utilized as a source of energy by the probiotics; the plant-based soluble dietary fibers have a prebiotic influence[47, 48]. From other studies, we know that polyphenols are commonly measured as prebiotic ingredients that enhance

commensal bacteria and human health [6,49-51].According to Duarte et al. [52], the phenol levels in seeds range from 2.3 to 5.7% when combined with other ingredients such as starch and fibers. Additionally, phenols could be affecting the adhesion, growth, and survival of probiotics. Phenolic compounds were not absorbed by the small intestine and instead reached the colon, where they were digested by the microbiota [53].

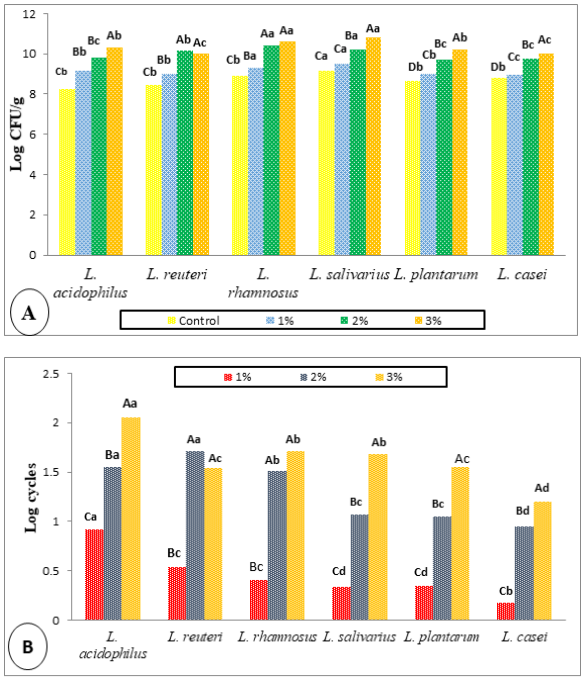


Figure 3: Evaluation the viability of probiotic strain in presence of nano-ASP.(A): the viable probiotic counts; (B); the Log cycles progress.

Data are represented as mean of three replicates. Means in the same capital superscript letters (effect between concentrations) are not significantly different ($p > 0.05$). Means in the same small superscript letters (effect between strains) are not significantly different ($p > 0.05$).

3.5. Chemical and nutritionalcomposition of fresh yogurt treatments and control

Table (4) shows the chemical and nutritional composition of nano-ASP and avocado seed nanopowdersupplemented stirred yogurt at levels 1-3. Nano-ASP contained 92.27 percent total solids as well as 4.41 percent total protein, 9.48 percent total fat, and 3.33% total ash. Nano-ASP demonstrated excellent quantities of carbohydrate (59.55%) and crude fiber (15.50%), along with DPPH scavenging activity (76.10 %). Moreover, the data on elements presented in nano-ASP showed that K, P, Ca, and Mg were the greatest abundant elements, while the other elements, in descending order by quantity, were Na, Fe, and Zn. The elemental values of nano-ASPwere potassium 776 mg/100 g, phosphorus 111 mg/100 g, calcium 60 mg/100 g, magnesium 37 mg/100 g, sodium 7.0 mg/100 g, iron 3.9 mg/100 g, and zinc 1.04 mg/100 g, which gave a high nutritional value to the nano-ASP. The changes in color measurements of nano-ASP were 50.60 for the l* value, 14.79 for the* value and 30.48 for the b* value (Table 4).

The total solids content of stirred yogurts supplemented with nano-ASP was slightly higher than in the control treatment, and this difference became more pronounced as the amount of avocado seed powder was increased. These findings, which agreed with those of El-Sayed et al. [54], confirmed that the total solids of yogurt increased as moisture content decreased (Table 4). The protein and fat content of nano-ASP supplemented yogurts and control treatments are not significantly different.

Table 4: Chemical, nutritional composition and colour measurements of nano-ASP, control stirred yogurt and stirred yogurt treatments *

Parameters	Nano-ASP	Control	T1	T2	T3
Moisture%	7.73	85.38	84.79	84.15	83.65
Total solids%	92.27	14.62	15.21	15.85	16.35
ASH%	3.33	1.13	1.15	1.17	1.20
Total protein%	4.41	4.51	4.15	4.13	4.09
Fat%	9.48	0.10	0.11	0.11	0.12
Carbohydrate %	59.55	8.88	9.63	10.12	10.46
Crude fiber%	15.50	-	0.17	0.32	0.48
(DPPH)%	76.10	43.33	55.41	69.22	71.71
Ca (mg/100g)	60.00	595	605	620	635
K (mg/100g)	776	630	640	655	681
Na (mg/100g)	7.00	181	183	189	192
P (mg/100g)	111	440	442	445	448
Mg (mg/100g)	37	43	45	51	62
Zn (mg/100g)	1.04	1.14	1.22	1.27	1.60
Fe (mg/100g)	3.90	0.47	0.58	0.67	0.96
l*	50.60	69.22	61.73	53.30	43.91
a*	14.79	-2.40	1.72	4.12	7.63
b*	30.48	16.20	17.69	18.60	20.01

Values are average of triplicate analysis with ± SD (0.001–4.8). l value represents from darkness (0) to lightness (100). a* value represents color ranging from redness (+) to greenness (−). b* value represents yellowness (+) to blueness. **Control**, yogurt without nano-ASP; **T1**, yogurt supplemented with 1% nano-ASP; **T2**, yogurt supplemented with 2% nano-ASP; **T3**, yogurt supplemented with 3% of nano-ASP

The ash,carbohydrate, crude fiber, DPPH scavenging activity, and mineral contents of stirred yogurt supplemented with nano-ASP were higher than control yogurt, in addition to increasing as the nano-ASP ratios increased. These were caused by the

high content of the previous components in the nano-ASP, as mentioned in Table (4). It was documented that avocado seeds have high levels of bioactive compounds such as polyphenols, minerals, and vitamins and contain 70% of the antioxidants found in the whole fruit of avocados[17, 18, 25, 55]. Moreover, it is a good source of fat, carbohydrate, and fiber for human consumption[56]. When compared to the control yogurt, the color parameters of nano-ASP supplemented stirred yogurt treatments decreased the white value (l*) of yogurt samples. Furthermore, the levels of nano-ASP supplementation significantly increased the red (a*) and yellow (b*) colors. It could be due to the high red color value in the nano-ASP which coincided with the results of [57].

3.6. Changes of pH and total solids for yogurts during storage

As shown in Figure (4A), pH values of stirred yogurt supplemented with nano-ASP were significantly lower ($P < 0.05$) than those of control yogurt when fresh and during their storage period. After four weeks of storage, the yogurt containing 3.0% nano-ASP (T3) had the lowest pH value. These modifications indicate that the acidity of the yogurt gradually increased during storage and was also increased with the addition of nano-ASP. This is mainly due to the increase in the growth of lactic acid bacteria and the production of acidic compounds that resulted from the fermentation process. Also, the nano-ASP had a good effect on the growth and activity of probiotic strains, as declared before in the part of microbiology (3.4).

The variations in total solids content of stirred yogurt as a result of nano-ASP addition ratios and storage time are depicted in Figure (4B). The total solid (TS) contents increased significantly (P >0.05) with the increased ratio of nano-ASP; they also increased significantly during storage, owing primarily to the high total solids content of nano-ASP (92.27%).

3.7. Microbiological evaluation of stirred yogurt treatments during storage

3.7.1. L. acidophilus counts

The data in Figure (5) shows the counts of the probiotic stain L. acidophilus in all stirred yogurt treatments during cold storage for 30 days. The results indicated that the viable counts of L. acidophilus gradually improved during storage until 15 days of storage. The higher viable counts were recorded for all treatments without significant differences between them, where the counts of L. acidophilus were recorded at 8.92, 8.84, and 9.00 log CFU/ml for T1, T2, and T3, respectively. Following that, a minor decrease in viable counts was observed after 21 days of storage. However, it was not expected that the viable cell counts in the treatment group would be lower than in the control group. At 30 days, the counts were 8.28, 8.00, 7.8 and 7.45 log CFU/mL for control, T1, T2, and T3, respectively. During storage, the drop in pH and excess organic acid production might have caused the decline in counts, as mentioned by other studies [58-60].

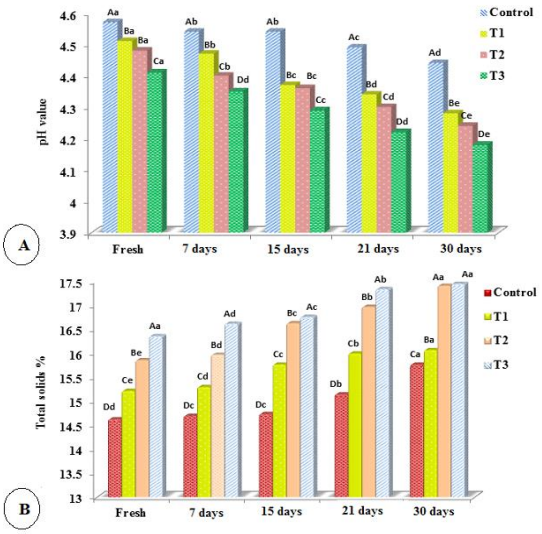


Figure 4 A, B: Changes in the pH values and total solidsof stirred yogurt treatments.

Control, yogurt without nano-ASP; **T1**, yogurt supplemented with 1% nano-ASP; **T2**, yogurt supplemented with 2% nano-ASP; **T3**, yogurt supplemented with 3% of nano-ASP. Data are represented as mean of three replicates. The same capital letters (difference between treatments) and the same small letters (differences between storage times) are not significantly different (P > 0.05).

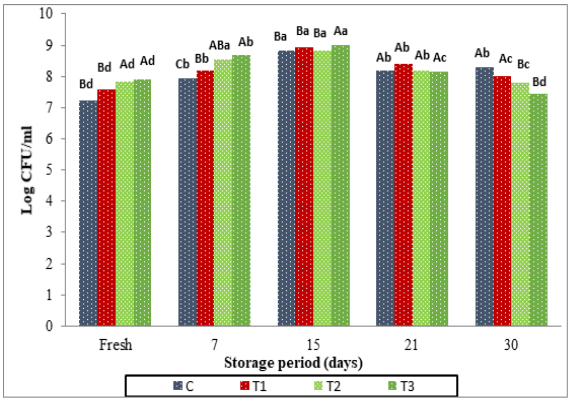


Figure 5: The *L. acidophilus* counts of stirred yogurt treatments during storage.

Control, yogurt without nano-ASP; **T1**, yogurt supplemented with 1% nano-ASP; **T2**, yogurt supplemented with 2% nano-ASP; **T3**, yogurt supplemented with 3% of nano-ASP. The data was expressed as the means of three replicates. The same capital letters (difference between treatments) and the same small letters (differences between storage times) are not significantly different (P > 0.05).

From the data, the addition of nano-ASP to stirred yogurt significantly enhanced the counts of *L. acidophilus* for two weeks, but the study did not observe a significantly different outcome between the treatments during storage. As a result, the 2% nano-ASP used was sufficient to keep the probiotic strain alive in the stirred yogurt for two weeks with sufficient counts to have a positive impact on humans. As Chouchouli et al. [61] found, fortifying yogurt with grape seed extracts filled with phenols did not cause a significant modification in the lactic acid bacteria populations compared with controls. Similarly, Ma et al. [62] demonstrated that the growth and metabolism of *L. casei* LC2W and *L. casei* 01 were enhanced by the integration of rich phenolic ingredients in cow milk.

3.7.2. The starter culture counts

As can be seen in Figures (6 A, B), the counts of the starter cultures (*L. bulgaricus* and *S. thermophilus*) used to make stirred yogurt were assessed during storage. In all treatments, the *L. bulgaricus* counts exhibited similar behavior (Figure 6A). The difference in counts between treatments and the control was caused by the fortification of treatments with nano-ASP. Whereas the *L. bulgaricus* in fresh products was recorded at 7.00, 7.21, and 7.33 log CFU/ml for T1, T2, and T3, respectively, but the count of the control was slightly decreased (6.70 log CFU/ml). The viability of the *L. bulgaricus* numbers grew over storage time to reach the highest counts at day 15 of storage, and then it decreased to reach 7.33, 7.00, 7.15 and 6.80 log CFU/ml for control, T1, T2 and T3, respectively. This reduction in *L. bulgaricus* counts was related to the acidity development, especially in the treatments that were fortified with nano-ASP. As mentioned by Donkor et al. [58], the proteolysis improved the survival of *L. delbrueckii* ssp. *Bulgaricus* Lb1466 during storage, causing pH lowering and the production of higher levels of organic acids that might have affected the low cell counts during storage.

Furthermore, the *S. thermophilus* counts demonstrated the same activities (Figure 6B). No significant differences in their counts were detected during the storage period for the fresh treatments. The S. thermophilus counts were recorded at 7.00, 7.15, 7.28, and 7.33 log CFU/ml for control, T1, T2, and T3, respectively, at fresh. However, when compared to the control, the viable counts for treatments increased over time for 15 days. The counts at 15 days were 7.90, 8.30, 8.78, and 9.00 log CFU/ml for control, T1, T2, and T3, respectively. The counts of S. thermophilus after that were slightly lower at the end of storage, but all counts for treatments were located in 7 log cycles. According to the findings, the addition of nano-ASP increased the lactic acid counts for 15 days of storage with no significant differences in concentrations. Also, the excessive acidity resulting from the metabolic activity of strains had a negative influence on the counts. But all in all, the lactic acid counts in final products were located between 6 and 7 log cycles.

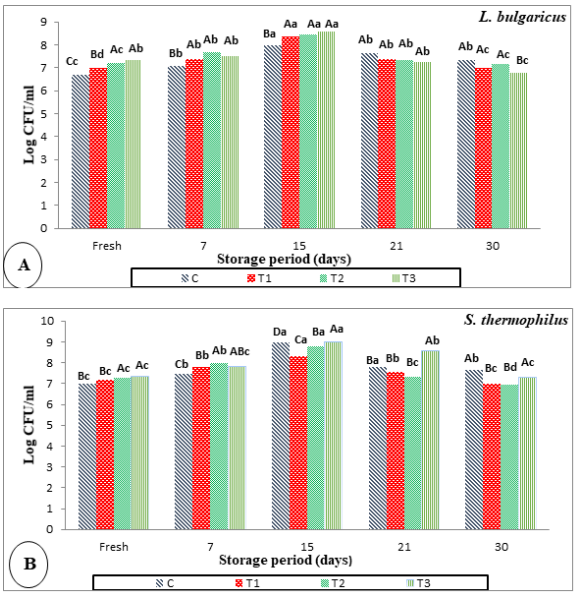


Figure 6 A, B: The starter cultures counts in stirred yogurt treatments during storage.

Control, yogurt without nano-ASP; **T1**, yogurt supplemented with 1% nano-ASP; **T2**, yogurt supplemented with 2% nano-ASP; **T3**, yogurt supplemented with 3% of nano-ASP.The data was expressed as the means of three replicates. The same capital letters (difference between treatments) and the same small letters (differences between storage times) are not significantly different ($P > 0.05$).

3.7.3. The total aerobic bacterial counts

In this study, the aerobic total bacterial counts were evaluated during storage, as shown in Figure (7)to confirm the effect of nano-ASPas a natural preservative agent. According to the data in the figure, total bacterial counts increased gradually during the controlled storage period. On the contrary, nano-ASPconcentrations in the treatments had every little bit bacterial counts. Total bacterial counts ranged between 5.6 and 4.8 log CFU/ml in fresh treatments, which it increased in the control but decreased in the other treatments after 15 days of storage. Total bacterial counts had increased slightly after 21 days of storage, and an even greater increase was observed at the end of storage. Where the total bacterial counts were recorded at 7.11, 4.00, 4.00 and 3.28 log CFU/ml for control, T1, T2, and T3, respectively. So, from the results, the addition of nano-ASPwas able to reduce undesirable bacterial counts during storage until 15 days, compared with the control. Also, minor but significant differences

were detected between the treatments with different nano-ASP concentrations.

Furthermore, during storage periods, all mold and yeast-free treatments count, based on the heat treatments and hygienic roles used during the stirred yogurt manufacturing process. Finally, the addition of nano-ASPwas a new trend in the microbiological evaluation of stirred yogurt as a natural agent full of nutrition components plus having mild antimicrobial activity as a bactericidal effect, which maintained the microbiological properties of stirred yogurt that were free of contamination with a sufficient probiotic count for 15 days. This preservative effect was related to the phenolic compounds found in nano-ASP. Besides, there was a slight but significant difference in microbiological counts between treatments (the stirred yogurt was fortified with 1, 2, and 3% nano-ASP); thus, the 2% concentration was optimal for product preservation during storage. Bahru, et al.[18]concluded that avocado seeds were considered good sources of protein, fat, ash, moisture, fiber, carbohydrates, minerals (Ca, Zn, K, Na, P and Co), phytochemicals (flavonoids, tanine, saponine, total phenols), and vitamins (A, B1, B2, B3, C, and E) with biological activities such as antioxidant, antihypertensive, fungicidal, and hypolipidemia. Also, according to Cardoso et al. [63], the avocado seeds are potential source of antimicrobial agents and were considered important in a new study with purified nano-powder that identified the compounds responsible for the antimicrobial activity.

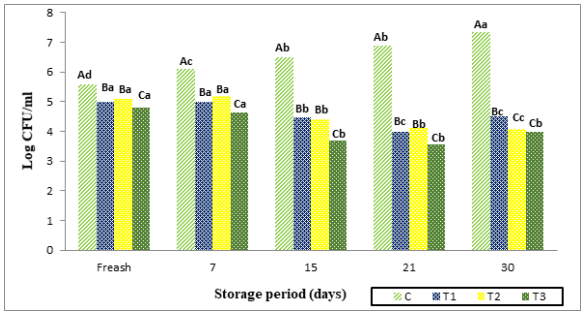


Figure 7: Total aerobic bacterial counts of stirred yogurt treatments during storage.

Control, yogurt without nano-ASP; **T1**, yogurt supplemented with 1% nano-ASP; **T2**, yogurt supplemented with 2% nano-ASP; **T3**, yogurt supplemented with 3% of nano-ASP.The data was expressed as the means of three replicates. The same capital letters (difference between treatments) and the same small letters (differences between storage times) are not significantly different ($P > 0.05$).

3.8. Sensory properties of nano-ASP supplemented yogurts

Figure (8) depicts the overall acceptability of yogurts supplemented with varying amounts of nano-ASP and control yogurts after 4 weeks of cold storage. The overall acceptability of supplemented yogurts was influenced by both the level of nano-ASP and the storage period. Yogurts containing 1.0% nano-ASP scored close to the control samples in terms of overall acceptability. After four weeks of cold storage, stirred yogurts containing 1.0% and 2% nano-ASP received higher overall acceptability scores than yogurtscontaining only 3.0% nano-ASP. Stirred yogurts with high levels of nano-ASP (3.0%) and stored for four weeks had the lowest overall acceptability. This is primarily due to the increased lactic acid bacteria count in this treatment, which had a negative impact on the taste and odor at the end of storage [64]. In both the control and supplemented yogurts, all sensory attributes gradually decreased during storage. The color and appearance, as well as the body and texture of the yogurts, were appropriate with the level of supplementation, so that the color became slightly brownies and the texture thicker. There was also no chalkiness in the flavor of yogurts containing levels 1.0 and 2.0% nano-ASP, except for stirred yogurts containing 3.0% nano-ASP, which had a slight chalkiness.

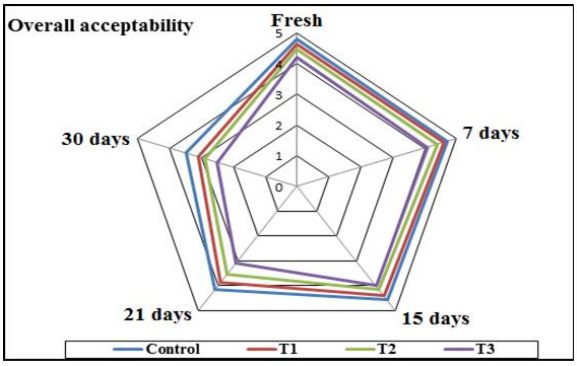


Figure 8: The overall acceptability of stirred yogurts treatments during storage period.

Control, yogurt without nano-ASP; **T1**, yogurt supplemented with 1% nano-ASP; **T2**, yogurt supplemented with 2% nano-ASP; **T3**, yogurt supplemented with 3% of nano-ASP.

3.9. *A. niger* counts in contaminated stirred yogurt during storage

Figure (9) shows the *A. niger* counts in the contaminated part of the stirred yogurt treatments. The study designated stirred yogurt treatments with A. niger as the model for fungi that may be present in the fermented dairy products during storage period. Therefore, the effect of nano-ASPas a preservative agent was confirmed and evaluated against A. niger by storage in contaminated treatments. According to the findings, the levels of *A. niger* increased more with time under control than with other treatments. Firstly, at fresh, the A. niger counts ranged from 2.00 to 2.25 log CFU/ ml. Following that, minor counts

were observed in treatments with nano-ASP, which acted as a preservative agent not permitted to advance the *A. niger* during storage, particularly for 15 days (the counts persisted in the 2 log cycles for all treatments, compared to the control, which exceeded the 4 log cycle at 15 days). But after 21 days of storage, a little progress was observed in the stirred yogurt with nano-ASPconcentrations and slightly increased at the 30 days of storage. The A. niger counts were recorded 6.28, 4.18, 3.67 and 3.00 log CFU/ml for control, T1, T2 and T3, respectively at 30 days of storage. So, the suitable shelf life for stirred yogurt with nano-ASPnot exceeds the 21 days.

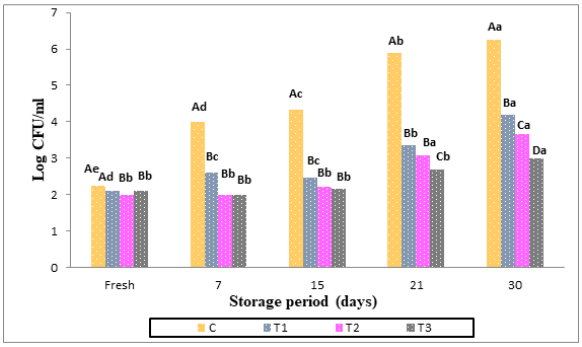


Figure 9: *A. niger* counts in contaminated stirred yogurt fortified with nano-ASPconcentrations during storage.

Control, yogurt without nano-ASP; **T1**, yogurt supplemented with 1% nano-ASP; **T2**, yogurt supplemented with 2% nano-ASP; **T3**, yogurt supplemented with 3% nano-ASP.The data was expressed as the means of three replicates. The same capital letters (difference between treatments) and the same small letters (differences between storage times) are not significantly different ($P > 0.05$).

4. Conclusion

This study was carried out on the basis of maximizing the utilization of avocado seed waste, which contains several food grade ingredients. So, the avocado seeds were converted to nano scale powder and integrated in stirred yogurt. The nano-avocado seeds powder (nano-ASP) was considered a significant source for polyphenols with respectable antioxidant activity, which had a positive effect on progress some probiotic strains and moderated antimicrobial activity against some pathogens. Also, nano ASP was demonstrated agriculture waste rich in carbohydrate, crude fiber, and elements (K, P, Ca, and Mg) that gave a high nutritional impact to stirred yogurt. The stirred yogurt was produced with different nano-ASP (1, 2, and 3 %) with *L. acidophilus* as a probiotic strain. From the final evaluation of the products during 30 days, the data concluded that stirred yogurt with nano-ASP was higher in ash, carbohydrates, crude fiber, DPPH scavenging activity, and mineral contents than control. In addition, The *L. acidophilus* and starter cultures counts were improved with different concentrations up to 15 days especially stirred yogurt with 1.0 and 2.0 %nano-ASP. The sensory evaluation for stirred yogurt was overall acceptable during storage with 1.0 and 2.0 % other than 3.0 %. Also, the presence of nano-ASP concentration in stirred yogurt was able to maintain the shelf life of products and control the progress of *A. niger* in contaminated treatments compared to control.

5. Disclosure statement

No potential conflict of interest was reported by the authors.

6. Authors' contributions

All authors contributed equally to that manuscript regarding design of experiments, experimental work, preparing and reviewing the manuscript before submission.

7. Data Availability Statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

8. Funding

Not applicable.

9. Ethics approval

This work was approved by the Medical Research Ethics Committee (MREC), National Research Centre, Cairo, Egypt with approval number 8661212022, and followed the recommendations of the National Institutes.

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