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Fortification of Cow Milk with *Moringa oleifera* Extract: Influence on Physicochemical Characteristics, Antioxidant Capacity and Mineral Content of Yoghurt

Katarina Lisak Jakopović [1], Maja Repajić [1,*], Ivana Rumora Samarin [2], Rajka Božanić [1], Marijana Blažić [3,4] and Irena Barukčić Jurina [1]

[1] Faculty of Food Technology and Biotechnology, Department of Food Engineering, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia
[2] Faculty of Food Technology and Biotechnology, Department of Food Quality Control, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia
[3] Department of Food Technology, Polytechnic Karlovac, Trg J.J. Strossmayera 9, 47000 Karlovac, Croatia
[4] Gastronomy Department, Aspira University College, Mike Tripala 6, 21000 Split, Croatia
* Correspondence: maja.repajic@pbf.unizg.hr

Abstract: Background: Fermented dairy products are known for their many positive effects on human health and are consumed worldwide. The supplementation of food with plant extracts as sources of valuable nutritional compounds has recently gained a lot of attention. Milk and fermented products are deficient in bioactive components such as phenolic compounds and iron. *Moringa oleifera* leaf extract is rich in vitamins, minerals (iron), polyphenols, flavonoids, tannins and proteins. Its addition to milk before fermentation might represent an excellent way to enrich fermented milk products. Methods: Yoghurts enriched with moringa extract (ME) (1, 3 and 4-%, *v*/*v*) were produced and compared to a control yoghurt without ME. In all samples, acidity, microbiological parameters, syneresis and water holding capacity, rheology parameters, total colour difference, mineral content, total phenols and antioxidant capacity (FRAP method) and sensory properties were determined. Results: The addition of ME to milk before fermentation resulted in a shorter fermentation time, lower yoghurt pH, increased growth of yoghurt bacteria, better rheological properties and an increased total phenols content as well as antioxidant capacity of yoghurts. Moreover, yoghurts with ME addition had a higher mineral content and gained a better sensory score when compared to the control sample.

Keywords: antioxidant capacity; phenols; fermentation; yoghurt; mineral content; *Moringa oleifera* extract

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1. Introduction

Fermented dairy products have been consumed for thousands of years; therefore, people have known their health benefits since ancient times. Yoghurt is certainly one of the most famous and most commonly consumed fermented dairy products. It is characterized by high nutritional value due to its valuable proteins, minerals and lactic acid bacteria, and it is important to point out that yoghurt, as with other fermented dairy products, is more easily digestible than milk itself [1–3]. The emergence of functional food has enabled a new approach to human diet and, at the same time, better care for human health. Plant extracts in dairy products might improve the physicochemical characteristics, which has a positive effect on consumers [4].

Moringa oleifera originates from India and is recognized for its rich nutritional composition in both medicine and the food sector as well. Although all parts of this plant can be utilised, moringa seed oil and moringa leaf are most often used for generating extracts that are added to foods in order to improve their nutritional and health value [5]. The popularity of moringa has risen sharply in recent years. Scientists have recognized the possibility

of the wide application and benefits of moringa both in agriculture and in the industrial production of food supplements [6,7]. Moringa is rich in bioactive components such as polyphenols and is a very good source of minerals such as iron, zinc or magnesium [8]. A very recent study by Ao et al. [9] has shown that selenium nanoparticles isolated from *Moringa oleifera* can effectively fight against pathogens such as *Listeria monocytogenes* and *Corynebacterium diphtheria* and, thus, exhibit great potential in food and medical science.

Such findings are particularly noteworthy for the dairy sector, whose production in the last few years has focused on the production of dairy products enriched by bioactive ingredients such as fruits, vegetables and various plant extracts [7]. Numerous scientific studies have been conducted to confirm the positive impact of moringa on food fortification and the improvement in technological properties during production, but also to determine the benefits of moringa on human health. Hassan et al. [10] published a study on yoghurt production with the addition of *Moringa oleifera* leaves. They examined the influence of the addition of dried and chopped moringa leaves (0.5%, 1% and 2%) on the sensory and chemical properties of fresh buffalo milk yoghurts. Yoghurt supplemented with 0.5% powder of *M. oleifera* leaves was considered as optimal according to the appearance, taste and aroma of the final product. The results also indicated that yoghurts enriched with moringa leaf powder had a higher dry matter content, higher protein content, higher acidity and lower pH value in comparison to the control sample. Hassan et al. [6] published a study on the effect of *M. oleifera* addition to soft white cheese. Cheeses were produced from buffalo milk, while moringa leaf powder was added to the cheeses immediately after coagulation in proportions of 1%, 2% and 3%. The results showed that the cheese enriched with 1% of moringa leaf powder had the best sensory properties in terms of the best rated appearance, texture and taste as well as a higher acidity and lower pH value in comparison with the control sample of cheese without the addition of moringa during 6 weeks of storage. Zhang et al. [7] produced a functional yoghurt with the addition of the aqueous extract of moringa leaf powder (0.05%, 0.1% and 0.2%) to investigate its impact on the fermentative, textural and bioactive properties. The obtained results showed that moringa extract reduced the fermentation time, increased viscosity, decreased the syneresis rate and showed a higher ability to "catch" free radicals. The accelerated fermentation time might be associated to the phytochemical components (phenolic acids, organic acids and flavonoids), which are known to promote the lactic acid bacteria (LAB) growth. Moreover, some authors claim that moringa extract acts as a prebiotic to LAB [7]. Furthermore, Shori [11] reported about the addition of nutmeg, black pepper and white pepper extracts into the plain yoghurts and concluded that polyphenol compounds derived from added extracts could enrich yogurt with antioxidant properties, where nutmeg extract addition shows the best effect [11].

Since fermented milk lacks some minerals such as iron, zinc, magnesium, cooper and bioactive polyphenols, the aim of the present study was to produce a yoghurt with the addition of moringa leaf extract and to compare it with a control yoghurt without the addition of the extract in terms of milk fermentation time, mineral element enrichment, total phenols content and the antioxidant capacity. Physicochemical, microbiological, rheological and sensory analyses and yoghurt colour were also determined.

2. Materials and Methods

2.1. Moringa Extract Production

M. oleifera extract (ME) was produced from 10 g of dry leaves powder (Bio&Bio, SuperFoods, Zagreb, Croatia), boiled with 200 mL of distilled water. Suspension was mixed and boiled on magnetic stirrer (Rotamix 550, Tehtnica, Železniki, Slovenia) for 30 min. After cooling to about 60 °C, suspension was filtrated trough filter paper (Whatman TM, 150 mm) and used as an extract addition into the milk prior the fermentation.

2.2. Yoghurt Production

Pasteurized and homogenized cow milk with 3.2% milk fat (Dukat Ltd., Zagreb, Croatia) was used for yoghurt production. Since experiments were repeated three times,

different milk batches were used every time. Milk was preheated to the fermentation temperature of approximately 43 °C, divided into smaller portions and supplemented by adding ME. The control sample was not supplemented with ME. After ME addition, milk samples were inoculated with thermophilic lyophilized yoghurt culture YoMix 10 DCU (Danisco-DuPont, Wilmington, NC, USA). Four different fermentations were performed: control (without ME addition); moringa 1% (addition of 1% ME (*v*/*v*)); moringa 3% (addition of 3% ME (*v*/*v*)) and moringa 4% (addition of 4% ME (*v*/*v*)). Fermentation was performed at 43 °C until pH value reached about 4.6 in all samples. Afterwards, fermentation was stopped with cooling and samples were stored at +4 °C for 28 days. All analyses were performed on the 1st, 7th, 14th, 21st and 28th day of storage, except for mineral content (1st day), microbiological analysis (beginning and end of fermentation, 28th day), syneresis and water holding capacity (1st and 28th day).

2.3. Acidity Measurements

Active acidity was measured by a pH meter (WTW, pH3110, Weilheim, Germany) described earlier [12].

2.4. Microbiological Analyses

All yoghurt samples were analysed for viable counts of lactobacilli and streptococci at the beginning and at the end of fermentation as well as at the end of storage period (28th day). The initial solution was prepared by diluting 20 g of yoghurt in 180 mL of sterile physiological solution (0.9% NaCl) [13]. The obtained suspension was used for preparation of further dilutions. The viable count of *Lactobacillus* sp. was enumerated at the De Man, Rogosa and Sharp (MRS) agar (37 °C/48 h), while M-17 agar (37 °C/48 h) was used for *Streptococcus* sp. enumeration (both Biolife, Milan, Italy).

2.5. Syneresis, Water Holding Capacity and Rheology Parameters

Syneresis was determined according to the modified method by Joung et al. [14]. Briefly, 20 g of yoghurt was weighed into 50 mL cuvettes and centrifuged at 5000 rpm/min (Rotina 380R Hettich, Tuttlingen, Germany) for 10 min. After the centrifugation, aliquot was divided, its mass was weighed and syneresis index was calculated according to Equation (1) [15]

$$\text{S (\%)} = [\text{weight (supernatant)}/(\text{weight (sample)}] \times 100 \quad (1)$$

Water holding capacity (WHC) was determined using a modified centrifugal method according to Feng et al. [16] and method described by Cardines et al. [15]. Briefly, 20 g of yoghurt was weighed and centrifuged at 5000 rpm for 10 min at 4 °C. The obtained supernatant was separated, the residual precipitate was weighed and WHC was calculated according to Equation (2)

$$\text{WHC (\%)} = [\text{weight (drained gel)}/(\text{weight (sample)}] \times 100 \quad (2)$$

A rotating rheometer Rheometric Scientific RM-180 (Rheometric Scientific, Inc., Piscataway, NJ, USA) was used to determine rheological properties of yoghurt samples at 20 °C. Thereby, shear stress (T) and apparent viscosity (μ in Pa s) were measured at shear rates ranging between 100 and 1290 m s^{-1}, and they were calculated by linear regression to the flow index (n), consistency coefficient (K, mPas) and related linear regression coefficient (R^2).

2.6. Colour Measurements

The colour of yoghurt samples was determined according to the CIElab system using a CM-3500d spectrophotometer (Konica Minolta, Tokyo, Japan) with D65 light source. The data were obtained in the SpectraMagic NX program. The total colour difference

was calculated in order to determine if ME-supplemented samples differed from control samples. The total colour difference (ΔE^*) was calculated according to Equation (3) [6]

$$\Delta E^* = \sqrt{[(L^* - L^*_{ref})^2 + (a^* - a^*_{ref})^2 + (b^* - b^*_{ref})^2]} \quad (3)$$

where L^*, a^* and b^* refer to the test samples and L^*_{ref}, a^*_{ref} and b^*_{ref} to the control sample [17].

2.7. Mineral Content Determination

Prior the mineral content determination, frozen samples (approx. 20 g) were lyophilized for 48 h at −50 °C (HETOSIC, Heto Ltd., Gydevang, Denmark) and stored at room temperature until decomposition. Samples were prepared for digestion in a microwave system UltraCLAVE IV (Milestone, Sorisole, Italy). Aliquots (~0.250 g) were weighed in quartz vessels and digested with purified concentrated nitric acid (HNO_3, conc. 65% p.a., Merck, Germany) and ultrapure water (purified in BarnsteadTMSmart2Pure 6 UV/UF, Thermo Scientific, Germany) (2:2). After digestion, all samples were adjusted to 6 g with ultrapure water. Reagent blanks (n = 4) and reference materials (n = 3) were prepared and analysed in the same way as samples. Blanks were digested in a microwave system UltraCLAVE IV (Milestone, Sorisole, Italy) and aliquots (~0.250 g) were weighed in quartz vessels and digested with purified concentrated nitric acid (conc. 65% p.a.) and ultrapure water (2:2). Reference material was skimmed milk powder BCR-150 and BCR-151 (from the European Institute for Reference Materials and Measurements, Belgium) and IAEA-153 milk powder (from the International Atomic Energy Agency) and they were used for quality control of measurements. Mineral elements were analysed by inductively coupled plasma mass spectrometry (ICP-MS) using Agilent 7500cx (Agilent Technologies, Santa Clara, CA, USA) equipped with a collision/reaction cell. The accuracy of measurements was checked using Certified Reference Materials (CRM) skim milk powder BCR-150 and BCR-151, Institute for Reference Materials and Measurements (IRMM), Belgium. Digested samples were additionally diluted to 1:20 with a solution containing 1% (v/v) HNO_3 and 3 µg/L internal standards (Ge, Rh, Tb, Lu and Ir) before analysis.

2.8. Total Phenols Content and Antioxidant Capacity

For the determination of total phenols content (TPC) and antioxidant capacity (AC), yoghurt samples were prepared as follows: 20 g of yoghurt sample was weighed into the 50 mL Falcon cuvette and centrifuged at 5000 rpm/min for 10 min. After centrifugation, supernatant was divided and filtered through Whatman filter paper pore size 0.45 µm and used for the analyses.

TPC was analysed by Folin–Ciocalteu method according to the modified method of Shortle et al. [18]. The results were expressed as milligrams of gallic acid equivalent (GAE) per litre. Equation (4) obtained from the standard curve was as follows

$$Y = 0.0035 \times X \ (R^2 = 0.9995) \quad (4)$$

Antioxidant capacity (AC) was determined by Ferric Reducing Antioxidant Power (FRAP) method. FRAP assay was performed according to the procedure described by Benzie [19] and Benzie and Strain [20]. The standard curve was plotted using 2 mM Trolox stock solution. Trolox concentrations used for the standard curve establishment were 25, 50, 75, 100, 125, 250, 500, 750, 1000 and 1500 µM. Equation (5) was obtained from the standard curve as follows

$$Y = 0.0014 \times X \ (R^2 = 0.9995) \quad (5)$$

FRAP results were expressed as µmol TE L^{-1}.

2.9. Sensory Evaluation

Sensory evaluation of yoghurt samples was performed by a group of five specially trained panellists using a scoring system of weighted factors on a 20-point scale [21,22].

Yoghurt samples were cool stored at 4 °C from the point of production, until the point of sampling and evaluation. In a room designed according to ISO standard 8589:2007 [23], samples were opened, encoded, divided into equal portions and presented simultaneously to each of the five assessors. Samples were evaluated for overall appearance, colour, odour, consistency, syneresis appearance and taste, whereby each attribute could be rated with notes from 1 to 5. The average note of every attribute was multiplied with a predetermined weighting factor, resulting in a score for each attribute as follows: taste, 10 scores; consistency, 4 scores; odour and syneresis, 2 scores each; and overall appearance and colour, 1 score each. By summarizing scores of each attribute, a final score for the particular sample was obtained. The maximum score that one sample could obtain was 20 [22].

2.10. Statistical Analysis

Fermentations were repeated three times, and the obtained results of all measurements were expressed as the mean ± standard deviation (SD). For statistical analysis, Shapiro–Wilk test and Levene's test were applied to test the normality and homoscedasticity of the data. Data were then analysed with ANOVA (for parametric data) or the Kruskal–Wallis test (for nonparametric data), and means within groups were compared with Tukey's HSD test or the Kruskal–Wallis test when appropriate. The significance level for all tests was $p \leq 0.05$, and the results of statistical analysis are presented as mean ± standard error (SE). All statistical tests were performed using Statistica ver. 12.0 software (StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Fermentation Time and Acidity

The acidity of control samples and samples with ME addition was measured during the fermentation period. pH value was measured in order to investigate if ME addition has an influence on the fermentation time. The initial pH values were almost the same in all samples, and it is evident that ME did not affect the initial pH (Figure 1). ME addition reduced the fermentation period for 1 h when compared to the control sample and fermentation lasted 4 h in all supplemented samples no matter the ME amount. On the other hand, fermentation time for the control sample was 5 h. A one-hour shorter fermentation time could be of great interest in the dairy industry due to the economical savings. Zhang et al. [7] also produced yoghurts with ME addition (0.05, 0.1 and 0.2%) and they also concluded that ME addition reduced fermentation time. Fermentation with 0.2% ME addition reduced the fermentation period for 3 h when compared to the control sample (6 h of fermentation). Some phytochemicals such as phenolic components and oligosaccharides can act as prebiotics and thus stimulate the growth of lactic acid bacteria (LAB). Therefore, it can be assumed that ME addition, rich in the listed phytochemicals, prior to the fermentation could promote LAB growth and thus reduce fermentation time. In our previous study [24], olive leaf extract (OLE) addition into the milk prior to the fermentation also reduced fermentation time by 36 min in comparison with the control sample without OLE addition. Fermentation with 3% OLE (v/v) addition lasted for 4 h, which is the same period as with the addition of ME in the present study. OLE composition also includes bioactive compounds, primarily polyphenols, which act as prebiotics and thus stimulate LAB growth and consequently reduce the fermentation time.

pH of the produced yoghurts was measured in 7-day intervals (Table 1) for 28 days of cold storage (+4 °C). On the first day of yoghurt production there was a pH drop in all samples ranging from 0.10 to 0.21 pH units compared to the end of the fermentation. As the concentration of ME increased, the pH drop also increased. The highest pH drop was observed on the 14th day of cold storage for the control and the M 1% samples, while in the M 3% and the M 4% samples, a pH decrease was observed on the 7th day of storage. The observed drop in the pH value during the first days of the storage period could be related to the post acidification occurring as a result of the residual activity of LAB digesting lactose

to lactic acid but less intensively than at optimal temperature conditions. Afterwards, a further increase between 0.1 and 0.2 pH units was observed until the end of storage.

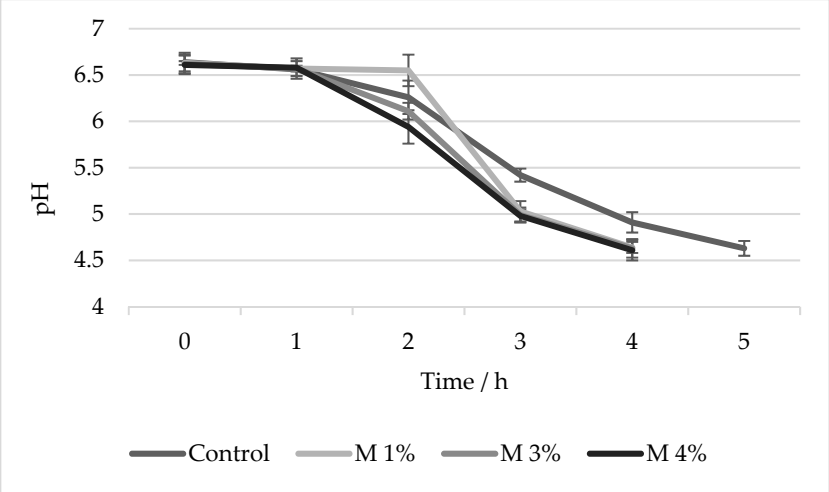


Figure 1. pH values measured during fermentation time (h) in control sample (Control) and samples with ME addition (1, 3 and 4% of extract addition, M 1%, M 3%, M 4%). Results are expressed as mean values and error bars show SD.

Table 1. pH values measured during cold storage (1st, 7th, 14th, 21st and 28th day) in control sample (Control) and samples with moringa extract addition (1, 3 and 4% of extract addition, M 1%, M 3%, M 4%).

Time (Days)	pH Value			
	Control	**M 1%**	**M 3%**	**M 4%**
1	4.53 ± 0.12	4.51 ± 0.12	4.43 ± 0.09	4.40 ± 0.11
7	4.32 ± 0.08	4.22 ± 0.02	4.21 ± 0.10	4.15 ± 0.09
14	4.27 ± 0.10	4.12 ± 0.10	4.24 ± 0.12	4.21 ± 0.11
21	4.34 ± 0.11	4.35 ± 0.13	4.36 ± 0.09	4.29 ± 0.10
28	4.37 ± 0.13	4.32 ± 0.11	4.36 ± 0.11	4.32 ± 0.04

Results are expressed as mean ± SD.

Final pH values after 28 days of cold storage were more or less similar, ranging between 4.32 and 4.37. The influence of extract content and storage time on pH value is shown in Table 2. The results presented in Table 2 show the average values of each source of variation (extract content and storage time) for each physical and chemical characteristics of yoghurt (pH, TPC, AC, flow index and consistency). According to statistical analysis, extract content had a significant influence (p = 0.001) on the pH of yoghurts, where control samples showed significantly higher pH values compared to the ME 1% and ME 4% samples (Table 2). The influence of storage time on the pH was also significant ($p < 0.001$) and significant differences were observed between the 1st day of cold storage in comparison to the rest of the cold storage period. Between the 7th and 14th day there was no difference, as well as between the 21st and 28th day of cold storage. In our previous research with OLE addition, results showed the same pH trend [24].

Table 2. Influence of extract content (0, 1, 3 and 4% ME) and storage time (1st, 7th, 14th, 21st and 28th day) on the physical and chemical characteristics of yoghurts.

Source of Variation	pH	Total Phenols (mg GAE L^{-1})	FRAP (μmol TE L^{-1})	Flow Index (n)	Consistency (K, mPas)
Extract content (%)	$p = 0.001$ *	$p < 0.001$ *	$p < 0.001$ *	$p = 0.524$	$p = 0.166$
0	4.37 ± 0.03 ^{b}	77.4 ± 1.6 ^{a}	503.3 ± 1.6 ^{a}	0.72 ± 0.04 ^{a}	5.77 ± 0.31 ^{a}
1	4.29 ± 0.04 ^{a}	86.5 ± 1.8 ^{b}	529.8 ± 1.8 ^{a}	0.66 ± 0.02 ^{a}	5.39 ± 0.18 ^{a}
3	4.32 ± 0.03 ^{ab}	89.2 ± 4.0 ^{b}	636.3 ± 4.0 ^{b}	0.70 ± 0.03 ^{a}	5.74 ± 0.20 ^{a}
4	4.28 ± 0.03 ^{a}	100.5 ± 2.9 ^{c}	633.2 ± 2.9 ^{b}	0.69 ± 0.01 ^{a}	5.81 ± 0.20 ^{a}
Storage time (day)	$p < 0.001$ *	$p = 0.214$	$p = 0.757$	$p < 0.001$ *	$p = 0.001$ *
1	4.46 ± 0.02 ^{c}	85.0 ± 3.0 ^{a}	587.8 ± 26.4 ^{a}	0.60 ± 0.01 ^{a}	4.64 ± 0.23 ^{a}
7	4.23 ± 0.03 ^{a}	84.7 ± 1.9 ^{a}	593.5 ± 21.2 ^{a}	0.66 ± 0.01 ^{ab}	5.92 ± 0.02 ^{ab}
14	4.21 ± 0.02 ^{a}	91.0 ± 5.1 ^{a}	570.5 ± 15.8 ^{a}	0.68 ± 0.01 ^{bc}	5.69 ± 0.09 ^{ab}
21	4.33 ± 0.01 ^{b}	97.0 ± 3.6 ^{a}	558.4 ± 27.3 ^{a}	0.74 ± 0.03 ^{cd}	5.95 ± 0.19 ^{b}
28	4.34 ± 0.01 ^{b}	84.2 ± 5.3 ^{a}	568.1 ± 32.2 ^{a}	0.80 ± 0.02 ^{d}	6.18 ± 0.21 ^{b}
Grand mean	4.31 ± 0.02	88.4 ± 1.9	575.7 ± 1.9	0.69 ± 0.01	5.68 ± 0.11

* $p \leq 0.05$. Results are expressed as mean ± SE. Values with different letters within column are statistically different at $p \leq 0.05$.

3.2. Microbiological Analysis

Lactobacillus sp. and *Streptococcus* sp. counts were determined at the beginning (prior to the incubation) and at the end of the fermentation time as well as at the end of the cold storage period (Table 3). The obtained results prior to fermentation showed that *Lactobacillus* sp. and *Streptococcus* sp. counts were more or less the same and ranged between 8.77 and 8.99 log CFU mL^{-1} and 6.92 and 6.97 log CFU mL^{-1}, respectively. At the end of the fermentation period, which lasted for 5 h in the control sample, viable counts of lactobacilli and streptococci were 10.48 and 9.89 log CFU mL^{-1}, respectively. The viable count of lactobacilli at the end of the fermentation period was the highest fermentation in the control sample, while the lowest count was observed in yoghurt supplemented with 1% ME (8.70 log CFU mL^{-1}). Since fermentation in ME-supplemented yoghurts lasted 1 h less (Figure 1), it was expected that viable counts of lactobacilli and streptococci would be higher in all supplemented yoghurts in comparison to the control. However, the number of *Streptococcus* sp. was higher only in the 3% ME-supplemented yoghurt when compared to the control sample. The assumption that ME enhances LAB growth, and thus produces more acid, which results in shorter fermentation, was not supported by the results of microbiological analyses. Zhang et al. [7] concluded that the addition of ME enhanced LAB growth and, thus, the fermentation time was reduced in supplemented yoghurts. In their research, *S. thermophilus*, *L. acidophilus* and *B. longum* were used as starter cultures and the addition of ME was 0.05, 0.1 and 0.2%. A possible reason for the results obtained in the present study could be an excessive amount of added ME, and it is possible that ME blocked LAB growth but still had an influence on fermentation time reduction.

In general, the lactobacilli count at the end of cold storage decreased when compared to the end of fermentation, and values were between 3.98 (M 1%) and 7.55 (Control) log CFU mL^{-1}. The highest drop was observed in yoghurt supplemented with 3% ME, and it amounted 6.11 log CFU mL^{-1}, while the lowest level was measured in the control sample (2.93 log CFU mL^{-1}). Such results indicate that the addition of ME to milk before the fermentation negatively affected lactobacilli survival since their count decreased more intensively than in the control sample. Regarding the streptococci count, an increase (by 0.19 and 0.13 log CFU mL^{-1}) could be determined in two yoghurt samples (M 1% and M 4%, respectively), but in two samples (control and M 3%) the streptococci count decreased (0.77 and 2.22 log CFU mL^{-1}, respectively). It is clear that the survival of lactobacilli during the 28 days of cold storage was reduced by 28% in the control sample and by 59% in the yoghurt supplemented with 3% of ME. In general, streptococci showed better survival than lactobacilli. In our previous work [24], the same trend was observed when milk was

supplemented with OLE before the fermentation. Additionally, one of the conclusions was that OLE supplementation did not affect the viability of *Lactobacillus* sp. and *Streptococcus* sp. The results indicate that both ME and OLE, regardless of their antimicrobial effect, did not inhibit the growth of LAB. Although statistical analysis showed no significant influence of extract addition on the lactobacilli and streptococci counts, numerical values for lactobacilli, especially, differed between the control sample and the 1% ME addition sample (Table 4).

Table 3. *Lactobacillus* sp. and *Streptococcus* sp. counts in control sample (Control) and samples with moringa extract addition (1, 3 and 4% of extract addition, M 1%, M 3%, M 4%) at the beginning and end of fermentation and at the last day of cold storage (28th day) expressed as log CFU mL^{-1}.

	***Lactobacillus* sp., log (CFU mL^{-1})**		
Sample	**Beginning**	**End**	**28th day of cold storage**
Control	8.77 ± 0.30	10.48 ± 0.08	7.55 ± 0.06
M 1%	8.87 ± 0.16	8.70 ± 0.07	3.98 ± 0.01
M 3%	8.99 ± 0.01	10.43 ± 0.35	4.32 ± 0.08
M 4%	8.98 ± 0.01	9.23 ± 0.07	5.79 ± 0.29
	***Streptococcus* sp., log (CFU mL^{-1})**		
Sample	**Beginning**	**End**	**28th day of cold storage**
Control	6.97 ± 0.03	9.89 ± 0.06	9.12 ± 0.15
M 1%	6.92 ± 0.04	9.15 ± 0.08	9.34 ± 0.16
M 3%	6.93 ± 0.03	11.60 ± 0.08	8.84 ± 0.05
M 4%	6.92 ± 0.05	9.17 ± 0.01	9.30 ± 0.06

Results are expressed as mean ± SD.

Table 4. Influence of extract content (0, 1, 3 and 4%) and storage time (fermentation start and end and 28th day) on the *Lactobacillus* sp. and *Streptococcus* sp. (log CFU mL^{-1}) counts.

Source of Variation	***Lactobacillus* sp. (log CFU mL^{-1})**	***Streptococcus* sp. (log CFU mL^{-1})**
Extract content (%)	$p = 0.464$	$p = 0.950$
0	8.9 ± 0.5 [a]	8.7 ± 0.6 [a]
1	7.2 ± 1.0 [a]	8.5 ± 0.5 [a]
3	7.9 ± 1.2 [a]	9.1 ± 0.9 [a]
4	8.0 ± 0.7 [a]	8.5 ± 0.5 [a]
Storage time	$p < 0.001$ *	$p < 0.001$ *
Fermentation start (0 day)	8.9 ± 0.1 [b]	6.9 ± 0.0 [a]
Fermentation end (0 day)	9.7 ± 0.3 [b]	10.0 ± 0.4 [b]
28th day	5.4 ± 0.5 [a]	9.1 ± 0.1 [b]
Grand mean	8.0 ± 0.4	8.7 ± 0.3

* $p \leq 0.05$. Results are expressed as mean ± SE. Values with different letters within column are statistically different at $p \leq 0.05$.

Considering the storage period, the obtained results showed a significant impact ($p < 0.001$) on both lactobacilli and streptococci count. By the end of fermentation, the streptococci count had increased significantly, while there was no significant increase in the lactobacilli count. On the other hand, the lactobacilli count further decreased significantly by 4.3 log CFU mL^{-1} (44%) by the end of cold storage, while there was no significant decrease in the streptococci count between the end of fermentation and the 28th day of cold storage, indicating that *Streptococcus* sp. had better survival during the storage period.

3.3. Syneresis and WHC

Syneresis implies the occurrence of whey on the surface of the product and it is common in fermented milk. Consumers find syneresis to be an undesirable property of fermented milk and, thus, the dairy industry is continuously seeking for natural hydrocol-

loids to prevent this phenomenon. Plant extracts have shown a good behaviour as natural hydrocolloids thanks to their dietary fibre composition [25]. WHC is the ability of the product to hold all or part of its own whey [16]. Syneresis and WHC were determined at the beginning (1st day) and at the end (28th day) of the cold storage, and the obtained results are presented in Table 5.

Table 5. Syneresis and WHC in control sample (Control) and samples with moringa extract addition (1, 3 and 4% of extract addition, M 1%, M 3%, M 4%) at the 1st and 28th day of cold storage.

	Syneresis (%)		WHC (%)	
Sample	**1 Day**	**28 Day**	**1 Day**	**28 Day**
Control	62.72 ± 0.49	50.48 ± 0.66	34.38 ± 0.57	46.55 ± 0.42
M 1%	58.36 ± 0.28	50.14 ± 0.35	38.50 ± 0.40	47.33 ± 0.30
M 3%	58.56 ± 0.39	49.94 ± 0.33	39.47 ± 0.49	48.34 ± 0.51
M 4%	56.95 ± 0.06	51.93 ± 0.19	42.24 ± 0.35	46.68 ± 0.30

Results are expressed as mean ± SD.

Accordingly, the syneresis was the highest in the control sample on both examined days of cold storage, and it was 62.72 and 50.48%, respectively. Generally, the syneresis ratio decreased as the amount of ME increased. On the 1st day of cold storage, syneresis decreased by about 10% in ME-enriched yoghurts and the lowest syneresis was observed in a sample supplemented with 4% of ME. At the end of the storage period, syneresis was the lowest in the 3% ME-supplemented sample, while the highest value was found in the 4% ME sample. Since moringa contains dietary fibres and has the ability to retain water, lower syneresis values were expected in yoghurts with higher amounts of added ME in comparison to the control sample. WHC showed the opposite trend to syneresis, which was expected. WHC on the 1st day of cold storage increased by about 18% in the 4% ME sample in comparison with the control, and it can be concluded that ME had an influence on the retention of water in the product. The results of the statistical analysis of the influence of the extract content, as well as storage time, on syneresis and WHC are presented in Table 6. The results indicate that there were no significant differences in syneresis or WHC upon extract content. However, influence of storage time showed a significant impact on syneresis and WHC ($p < 0.001$), where syneresis significantly increased by the end of storage, while in the same period, WHC decreased significantly.

Table 6. Influence of extract content (0, 1, 3 or 4% ME) and storage time (1st and 28th day) on the syneresis and WHC of the produced yoghurts.

Source of Variation	**Syneresis (%)**	**WHC (%)**
Extract content (%)	$p = 0.897$	$p = 0.544$
0	56.6 ± 3.5 ^{a}	40.5 ± 3.5 ^{a}
1	54.3 ± 2.4 ^{a}	42.9 ± 2.6 ^{a}
3	54.3 ± 2.5 ^{a}	43.9 ± 2.6 ^{a}
4	54.4 ± 1.5 ^{a}	44.5 ± 1.3 ^{a}
Storage time (day)	$p < 0.001$ *	$p < 0.001$ *
1	59.1 ± 0.8 ^{b}	38.6 ± 1.1 ^{a}
28	50.6 ± 0.3 ^{a}	47.2 ± 0.3 ^{b}
Grand mean	54.9 ± 1.2	42.9 ± 1.2

WHC = water holding capacity. * $p \leq 0.05$. Results are expressed as mean ± SE. Values with different letters within column are statistically different at $p \leq 0.05$.

Zhang et al. [7] also calculated WHC only on the 1st day of storage and obtained similar results as in the present research. By increasing the ME addition (from 0.05 to 0.2%), WHC also increased. Furthermore, El-Gamal et al. [26] reported that by increasing the extract (moringa) concentration, WHC also increased during storage. Similar results

were obtained in our previous work [24] where the same trend of syneresis and WHC was observed. A possible explanation for the syneresis decrease and WHC increase on the 1st day of storage could be that dietary fibre and polyphenols together with casein form the protein network, which has a greater water binding capacity [27,28]. The same trend was not observed on the last day of storage, and it is possible that connections between casein and polyphenols became weak and, consequently, water came out from the protein network.

3.4. Rheology Parameters and the Total Colour Difference

Fluids such as yoghurts do not succumb to Newton's law of viscosity and therefore are called non-Newtonian fluids. The most common rheological model for non-Newtonian fluid is the power-law or Ostwald–de Waele model, which provides an adequate representation of several non-Newtonian fluids over the entire range of shear rates [29]. In the control and supplemented ME samples' flow index (n), consistency coefficients (K) with the associated linear regression coefficients (R2) were calculated (Table 7) in order to determine the non-Newton behaviour of yoghurts (non-Newton shear-thinning fluids $n < 1$ and for Newtonian fluids $n = 1$). According to the obtained results for the flow index, all yoghurts belong to pseudoplastic non-Newton fluids since the values for flow indexes were below 1 (Table 7). Statistical analysis showed no significant differences in the flow index values between samples with respect to the extract content (Table 2). After 28 days of cold storage, flow indexes were the highest, ranging between 0.75 in ME 1% and ME 4% and 0.89 in the control sample. The consistency coefficients showed the same trend as the flow indexes. There were no noticeable differences between samples during the cold storage period regardless of the supplementation with ME. However, K values increased till the end of storage and were highest on the 28th day, ranging from 5.39 in the 1% ME sample to 6.93 mPas in the control sample. Regression coefficients (R^2) revealed the method's accuracy, and they corresponded relatively well to the Ostwald–de Waele model, as all values ranged above 0.900 (Table 7).

Table 7. Parameters of rheological behaviour (flow index (n); consistency coefficient K (mPas); coefficient of regression (R^2) according to Ostwald–de Waele model in the control sample (Control) and samples with moringa extract addition (1, 3 and 4% of extract addition, M 1%, M 3%, M 4%) during 28 days of cold storage.

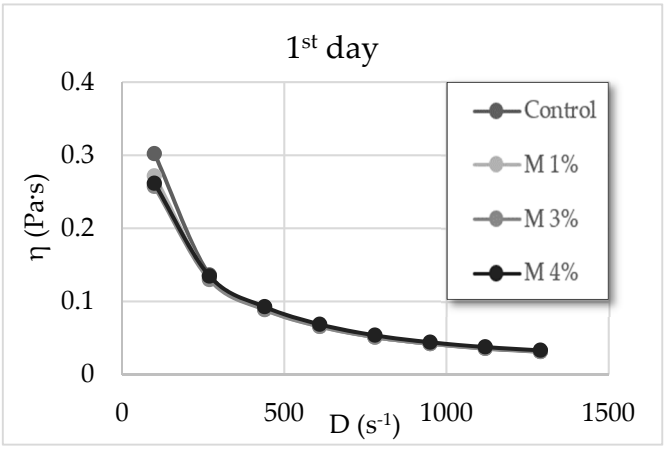
Days of Storage	1	7	14	21	28
Sample			**Flow index (n)**		
Control	0.56 ± 0.06	0.68 ± 0.14	0.70 ± 0.07	0.79 ± 0.05	0.89 ± 0.01
M 1%	0.58 ± 0.14	0.65 ± 0.06	0.68 ± 0.06	0.65 ± 0.06	0.75 ± 0.02
M 3%	0.62 ± 0.01	0.63 ± 0.14	0.65 ± 0.01	0.79 ± 0.01	0.81 ± 0.01
M 4%	0.64 ± 0.14	0.68 ± 0.01	0.68 ± 0.00	0.71 ± 0.01	0.75 ± 0.05
Sample			**Consistency coefficient (K)/mPas**		
Control	4.14 ± 0.01	5.89 ± 0.07	5.6 ± 0.07	6.27 ± 0.01	6.93 ± 0.05
M 1%	4.15 ± 0.02	5.89 ± 0.01	5.44 ± 0.04	5.08 ± 0.07	5.39 ± 0.01
M 3%	4.62 ± 0.07	5.97 ± 0.01	5.66 ± 0.03	6.26 ± 0.07	6.22 ± 0.01
M 4%	4.65 ± 0.04	5.95 ± 0.02	6.08 ± 0.07	6.18 ± 0.02	6.20 ± 0.07
Sample			**Coefficient of regression (R^2)**		
Control	0.9965 ± 0.0007	0.9675 ± 0.0007	0.9965 ± 0.0007	0.9955 ± 0.0014	0.9875 ± 0.0014
M 1%	0.9970 ± 0.0000	0.9907 ± 0.014	0.9945 ± 0.0007	0.9845 ± 0.0055	0.9955 ± 0.0007
M 3%	0.9985 ± 0.0007	0.9935 ± 0.0007	0.9935 ± 0.0007	0.9675 ± 0.0021	0.9905 ± 0.0014
M 4%	0.999 ± 0.0000	0.9875 ± 0.0007	0.9905 ± 0.0007	0.9990 ± 0.0000	0.9925 ± 0.0007

Results are expressed as mean ± SD.

Figure 2 shows the changes in viscosity (μ, Pa s) of the yoghurt samples without and with the ME addition (M 1%, M 3% and M 4%) in relation to the shear rate (D, s^{-1}) after the 1st, 14th, and 28th day. The viscosity of the samples was measured at the different shear rates ranging between 100 and 1290 m s^{-1}, and it can be observed that by increasing

the shear rate, the viscosity decreased on all analysed days of storage. On the 1st day of analysis, the control sample had the highest viscosity (0.302 Pa s), while the lowest value was measured in the 3% ME-supplemented sample (0.257 Pa s). Comparing the values of viscosity on the 14th day and the 1st day of storage, the control sample had the lowest viscosity (0.225 Pa s), while the highest viscosity was obtained for the 3% ME-supplemented sample (0.313 Pa s). At the end of the storage period (28th day), the yoghurt with 4% ME had the highest viscosity (0.351 Pa s), while the lowest viscosity was observed in the 3% ME-supplemented yoghurt (0.310 Pa s). The obtained viscosity values are in accordance with the calculated flow indexes and consistency coefficients.

a



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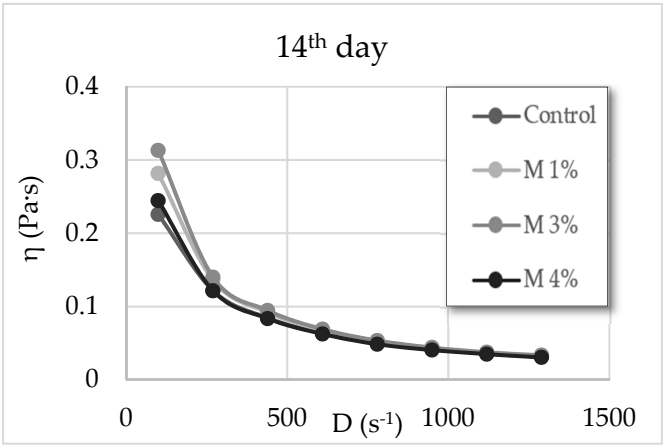

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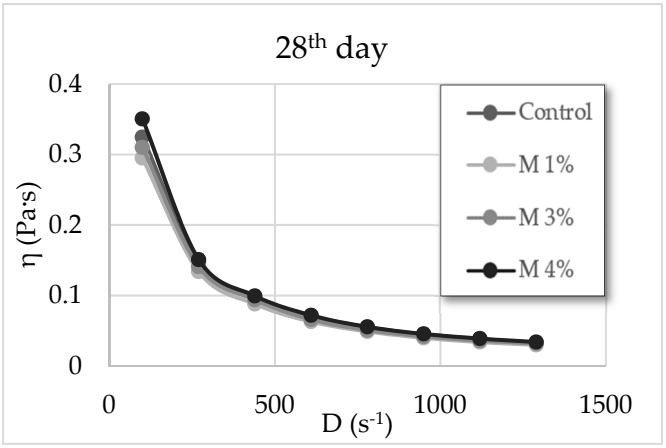


Figure 2. Changes in viscosity (μ, Pa s) of yoghurt samples without (Control) and with the addition of ME (M 1%, M 3% and M 4%) in relation to the shear rate (D, s^{-1}) after (**a**) 1st, (**b**) 14th and (**c**) 28th day of cold storage.

Zhang et al. [7] reported that in the ME-supplemented yoghurt, viscosity was higher when compared to the control yoghurt, and the addition of 0.2% ME increased the viscosity approximately five-fold during the storage in comparison with the control yoghurt. In the present work, the viscosity of the yoghurts with 4% ME increased from 0.262 to 0.351 Pa s or approximately 25% during the storage. Meanwhile, the viscosity of the control sample slightly increased from 0.302 to 0.324 Pas. A possible explanation for such a slight viscosity increase could be the dilution of the milk by the addition of moringa water extract prior to the fermentation.

The moringa water extract used for supplementation had a dark green colour and the assumption was that it could have an impact on the final colour of the yoghurt. In order to determine the difference between the colour of the control sample and the ME supplemented samples, the total colour difference was calculated (ΔE^*) [17] (Figure 3).

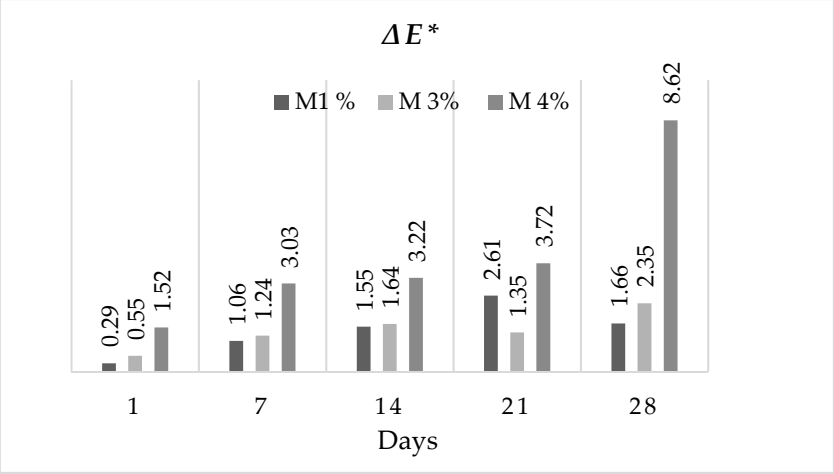


Figure 3. The total colour difference (ΔE^*) of yoghurt samples without (Control) and with the addition of moringa extract (M 1%, M 3% and M 4%) after 1st, 7th, 14th, 21st and 28th day of cold storage.

The total colour difference (ΔE^*) is a psychophysical difference noticeable by the observer, determined by the observation of two samples. When ΔE^* values are >3.00, the difference from the control sample is visible to the human eye [17]. On the 1st day of cold storage, there were no noticeable differences between the samples when compared to the control sample. Until the end of the cold storage period, samples with 1% and 3% ME did not differ from the control sample. However, the yoghurt with 4% ME was noticeably different from the 7th day till the end of the storage when the ΔE^* was the highest (8.62). Zhang et al. [7] measured L^*, a^* and b^* on the 1st day of storage and their data showed decreased redness and increased yellowness in ME-supplemented yoghurt without any changes in lightness. Thereby, the amounts of added ME ranged from 0.05% to 0.2% and did not induce colour changes in the yoghurts. Furthermore, Bikheet et al. [30] reported that the addition of moringa water and ethanol extract (1%, 4% and 5%) to yoghurt significantly lowered the lightness (L^*), while a^* shifted to a positive range with higher values of the b^* parameter.

3.5. Mineral Content

Fermented dairy products are the most popular milk products worldwide due to their nutritional and functional properties. While having excellent nutritional properties, there is still a lack of some mineral components, such as iron, copper and zinc, which play important roles in the human immune system. Moringa contains significant amounts of minerals such as calcium, magnesium, iron, copper and zinc in quantities of 2, 0.37, 0.03, 0.001 and 0.003 g per 100 g of moringa leaf powder, respectively. The powder is often used in alternative medicine for the treatment of iron deficiency, osteoporosis, immune system

improver, cardiovascular diseases, etc. [31–33]. Therefore, moringa powder is often called a superfood.

From the results shown in Table 8 it is evident that supplementation with ME significantly increased the amounts of mineral elements in the moringa-enriched yoghurts. By increasing the ME content, all examined minerals were found in increased amounts. In the control sample, magnesium amounted 84 mg kg^{-1}, and the addition of 4% ME increased its amount by 50% (168 mg kg^{-1}). Milk and dairy products are excellent sources of calcium, but with the addition of ME, calcium also significantly increased by about 50% (from 891 mg kg^{-1} in the control sample to 1768 mg kg^{-1} in the yoghurt with 4% ME). Moreover, yoghurt generally lacks from iron, and thus fortification can improve its functional characteristics. When compared to the control sample, the amount of iron amount in the supplemented sample (4% M) increased by about 60%, and thus fortification of milk with ME prior to the fermentation could be a good iron fortification method. Copper and zinc levels also increased in the sample with the 4% ME addition by about 50% in comparison with the control.

Table 8. Mineral elements in control sample (Control) and samples with moringa extract addition (1%, 3% and 4% of extract addition, M 1%, M 3%, M 4%) at the 1st day of analysis.

	Mineral Elements (mg kg^{-1})				
Sample	**Mg**	**Ca**	**Fe**	**Cu**	**Zn**
Control	83.0 ± 1.4^{ab}	888.5 ± 3.5^{a}	0.172 ± 0.002^{a}	0.038 ± 0.000^{a}	0.003 ± 0.000^{a}
M 1%	101.0 ± 1.4^{b}	1092.0 ± 7.1^{a}	0.245 ± 0.007^{b}	0.051 ± 0.000^{b}	0.004 ± 0.000^{a}
M 3%	102.0 ± 4.2^{b}	1047.0 ± 97.6^{a}	0.290 ± 0.001^{c}	0.047 ± 0.000^{b}	0.004 ± 0.000^{a}
M 4%	167.0 ± 1.4^{c}	1753.5 ± 20.5^{b}	0.420 ± 0.014^{d}	0.071 ± 0.004^{c}	0.007 ± 0.000^{b}

* $p \leq 0.05$. Results are expressed as mean ± SD. Values with different letters within column are statistically different at $p \leq 0.05$.

3.6. TPC and AC

The TPC and AC of the control sample and ME-supplemented samples during the cold storage period are shown in Table 9. Moringa, in its composition, contains bioactive components such as polyphenols and also has AC [30]. Generally, by increasing the ME amount from 1 to 4%, an increase in the TPC was noticed over the cold storage period. On the 1st day of analysis, an increase of about 22% between the control and 4% ME addition was observed. On the 7th day of cold storage, the TPC in the ME-supplemented yoghurts was similar, but in the rest of the cold storage period, the TPC values further increased (Table 9). Expectedly, the extract content had a significant impact ($p < 0.001$) on the TPC, where an increase in the TPC in yoghurts was in line with the increase in ME addition (Table 2). However, no significant influence of the storage period on the TPC was found. Zhang at al [7] also obtained similar results since supplementation with ME significantly increased the TPC in yoghurts. Moringa leaves are well known as a rich source of polyphenols (such as quercetin and kaempferol). Thus, the obtained results were expected and moringa could be used for the fortification of fermented dairy products.

The TPC enhances the AC when added into foods, so it is expected that the AC will be also increased in ME-supplemented yoghurts. During the storage time, proteolysis can lead to the bioactive peptides' formation or to enhanced activity of the starter culture (streptococci), which can increase the AC and, consequently, the TPC [34]. As can be seen in Table 9, on the 1st day of storage, the AC increased from 495.79 in the control to 664.96 μmol TE L^{-1} in the M 4% sample, which was an increase of about 25%. According to statistical analysis, ME addition had a significant influence on the AC of the yoghurts ($p < 0.001$) where samples with 3 and 4% of ME showed the highest AC values (Table 2). Up to the end of storage, the AC slightly increased in all samples except in M 1% (Table 9), but these differences were not significant (Table 2). In our previous study with OLE addition in yoghurt production, it was observed that the AC decreased in all supplemented samples

during the 35-day cold storage period. The reason for the observed degradation could be the lactic acid activity and the degradation of phenolic components [24].

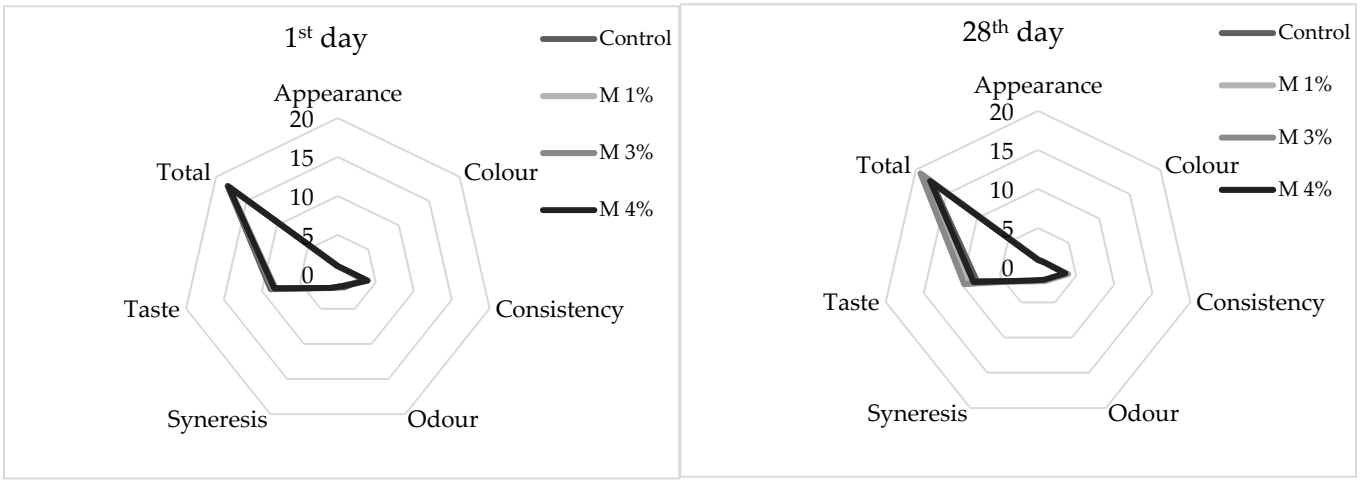
Table 9. TPC and AC (FRAP) in control sample (Control) and samples with moringa extract addition (1, 3 and 4% of extract addition, M 1%, M 3%, M 4%) at the cold storage period.

Days of Storage	1	7	14	21	28
Sample			**TPC (mg GAE L^{-1})**		
Control	73.85 ± 0.73	76.09 ± 0.05	76.36 ± 0.78	86.89 ± 0.04	73.67 ± 0.14
M 1%	82.22 ± 0.71	87.78 ± 0.49	78.64 ± 0.49	91.31 ± 0.64	92.50 ± 0.52
M 3%	89.56 ± 0.59	87.78 ± 0.49	102.74 ± 0.57	98.05 ± 0.67	67.79 ± 0.71
M 4%	94.45 ± 0.63	87.27 ± 0.57	106.09 ± 0.93	111.93 ± 1.02	102.88 ± 0.63
Sample			**FRAP (µmol TE L^{-1})**		
Control	495.79 ± 0.61	532.41 ± 7.20	499.10 ± 0.24	464.20 ± 2.40	524.92 ± 1.39
M 1%	545.64 ± 1.41	543.26 ± 7.14	594.00 ± 3.16	516.52 ± 1.63	449.79 ± 7.28
M 3%	644.87 ± 3.34	656.77 ± 3.01	584.67 ± 7.04	648.49 ± 1.40	646.75 ± 7.13
M 4%	664.96 ± 1.45	641.45 ± 6.58	604.20 ± 1.40	604.31 ± 1.49	650.93 ± 1.82

TPC = total phenols content. AC = antioxidant capacity. TE = Trolox equivalent. Results are expressed as mean ± SD.

3.7. Sensory Analyses

The results of the sensory evaluation of yoghurt samples, including the appearance, colour, consistency, odour, syneresis, taste and total sensory score on the 1st and 28th day of storage are presented in Figure 4. On the 1st day of analysis, panellists scored all examined samples and all tested properties with very similar scores, and there was no great difference between the samples. The most important property, taste, was graded between 8.4 and 8.8 (out of max 10) in the M 4% and control samples, respectively (Figure 4a). Panellists characterized the odour and taste of the ME-supplemented yoghurts as the odour and taste of green tea or herbal flavour. The total sensory score on the 1st day of cold storage was high for all yoghurts and it ranged between 17.8 and 18.1 (out of max 20) for the M 1% and control samples, respectively.



(**a**) (**b**)

Figure 4. Total sensory scores of yoghurt samples without (Control) and with the addition of moringa extract (M 1%, M 3% and M 4%) at the 1st (**a**) and 28th (**b**) day of cold storage period.

Sensory analysis was performed every 7th day of the cold storage period, but the results are shown only for the 1st and last day since there were no remarkable differences in scores between the other days. Figure 4b shows the sensory scores for the yoghurts on the 28th day of cold storage. As can be seen, the results differ from the 1st day of storage. Yoghurt with 3% ME had the highest scores for taste and the total score (9.7 and

19.2, respectively), and the control sample had the lowest scores for taste and the total score (8.0 and 17.4, respectively). Diacetyl and acetaldehyde give the typical flavour to fermented dairy products and their amount during the cold storage period decreased and a lack of the typical yoghurt flavour was present [35]. El-Gammal et al. [26] reported that the supplementation of yoghurt with ME had an influence on acetaldehyde retention during the cold storage period and yoghurts with ME had more acetaldehyde when compared to the control sample without ME addition. Similarly, Saad and Elkhtab [36] in their research documented that yoghurts with moringa addition achieved better scores on the 7th day of cold storage in comparison with the control sample. Furthermore, the consistency of the yoghurts in the present study was better scored in the ME samples, where panellists described ME-added samples as thicker in comparison with the control. They also noticed syneresis in a lesser amount only in the control sample. Sensory scores are in accordance with the rheology analyses (Table 8).

4. Conclusions

The addition of ME into milk prior to fermentation could be effective for the improvement in some functional and nutritional as well as health benefits of yoghurt. ME addition resulted in the reduction in the fermentation time by 1 h. Moreover, ME addition decreased syneresis, while the water holding capacity increased compared to the control sample. On the first day of cold storage, the control sample had the highest viscosity, but at the end of the cold storage period, the 4% ME sample had the highest viscosity. The contents of all determined minerals (Mg, Ca, Fe, Cu, Zn) were highest in the 4% ME, and showed significant differences compared to the control sample, and their amounts increased by more than 50%. In addition, it was observed that the ME addition increased the concentration of total phenolic compounds as well as antioxidant activity and, thus, supplementation by *Moringa oleifera* leaves could be considered a potential source of antioxidant supplements. The total sensory score after the cold storage period was higher for the ME-supplemented yoghurts compared to the control sample. According to all examined physicochemical characteristics and antioxidant capacity as well as the highest observed mineral content, yoghurt with the addition of 4% ME showed the greatest potential for further optimisation and scaling up of the production process. Generally, moringa extract can be regarded as an efficient ingredient (by mineral elements and polyphenol components) in functional food fortification and for obtaining products with added value.

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Conflicts of Interest: The authors declare no conflict of interest.

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