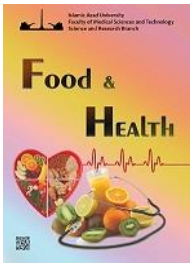

Food & Health Journal
Journal homepage: fh.srbiau.ac.ir

Physico-chemical properties and viability of *Lactobacillus rhamnosus* LGG in inulin-enriched Doineh (a traditional Iranian food) during 21-day storage at 4°C

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ARTICLE INFO

Original Article

Article history:
Received 11 August 2022
Revised 21 November 2022
Accepted 26 November 2022
Available online 20 December 2022

Keywords:
Doineh
Probiotic
Lactobacillus rhamnosus
Inulin
Synbiotics

ABSTRACT

The aim of this study was to investigate the physicochemical properties and survival of *Lactobacillus rhamnosus* LGG in synbiotic wet Doineh, a traditional Iranian fermented food enriched with different ratios of inulin. Milk and wheat bulgur were cooked in the conventional method and then mixed with LGG strain and different ratios of inulin (0, 2.5, 5, 7.5, and 10 percent). After 24 h of fermentation at 37ºC, the samples were stored at 4ºC for 21 days. pH, acidity, sensory evaluation, and probiotic culture were measured at 24 h after fermentation and on days 7, 14, and 21, using a pH meter, potentiometric, 5-point hedonic scale, and pour-plate methods, respectively. Protein, fat, solids content, and ash were determined by Kjeldahl, Soxhlet, dry weight at 100–105°C and 550ºC oven methods after 24 h of fermentation. The samples containing inulin showed significantly slower changes in pH and acidity. The viability and survival of LGG increased in the samples with higher amounts of inulin due to its prebiotic effect, and these changes were significant. The 2.5%, 5%, and 7.5% inulin models had better sensory characteristics than the others. Traditional foods and their preparation methods are suitable targets for developing health-oriented products, and functional foods with nutraceutical capabilities can be designed and produced based on them.

1. Introduction

A functional food is a food that has an additional function beyond its basic nutritional value, often related to health promotion or disease prevention. Functional foods can include natural ingredients rich in beneficial compounds, such as antioxidants, fiber, and omega-3 fatty acids. They can also include foods that have been fortified or modified with added ingredients, such as vitamins, minerals, probiotics, or prebiotics. Functional foods are intended to enhance health and well-being and may reduce the risk of chronic diseases or improve specific physiological functions (1). A functional food is a food that has an additional function beyond its basic nutritional value, often related to health promotion or disease prevention. Functional foods can include natural ingredients that are rich in beneficial compounds, such as antioxidants, fiber, and omega-3 fatty acids. They can also include foods that have been fortified or modified with added ingredients, such as vitamins, minerals, probiotics, or prebiotics. Functional foods are intended to enhance health and well-being and may reduce the risk of chronic diseases or improve specific physiological functions (2). Doineh is a traditional food of western Iran, which, according to the experiences of the natives of western Iran, is effective in treating and preventing respiratory infections, especially colds. This food is known as Tarhana, with a slight difference in Turkey's ingredients and preparation steps. Doineh is a traditional fermented food that is made from a mixture of concentrated Dough (Drink yogurt) or yogurt and wheat Bulgur. After fermentation for 1 to 10 days at an ambient temperature of about 25 to 30 degrees Celsius, it is prepared and consumed dry, semi-dry, and wet. Soup is prepared from it in the cold seasons of the year. Its main microbial content is lactic acid bacteria and yeast, and it is considered one of the oldest probiotic foods (3-5). One of the known probiotic strains that have attracted the attention of many researchers is *Lactobacillus rhamnosus* LGG, which is

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clinically known to improve and prevent diarrhea, childhood infections, allergies, and atopic eczema (6). It also improves glucose metabolism, inflammatory factors, insulin levels, and blood serum lipid profile (7). It increases leptin levels in the intestinal epithelium, which increases metabolism (8). However, the fermentation time of *L. rhamnosus* LGG in milk is long, and this can be due to the need of Lactobacillus for amino acids and essential nutrients required for initial growth. Although *L. rhamnosus* LGG can hydrolyze milk proteins to obtain the necessary amino acids, this ability is weak in the early stages, and the fermentation time is long (9). Meanwhile, much attention has been paid to cereals as a suitable substrate for the growth and survival of probiotics (10) because they are a good source of indigestible carbohydrates (wheat fiber, arabinoxylan-oligosaccharide) and are effective in the survival of probiotics. On the other hand, inulin is also a common prebiotic as an indigestible dietary fiber in the digestive system. It increases the growth of probiotics (11), whose prebiotic effects are observed in amounts (5-8 grams) per day (12). During fermentation, probiotics synthesize or release oligopeptides with a different composition and amino acid sequence from grain proteins called bioactive peptides. They have health-promoting and hormone-like effects. And they have anti-hypertensive, anti-diabetic, and antioxidant properties (13). Also, bioactive peptides from the fermentation of milk proteins have anti-free radical and anti-inflammatory activities, increase insulin secretion, reduce cholesterol and blood sugar, and potentially treat diabetes (14). The purpose of the present study is to investigate the physicochemical properties and survival of *L. rhamnosus* LGG in the synbiotic composition containing wheat Bulgur and milk according to the traditional method of preparation of Doineh prepared in the presence of different amounts of inulin during a 21-day storage period at 4°C in the refrigerator.

2. Materials and methods

2.1. Preparation of microbial inoculum

0.2 g of lyophilized powder of LGG probiotic strain (Chr. Hansen, Hørsholm, Denmark) was added to 50 ml of milk (autoclaved at 121º C for 20 minutes) and activated for 30 minutes at 42 ° C. It was then cultured on an MRS agar culture medium to obtain single colonies by surface culture method. A dilution equal to half of McFarland was prepared before inoculation.

2.2. Preparation of cooked Wheat Bulgur and milk

The wheat was cooked in hot water until it was easily chewable. The boiled wheat was drained and dried at room temperature, and Wheat Bulgur was made from the dried wheat with an electric mill. Raw bovine milk was obtained from a local market in Tehran and preheated to 50°C before inulin was added to it. The batch was divided into 5 portions in the next step. They were supplemented with inulin with high-DP (DP>23), which was supplied by Beneo (Beneo TM, ORAFTI, Oreye, Belgium) individually, at a ratio of 2, 5, 7, and 10 percent (w/v). The last portion was used as a control sample with no added inulin. All milk portions were heat-treated at 100°C for 30 minutes and cooled at room temperature.

2.3. Doineh preparation according to the traditional method

Doineh was prepared in 500 ml volumes inside polypropylene containers. 30% (w/v) (120 g) of prepared Wheat Bulgur was transferred to containers. In the next step, milk containing ratios of 0, 2, 5, 7, and 10 percent (w/v) of inulin at 40 º C was added to the containers with Wheat Bulgur, and they were brought to a volume of 500 ml. Then each portion was inoculated with 300µl of *L. rhamnosus* LGG (LGG) suspension (6.7 log CFU⁄ml). The prepared Doineh was incubated at 37 º C for 24 h following this step. All productions were made in duplicate, and after fermentation, they were kept in a refrigerator at 4ºC. In the end, five different samples (s1-s5) were obtained according to Table 1.

Table 1. General composition of the Doineh in a volume of 500 ml.

Samples	Inulin	300 µl of LGG suspension	Wheat Bulgur	Milk
S1	0 %	+	30 %	+
S2	2.5 %	+	30 %	+
S3	5 %	+	30 %	+
S4	7.5 %	+	30 %	+
S5	10 %	+	30 %	+

2.4. Measurement of pH and acidity

pH and acidity were measured 24 hours after fermentation on the 7th, 14th, and 21st days. The pH values of Doineh samples were measured with a digital pH meter model 827 (Metrohm, Switzerland). The acidity was measured using the potentiometric method, and the results were calculated using the following formula. It was expressed as mmol/100g (ISO 11869:2012).

$$I=V\times 10/m$$

V=volume of NaOH, m=Sample weight (g)

2.5. Counts of probiotic bacteria

Lactobacillus rhamnosus LGG was counted in three replicates 24 hours after fermentation, day 7, day 14 and day 21 of the samples stored at 4 º C. To prepare the initial dilution, 10 g of each sample was diluted with 90 ml of sterile peptone water. Then serial dilutions were prepared by adding 1 ml of the initial dilution to 9 ml of peptone water. Probiotics were cultured by the pour-plate technique using MRS agar (Merck, Germany) culture medium. Probiotic bacteria were counted after anaerobic incubation at 37ºC for 48 h. The cell concentration was given in log CFU/mL (11, 15).

2.6. Physicochemical analysis

The Physicochemical analysis factors of the samples were

measured 24 hours after fermentation. Protein content was measured according to the AOAC 978.04 standard using Kjeldahl (Kjeltec TM2300, Sweden). Factor 6.25 was used for the conversion of nitrogen to crude protein. Fat content was measured by the Soxhlet method using a Soxtec system (Soxtec 2050, Sweden) with Hexane solvent at a ratio (1: 10) at a temperature of 80°C and according to the AOAC 920.39 standard. The total solids content was determined by dry weight at 100–105 ° Cand in 10 grams of the sample. Total ash was measured according to the AOAC930.05 standard. It was done using an electric furnace (Stuart, England) at a temperature of 550 ° C.

2.7. Sensory evaluation

Sensory evaluation of wet Doineh samples was performed by 10 native and trained panelists from Qorve city (20-35 years old). The panelists evaluated the intensities of the various sensory characteristics of the samples. The samples were randomly coded using the same containers and random numbers and were provided to the panelists on days 1, 7, 14, and 21. The samples' taste, texture, flavor, and mouthfeel were evaluated on a five-point scale, with a score of 5 indicating excellent and 1 representing poor. Overall acceptability was also obtained from the average of the investigated indicators.

2.8. Statistical analysis

Statistical analyzes were performed by SPSS 26 software. Data analysis was performed using one-way ANOVA, and when significant differences were detected ($p<0.05$), Duncan's post hoc test was used to determine different groups.

3. Results and discussion

The samples' fat, protein, and ash content were compared 24 hours after fermentation (Table 2). These parameters did not differ significantly in the samples ($p>0.05$), except for the dry matter content, which was affected by the different amounts of inulin ($p<0.05$). The lowest protein content was 5.24% in sample S5, and the highest was 5.64% in sample S1. The changes in pH and acidity of the symbiotic samples fermented by *Lactobacillus rhamnosus* LGG bacteria are shown in Table 3. A decrease in pH and an increase in acidity ($p<0.05$) were observed for each sample after 24 hours of fermentation at 37 °C and during 21 days of storage at 4 °C. The lowest pH (3.87±0.045) and the highest acidity (16.00 mmol/100g) on day 21 were found in sample S1, while the highest pH (4.63±0.045) and the lowest acidity (6.20 mmol/100g) 24 hours after fermentation were found in sample S5. The effect of inulin on pH and acidity was not significant($p>0.05$) across the samples after 24 hours of fermentation and during storage. However, on day 7, the lowest pH (4.18) was found in sample S1 and the highest pH (4.41) was found in sample S5. The results indicated that the samples with inulin had higher pH values than the sample without inulin ($p<0.05$) 24 hours after fermentation, on day 7, day 14, and day 21. Moreover, the pH value increased with the amount of inulin in the samples. These changes were consistent with Ozturkoglu-Budak et al. (11) and De Souza et al. (16) results. The increase in the amount of inulin did not cause a proportional decrease in pH and acidity as the percentage of inulin increased. A similar study by Guven et al. (17) found that the yogurt with 1% inulin had the highest acidity among the samples with different amounts of 1, 2, and 3% inulin.

Table 2. The composition of the samples prepared contained different amounts of inulin after 24 hours of fermentation (mean ± SE; n = 2).

Samples	Component			
	Protein %	Ash %	Fat %	Solid matter %
S1	$5.64\pm0.060^{c*}$	1.74 ± 0.012^{b}	3.60 ± 0.035^{c}	31.61 ± 0.91^{a}
S2	5.54 ± 0.075^{bc}	1.72 ± 0.030^{ab}	3.61 ± 0.055^{c}	32.58 ± 0.15^{a}
S3	5.48 ± 0.090^{abc}	1.67 ± 0.022^{ab}	3.56 ± 0.050^{b}	33.86 ± 1.06^{ab}
S4	5.33 ± 0.065^{ab}	1.64 ± 0.025^{a}	3.49 ± 0.075^{a}	36.08 ± 1.05^{bc}
S5	5.24 ± 0.065^{a}	1.65 ± 0.012^{a}	3.53 ± 0.065^{ab}	38.28 ± 1.04^{c}

*Values with different letters in the same column are significantly different ($p< 0.05$).
S1: non-supplemented Doineh, S2: Doineh supplemented with 0.025 g of inulin, S3: Doineh supplemented with 0.05 g of inulin, S4: Doineh supplemented with 0.074 g of inulin S5: Doineh supplemented with 0.1 g of inulin.

Table 3. Post-acidification and acidity of Doineh fermented by *Lactobacillus rhamnosus*-LGG (LGG) (mean ± SE; n = 2).

Parameter	Samples	Day of storage			
		D1	***D7***	***D14***	***D 21***
pH	S1	4.47 ± 0.015^{aC}	4.18 ± 0.070^{aB}	4.04 ± 0.045^{aAB}	3.87 ± 0.045^{aA}
	S2	4.59 ± 0.010^{cdD}	4.32 ± 0.020^{bC}	4.14 ± 0.020^{aB}	3.91 ± 0.050^{aA}
	S3	4.56 ± 0.015^{bcD}	4.30 ± 0.010^{abC}	4.12 ± 0.025^{aB}	3.92 ± 0.015^{aA}
	S4	4.51 ± 0.015^{abD}	4.28 ± 0.025^{abC}	4.12 ± 0.020^{aB}	3.99 ± 0.020^{aA}
	S5	4.63 ± 0.015^{dD}	4.41 ± 0.015^{bC}	4.07 ± 0.020^{aB}	4.00 ± 0.020^{aA}
Acidity (mmol/100g)	S1	8.00 ± 0.200^{cA}	10.85 ± 0.450^{cB}	12.75 ± 0.050^{cC}	16.00 ± 0.300^{bD}
	S2	6.70 ± 0.100^{abA}	9.25 ± 0.150^{bB}	11.60 ± 0.200^{aC}	14.85 ± 0.850^{abD}
	S3	6.75 ± 0.250^{abA}	9.30 ± 0.100^{bB}	11.75 ± 0.250^{abC}	15.65 ± 0.150^{bD}
	S4	7.20 ± 0.100^{bA}	9.55 ± 0.250^{bB}	12.15 ± 0.150^{abcC}	13.60 ± 0.500^{aD}
	S5	6.20 ± 0.100^{aA}	8.15 ± 0.350^{aB}	12.30 ± 0.100^{bcC}	13.25 ± 0.250^{aD}

*Values with different letters in the same column are significantly different ($p< 0.05$).
S1: non-supplemented Doineh, S2: Doineh supplemented with 0.025 g of inulin, S3: Doineh supplemented with 0.05 g of inulin, S4: Doineh supplemented with 0.074 g of inulin S5: Doineh supplemented with 0.1 g of inulin.

Another study by Khorasany and Shahdadi (18) showed that yogurt with 1.5% inulin had a slightly lower pH than samples with 0.5 and 1% inulin. These results were comparable to ours. The buffering capacity is influenced by the production of CO_2 from the metabolism of proteins and carbohydrates, proteolysis, and the production of peptides and free amino acids (19). Foods with higher protein content have a higher buffering capacity (20). Adding protein to fermented milk enhances buffering properties and reduces changes in acidity and pH (21). In a protein mixture, pH measurement depends on the protein's spatial structure, which changes after a change in pH or denaturation. Hidden and hydrophobic proteins are exposed to enzymes (22). Wheat contains significant amounts of essential amino acids and has 8-15% protein (23). Probiotics can synthesize or release bioactive peptides from cereal proteins during their proteolytic activity (24). Wheat Bulgur that was cooked and treated under high-temperature conditions was used by us to prepare Doineh. It was shown by Zhang et al. (25) that enzymatic hydrolysis was increased by 10% by heat treatment of wheat gluten at 120 °C for 5 minutes due to changes in protein conformation (alpha-helix structure transformed to beta-sheet) and the creation of active sites in the gluten protein. Proteolysis was increased because of the enzyme access. Therefore, proteolysis can be enhanced by heat treatment in the presence of probiotics, and the number of probiotics and proteolysis by lactic acid bacteria (26) can be increased by adding inulin. These results explain the imbalance in the microbial population and the changes in pH and acidity in the Doineh composition. Milk that was affected by intense heat treatment was also used by us to prepare Doineh. The buffering capacity was increased, and the maximum pH peak was changed from 5.2-5 to 4.3-4.5 by heat treatment of milk at 95°C for 10 min, according to Lucey et al. (27). The pH values of the samples ranged from 4.3 to 4.5 24 hours after fermentation and on day 7, which can indicate the buffering effect of the heat treatment of the milk used in the preparation of Doineh. The results of counting *L. rhamnosus* LGG bacteria in fermented samples with different amounts of inulin and without inulin 24 hours after fermentation and on days 7, 14 and 21 storage periods at 4°C are shown in Table 4 and Fig. 1. Samples with different amounts of inulin and without inulin were compared at 24 hours after fermentation and on days 7, 14 and 21 storage period at 4°C, and the changes in the number of active bacteria were significant. The lowest growth rate was found in sample S1 (7.16±0.05 log CFU/ml), and the highest growth rate was found in S4 on the first day (9.61±0.01 log CFU/ml). By increasing the amount of inulin, the growth and viability of *Lactobacillus* were affected, and the changes were significant. However, these changes occurred not only because of the growth-stimulating effect of inulin on LGG but also because of the buffering effect created by the combination of milk and Bulgur due to the traditional preparation method of Doineh and the delay caused by inulin on the changes in pH and acidity of milk. An effect on the growth and survival of LGG was had by the addition of inulin when samples with different amounts of inulin and without inulin were compared, and these results were significant. Until the end of the storage period at 4 degrees, the number of LGG was always higher than the minimum acceptable level of probiotics in dairy products for creating health benefits (more than 10^7 CFU/mL). The growth and survival of probiotics were improved by heat treatment of milk and wheat Bulgur. The release of sulfur-containing amino acids and the increase of sulfhydryl groups were caused by high temperatures, which affected the reduction of oxidation and reduction potential, and increased the growth of probiotics (28). In addition, cereals are a good source of indigestible carbohydrates (wheat fiber, arabinoxylan-oligosaccharide) and are effective in the survival of probiotics (10, 29). Acidity and pH tolerance were increased by fermented substrates containing grains. The growth and survival rate of LA-5 and BB-12 in fermented puddings containing milk, corn, and rice flour for 21 days was observed (~9 log CFU/g) by Helland et al. (30). The effect of inulin along with wheat fiber on the growth and viability of probiotics was confirmed by HabibiNajafi et al. (31). These results were similar to ours, and the growth and survival of probiotics were affected by the use of wheat Bulgur, and significant growth was also observed in Doineh without inulin. The evaluators rated five samples' oral sensation, odor, texture, and overall acceptability (S1 to S5) on different days. The results showed that S3 had the highest scores for all attributes except for odor on day 21, while S1 and S5 had the lowest scores for most attributes (Table 5). The ratings of the samples varied over time, indicating changes in their sensory quality.

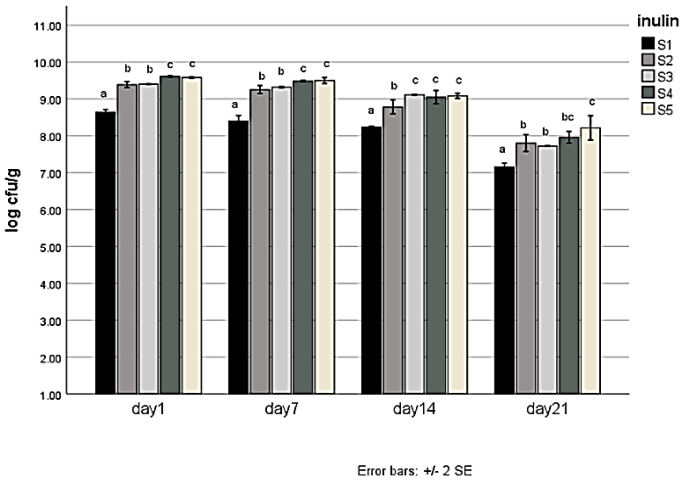

Fig. 1. The results of counting *Lactobacillus rhamnosus* LGG in fermented samples with different amounts of inulin and without inulin at 24 hours after 0, 7, 14, and 21 days of storage at 4°C.

4. Conclusion

The functionality of fermented foods can be enhanced by selecting probiotic strains with therapeutic effects and using traditional fermented foods as suitable substrate for their growth and survival. These foods can provide health benefits beyond meeting the nutritional needs of consumers. The traditional fermented food of Doineh, with the addition of inulin, was used as a culture medium for probiotic strains. The presence of inulin and the traditional preparation method of Doineh increased the buffering properties and tolerance of pH and acidity by probiotics and improved their viability

compared to the sample without inulin (S1). In all samples, even the sample without inulin, the number of probiotic bacteria on day 21 was higher than the recommended minimum amount for probiotic consumption. However, the fermentation time of 24 hours was too long for optimal probiotic activity. Reducing the fermentation time might increase the number of probiotics and extend their survival time.

Table 4. Viable cell counts (log CFU/g) of fermented Doineh by *Lactobacillus rhamnosus*-LGG (mean ± SE; n = 2).

Samples	Day of storage			
	D1	***D7***	***D14***	***D 21***
S1	8.64 ± 0.035^{aC}	8.40 ± 0.075^{aB}	8.23 ± 0.015^{aB}	7.16 ± 0.050^{aA}
S2	9.39 ± 0.040^{bC}	9.25 ± 0.055^{bC}	8.78 ± 0.095^{bB}	7.80 ± 0.115^{bA}
S3	9.40 ± 0.005^{bD}	9.32 ± 0.010^{bC}	9.11 ± 0.005^{cB}	7.72 ± 0.005^{bA}
S4	9.61 ± 0.010^{cC}	9.49 ± 0.010^{cC}	$9.05\pm.090^{cB}$	7.96 ± 0.080^{bcA}
S5	9.58 ± 0.010^{cC}	9.50 ± 0.040^{cC}	9.08 ± 0.035^{cB}	8.21 ± 0.165^{cA}

*Values with different letters in the same column are significantly different ($p< 0.05$).
S1: non-supplemented Doineh, S2: Doineh supplemented with 0.025 g of inulin, S3: Doineh supplemented with 0.05 g of inulin, S4: Doineh supplemented with 0.074 g of inulin S5: Doineh supplemented with 0.1 g of inulin.

Table 5. Mean±SEM sensory scores of various Doineh samples during storage up to 21 days, n =10.

Days	Samples	Sensory parameters			
		Mouthfeel	**Texture**	**Flavor**	**General acceptability**
Day 1	S1	2.5 ± 0.22^{a}	2.50 ± 0.16^{a}	3.10 ± 0.17^{a}	2.69 ± 0.10^{a}
	S2	3.8 ± 0.32^{b}	3.90 ± 0.23^{b}	3.80 ± 0.24^{b}	3.82 ± 0.16^{b}
	S3	4.2 ± 0.20^{b}	4.40 ± 0.22^{b}	4.20 ± 0.24^{b}	4.26 ± 0.11^{c}
	S4	4.0 ± 0.25^{b}	3.80 ± 0.24^{b}	3.80 ± 0.20^{b}	3.96 ± 0.15^{bc}
	S5	2.6 ± 0.26^{a}	2.50 ± 0.16^{a}	3.00 ± 0.21^{a}	2.69 ± 0.15^{a}
Da 7	S1	3.0 ± 0.21^{a}	2.90 ± 0.17^{a}	3.50 ± 0.22^{ab}	3.13 ± 0.11^{a}
	S2	4.2 ± 0.20^{b}	4.0 ± 0.21^{b}	4.00 ± 0.21^{bc}	4.06 ± 0.12^{bc}
	S3	4.3 ± 0.21^{b}	4.50 ± 0.16^{b}	4.40 ± 0.22^{c}	4.39 ± 0.11^{c}
	S4	3.8 ± 0.20^{b}	4.0 ± 0.25^{b}	3.80 ± 0.13^{abc}	3.86 ± 0.10^{b}
	S5	3.1 ± 0.17^{a}	3.10 ± 0.17^{a}	3.30 ± 0.21^{a}	3.16 ± 0.14^{a}
Day 14	S1	3.5 ± 0.16^{ab}	3.20 ± 0.13^{a}	3.70 ± 0.21^{ab}	3.46 ± 0.07^{ab}
	S2	3.8 ± 0.20^{b}	3.80 ± 0.20^{bc}	3.90 ± 0.23^{ab}	3.83 ± 0.07^{c}
	S3	4.40 ± 0.22^{c}	4.30 ± 0.21^{c}	4.30 ± 0.21^{c}	4.32 ± 0.14^{d}
	S4	3.70 ± 0.21^{b}	3.70 ± 0.21^{ab}	3.60 ± 0.16^{a}	3.66 ± 0.11^{bc}
	S5	3.0 ± 0.14^{a}	3.20 ± 0.13^{a}	3.40 ± 0.22^{a}	3.19 ± 0.12^{a}
Day 21	S1	3.1 ± 0.17^{ab}	3.10 ± 0.10^{ab}	2.90 ± 0.23^{ab}	3.03 ± 0.11^{b}
	S2	3.5 ± 0.16^{bc}	3.70 ± 0.21^{cd}	3.50 ± 0.16^{b}	3.56 ± 0.12^{cd}
	S3	4.0 ± 0.21^{c}	4.0 ± 0.22^{d}	3.50 ± 0.22^{b}	3.83 ± 0.14^{d}
	S4	3.4 ± 0.16^{b}	3.30 ± 0.21^{bc}	3.20 ± 0.20^{ab}	3.29 ± 0.13^{bc}
	S5	2.7 ± 0.15^{a}	2.60 ± 0.16^{a}	2.70 ± 0.15^{a}	2.66 ± 0.09^{a}

*Values with different letters in the same column are significantly different ($p< 0.05$).
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