Damanhour Journal of Veterinary Sciences 7 (1), (2021) 14-18

Damanhour Journal of Veterinary Sciences

Journal homepage: https://djvs.journals.ekb.eg/

Assessing the use of chitosan and nano-chitosan for prolonging the shelf life of fresh tilapia fish

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A B S T R A C T

This study aimed at investigation of the dipping effects of chitosan and nano chitosan (chitosan nanoparticles) (with particles sizes of 37, 78, and 222 nm) on extension of the shelf life of fresh tilapia fish (Oreochromis niloticus) at chilling storage. In addition, the effects of the chitosan and its nanoparticles on improvement of the microbiological quality of tilapia were further examined via evaluation of the hygiene indicators (total bacterial count, total psychrophilic count, and total mould and yeast counts). Furthermore, the effects of the chitosan and its nanoparticles against specific foodborne pathogens including Staphylococcus aureus, E. coli, Salmonella Typhimurium, Vibrio parahaemolyticus and Aspergillus flavus were screened. The obtained results indicated that chitosan nanoparticles were effective for elongation of the shelf life of tilapia, particularly chitosan nanoparticles with particle size of 37 nm. In addition, chitosan and its nanoparticles had significant antimicrobial effects either against the hygiene indicators, or foodborne-specific pathogens. In conclusion, the use of chitosan and its nanoparticles is highly recommended to extend the shelf life and improve the microbiological quality of tilapia fish.

Keywords: Tilapia; chitosan, chitosan nanoparticles; shelf life; microbiological quality.

1. Introduction

Fish is considered as an important source of essential amino acids, and high biological value protein and therefore is considered as a reasonable solution to overcome the shortage in the red meat with relatively lower cost. In addition, fish is a rich source of poly unsaturated fatty acids, vitamins, and minerals (Morshdy et al., 2019).

The common fish preservation methods include refrigeration with relatively short preservation time, and freezing which provides long preservation time, but it affects both the nutritive and sensory qualities of the fish. Chemical preservation methods are of value to increase the shelf life of the fish, but there is an increasing concern regarding the adverse effects of the chemical residues introduced to the fish. Therefore, there is an increasing demand to find friendly alternatives to extend the shelf life of the fish with no change on the sensory, nutritive, and safety of the end products (Imaida et al., 1983).

One of the newly emerging natural preservatives is chitosan. The latter fulfills the requirements of an ideal food preservative including antimicrobial, antifungal, nontoxic and biodegradable (Chen et al., 1998; Shahidi et al., 1999). While chitosan proved to be effective in its conventional solution, recent studies revealed that chitosan is more potent as antimicrobial and antifungal if it is applied as a disperses nanoparticles. Nano chitosan (chitosan nanoparticles) was proven to be denser and more effective antibacterial agent, as reported by Ramezani et al. (2015), when comparing the effectiveness of both chitosan and nano-chitosan coatings on silver carp fillets preserved under chilling.

In sight of the previous facts, this study was designed to investigate the effects of chitosan and its nanoparticles to extend the shelf life of tilapia at chilling condition (4°C). Besides, the antimicrobial effects of chitosan and its nanoparticles were evaluated either against the hygiene indicators (total bacterial count, total psychrophilic count, and total mould and yeast counts), or against specific foodborne pathogens (Staphylococcus aureus, E. coli, Salmonella Typhimurium, Vibrio parahaemolyticus and Aspergillus flavus) using tilapia as a food matrix.

2. Materials and Methods

1. Chitosan preparation

Different concentrations of chitosan (0.5 and 1.5%) were prepared in aqueous 1% acetic acid solution under mechanical stirring for 15 min (Qi et al., 2004).

2.2. Preparation of Chitosan nanoparticles

Chitosan nanoparticles were formulated (Fig. 1) according to Qi et al. (2004). In brief, chitosan nanoparticles were synthesized via the ionotropic gelation of chitosan and sodium tripolyphosphate (TPP) anions. Chitosan was dissolved in 1% acetic acid, nanoparticles were spontaneously obtained by the addition of 0.2%, 0.3% and 0.5% solutions of TPP aqueous basic solution to 0.2%, 0.3% and 0.5% of the chitosan acidic solution respectively under magnetic stirring at room temperature (the ratio of TTP to chitosan was 1:1).

2.3. Collection of samples:

A total of 180 tilapia fish samples (each fish weight is 200 ± 20 gram) were purchased from local fish markets in Zagazig City. Instantly all collected samples were transferred in ice box container, aseptically handled, and moved promptly to Microbiology Laboratory at Animal Health Research Institute, Zagazig branch for further examination.

2.4. Treatment groups

Fish samples used in each experiment throughout this study were divided into 6 groups (n = 5 fish/group). These groups were assigned as C group which was the control group which was immersed in distilled water for 30 min; T1 which was immersed in chitosan 0.5% for 30 min; T2 which was immersed in chitosan 1.5%; T3 which was immersed in nanochitosan of 222 nm for 30 min; T4 which was immersed in nanochitosan of 78 nm for 30 min; and T5 which was immersed in nanochitosan of 37 nm for 30 min, respectively. Samples were retrieved from the solution after 30 min and dried away on the bench at room temperature for 5 min. All fish samples were wrapped with polyethylene sheets, well-marked, and stored at 4ºC. Muscle samples from each fish were collected aseptically for organoleptic and microbiological analysis periodically, day after day.

2.5. Organoleptic examination

The color, odor, and consistency of each fish were evaluated according to Connell (1990).

2.6. Bacteriological examination

Samples were prepared according to APHA (2001). In brief, ten grams from each fish sample were taken under aseptic conditions and blended in 90 ml of 0.1% sterile buffered peptone water (Oxoid CM9) to provide a dilution of 10-1. Then decimal serial dilutions were prepared for each sample.

2.6.1. Enumeration of total bacterial count (TBC):

Total bacterial counts were enumerated using plate count agar (Oxoid, UK) according to APHA (2001) using the pour platting method followed by incubating the plates for 48 h at 35 ± 2°C.

2.6.2. Total psychrophilic count (TPsC)

The same protocol as in TBC was carried out with the exception of the incubation at 7°C for 10 days (Greer, 1982).

2.6.3. Total mould and yeast count (TMYC)

Total mould and yeast counts were enumerated using Sabouraud's dextrose agar supplemented with chloramphenicol and oxytetracycline (Oxoid, UK) according to APHA (2001) using the pour platting method followed by incubating the plates at 25°C for 7 days with the count every day.

2.6.4. Preparation of specific foodborne pathogens

Staphylococcus aureus, Vibrio parahaemolyticus, E. coli serotype O111, Salmonella Typhimurium, and Aspergillus flavus reference isolates were kindly obtained from the Microbiology Laboratory, Animal Health Research Institute, Cairo branch. Each pathogen was refreshed and cultured on its specific medium. Staphylococcus aureus was grown on Baird Parker supplemented with egg yolk-tellurite emulsion, Vibrio parahaemolyticus was grown on thiosulphate citrate bile salts sucrose (TCBS) media, E. coli serotype O111 was grown on eosin methylene blue (EMP), Salmonella Typhimurium was grown on xylose lysine deoxycholate (XLD) agar, Aspergillus niger was grown on Sabouraud's dextrose agar supplemented with chloramphenicol and oxytetracycline, respectively. Bacterial cells were purified and grown on brain heart infusion broth. Then the bacterial and fungal cells were pelleted by centrifugation for 15 minutes at 3000 rpm, washed twice in 10 ml of 0.01 phosphate buffered saline (PBS), pH 7.0 and diluted to 1.0 x 106 cfu/ ml in PBS for inoculation of the samples (Govaris et al., 2010).

2.6.5. Inoculation of tilapia samples by different pathogens

For each pathogen, a separate experiment was designed including six groups (n =5/group) as mentioned before. Where the control group was inoculated with the tested pathogen and immersed in distilled water for 30 min. Other groups were inoculated with the tested pathogen and treated with either chitosan or chitosan nanoparticles as mentioned before. Treatment groups were subjected to bacteriological examination following APHA (2001) protocols at zero time, and periodically each 48 hours during refrigeration to investigate the effect of both chitosan and chitosan nanoparticles at different concentrations.

2.7. Statistical analysis

All microbial counts were transferred into log 10 cfu/g. Results were recorded as mean ± standard errors (SE) also minimum (Min) and maximum (Max) were calculated. Statistical analysis of the obtained data was performed using analysis of variance (ANOVA) test and comparative of means were performed according to Duncan Multiple Range test according to Snedecor and Cochran (1969) using (SPSS14 2006). The P value less than 0.05 was used to indicate statistical significance.

4. Discussion

The obtained results in Table 1 revealed that treatment of tilapia with chitosan and chitosan nanoparticles could extend its shelf life till the 7th, 9th, 11th, 13th, and 15th days at chilling temperature after treatment with chitosan 0.5%, chitosan 1.5%, nanochitosan 222 nm, nanochitosan 78 nm, and nanochitosan 37 nm, respectively. These results go in agreement with Nowzari et al. (2013) who demonstrated a significant extension of the shelf life of rainbow trout fillets coated with chitosan gelatin and stored at chilling temperatures.

Total bacterial count is the most widely used microbiological test as hygiene indicator in the food industry. Egyptian Organization for Standardization (EOS, 2005) set a maximum permissible limit (MPL) for TBC at 6 log10 cfu /g. Accordingly, all examined fish samples had acceptable TBC as recorded in Table 2 at zero time. While fish showed higher TBC on the 5th day of the experiment in the control group. Chitosan and nanochitosan could reduce TBC in the treated groups, particularly nanochitosan 37 nm treated group could have acceptable TBC values till the 15th day at chilling temperature. These clear antimicrobial properties of chitosan and its nanoparticles agree with previous literatures (Tsai et al., 2002; Lopez-Caballero et al., 2005; Ojagh et al., 2010). Nanochitosan, particularly with smaller particle sizes had significant antibacterial effects compared with chitosan. This agrees with the results obtained by De-Azeredo (2013) who stated that nanochitosan suspension showed a clear retardation effect on total viable bacteria compared with chitosan.

Psychrophilic bacteria are the major group of microorganisms responsible for spoilage of aerobically stored fresh fish at chilling temperatures (Sallam, 2007). Total psychrophilic count at the zero time in all fish samples did not exceed 2.78 ± 0.37 log10 cfu/g, which indicates an acceptable fish quality (Table 3). This result was lower than the levels detected by Chamanara et al. (2013) in the fillet of Cynoglossus arel. Chitosan and nanochitosan treatment of fish significantly reduced TPsC compared with the control group and subsequently extended the shelf life of the fish. This agrees with Tsai et al. (2002) who reported that pretreatment of fish fillets (Oncorhynchus nerka) for 3 h with 1% chitosan solution reduced TPsC.

Mould and yeast include a large group of microorganisms which are ubiquitous in nature and widely contaminate the food supply. Presence of mould and yeast in fish could be due to improper sanitation during catching, handling, processing, salting, storage, transportation, and distribution rendering it unsuitable for human consumption (Youssef, 1998). The obtained results in Table 4 showed significant reductions in TMYC in chitosan and nanochitosan treated groups compared with the control, and subsequently extended the shelf life of the fish at chilling temperatures. This result agrees with Ahmed (2016) who reported the extension of the shelf life of sausage treated with chitosan and the extension of its shelf life to 28 days post treatment. The antifungal effects of chitosan and its nano particles were explained by Rogis et al. (2007) who stated that such activities of chitosan exists due to the enzyme activity of chitinase (β-1,3-glucanase) produced by the action of some moulds. The β-1,3-glucanase enzyme causes chitosan to break down into D-glucosamine compounds that decompose chitin on the hyphae and sporangium walls of the fungus leading to the inhibition of the mould growth.

Staphylococcus aureus (S. aureus) is major foodborne pathogen that might lead to foodborne intoxication via production of several types of enterotoxins (Darwish et al., 2018). Table 5 showed that chitosan and chitosan nanoparticles could significantly reduce S. aureus counts, particularly at nanochitosan 37 nm. Chitosan caused ultra-structural changes in the Staphylococci as the cell membrane became locally splitted from the cell wall leading to the presence of a "vacuole-like" structures beneath the wall. The detachments generate ions and water efflux, arouse decreases of the pressure inside the bacterial cell (Raafat et al., 2008; Silva et al., 2010).

Contamination of fish with E. coli originates mainly due to contamination of various water bodies with human and animal excreta. E. coli infection in human includes foodborne illnesses that may range from diarrheal disease to life threatening hemorrhagic colitis, hemolytic uremic syndrome due to shiga toxic E. coli (James et al., 2001). Table 6 showed significant reduction in E. coli O111 counts among chitosan and nanochitosan treated groups compared with the control. This result agrees with those obtained by Fernandes et al. (2008) who stated that at least 3 log cycles of E. coli initial population was reduced upon the use of chitosan 0.25% (w/v).

Salmonella is a main reason of a number of different disease syndromes including gastroenteritis, bacteremia, and typhoid fever, with the most common being gastroenteritis, which is characterized by abdominal pain, nausea, vomiting, diarrhea, and headache (Coburn et al., 2007). Table 7 showed clear and significant effects for chitosan and nanochitosan against Salmonella Typhimurium. Similarly, chitosan significantly reduced Salmonella Typhimurium counts in shrimp (Tayel et al., 2010).

The antibacterial effects of chitosan against E. coli and Salmonella Typhimurium were suggested to be related to disarranging of the lipopolysaccharide layer of the outer membrane of the Gram-negative bacteria leading to altering its function as a barrier against oxygen transfer (No et al., 2002; Silva et al., 2010).

Vibrio spp. are vastly spread out in the coastal waters of many regions of the world (Eyisi et al., 2013). Ingestion of foods contaminated with Vibrio parahaemolyticus might lead to gastrointestinal disturbance or septicemia that can cause fatal complications (Faruque and Nair, 2006). The recorded results in Table 8 showed clear antibacterial effects for chitosan and nanochitosan against Vibrio parahaemolyticus. These results agree with previous reports (Fang et al., 2015; Jeon et al., 2014). The anti-vibrio effects of chitosan and its nanoparticles might be attributed to the fact that chitosan possesses positively charged molecules that bind to negatively charged structures on bacterial cell surfaces that lead to the leakage of intracellular material from bacterial cells (Raafat et al., 2008). Besides, the metal-binding capacity of chitosan was also considered to block pathogens by disrupting the synthesis of proteins consisting of virulence factors, such as cytolysin, elastase, and metalloproteinase (Lee et al.,

2009). Chitosan was found to inhibit vibrio cell-to-cell communication through the suppression of intracellular reactive oxygen species generation, which is the reason to cause cell death (Lee et al., 2009).

Aspergillus flavus is a major aflatoxigenic fungi that might contaminate fish and fish products. The obtained results in Table 9 showed that chitosan and its nanoparticles could significantly reduce A. flavus counts compared with the control group. Similarly, Rinto (2017) stated that chitosan at 1.5% retarded the growth of A. flavus at 50%. He added that chitosan damage the mycelia which in turn causes inhibition of conidia formation. Besides, chitosan contains lysozymes such as chitinase and glucanase enzymes that can break the cell walls of moulds, resulting in impaired mold growth.

5. Conclusion

On the basis of the obtained results food industries would benefit from the use of nanotechnology and the use of nanochitosan as a cheap and efficient food preservative to prolong the shelf life of the tilapia fish and to maintain the microbiological quality of the fish during storage period.

Funding: Not applicable

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

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Table (1): Organoleptic acceptability of treated and untreated fresh tilapia fish

	Control samples	Treated tilapia fish samples				
		T1	T2	T3	T4	T5
*Acceptable till	5th day	7th day	9th day	11th day	13th day	15th day
**Unacceptable	After the 5th day	After 7th day	After 9th day	After 11th day	After 13th day	After 15th day

*Acceptable means fresh good odor, grey to white color, bulged shinny eye, and firm consistency.
**Unacceptable means rot odor, greenish color, sunken cloudy eyes, and soft consistency.

Table (2): Total bacterial count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

Treated groups / Durations	Control	T1	T2	T3	T4	T5
1st day	2.80 ± 0.12^{c}	2.77 ± 0.15^{c}	2.77 ± 0.33^{d}	2.80 ± 0.12	2.68 ± 0.32	2.47 ± 0.26^{c}
3rd day	4.49 ± 0.24^{b}	3.47 ± 0.26^{c}	3.17 ± 0.21^{d}	2.75 ± 0.14^{d}	2.53 ± 0.02^{e}	2.27 ± 0.03^{c}
5th day	6.87 ± 0.52^{a}	4.83 ± 0.17^{b}	4.41 ± 0.44^{c}	3.91 ± 0.45^{c}	3.31 ± 0.30^{d}	2.50 ± 0.27^{c}
7th day	R	6.23 ± 0.23^{a}	5.38 ± 0.35^{b}	4.44 ± 0.28^{bc}	4.31 ± 0.34^{c}	2.12 ± 0.07^{c}
9th day	R	R	6.30 ± 0.32^{a}	5.06 ± 0.47^{b}	4.05 ± 0.26^{c}	2.72 ± 0.26^{c}
11th day	R	R	R	6.29 ± 0.36^{a}	5.03 ± 0.55^{b}	3.79 ± 0.23^{b}
13th day	R	R	R	R	6.10 ± 0.21^{a}	4.86 ± 0.39^{a}
15th day	R	R	R	R	R	5.48 ± 0.32^{a}

Different superscripts between values within the same column indicate significant differences (P<0.05).

Table (3): Total psychrophilic count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

Treated groups / Durations	Control	T1	T2	T3	T4	T5
1st day	3.21 ± 0.18^{c}	3.00 ± 0.12^{c}	3.00 ± 0.12^{d}	3.00 ± 0.17^{c}	3.00 ± 0.58^{d}	3.03 ± 0.13^{c}
3rd day	4.77 ± 0.15^{b}	3.83 ± 0.38^{b}	3.04 ± 0.08^{d}	2.89 ± 0.09^{c}	2.56 ± 0.26^{d}	1.81 ± 0.16^{de}
5th day	5.95 ± 0.33^{a}	4.67 ± 0.49^{b}	4.18 ± 0.37^{c}	3.28 ± 0.35^{c}	3.13 ± 0.18^{d}	1.57 ± 0.27^{e}
7th day	R	5.58 ± 0.48^{a}	5.13 ± 0.10^{b}	4.45 ± 0.28^{b}	4.01 ± 0.05^{c}	2.33 ± 0.31^{d}
9th day	R	R	6.43 ± 0.23^{a}	5.10 ± 0.15^{b}	4.31 ± 0.32^{bc}	3.31 ± 0.34^{c}
11th day	R	R	R	6.17 ± 0.09^{a}	4.80 ± 0.40^{b}	4.13 ± 0.36^{b}
13th day	R	R	R	R	6.04 ± 0.0.8^{a}	4.50 ± 0.29^{b}
15th day	R	R	R	R	R	6.17 ± 0.09^{a}

Table (5): Total S. aureus count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

Treated groups / Durations	Control	T1	T2	T3	T4	T5
Zero day	6.10 ± 0.21^{b}	5.90 ± 0.20^{ab}	5.93 ± 0.07^{a}	5.88 ± 0.12^{a}	5.89 ± 0.14^{b}	5.80 ± 0.19^{a}
24 hours	6.33 ± 0.24^{ab}	6.57 ± 0.28^{a}	5.53 ± 0.18^{ab}	3.83 ± 0.20^{b}	3.64 ± 0.11^{a}	3.12 ± 0.06^{b}
48 hours	6.80 ± 0.17^{a}	5.57 ± 0.12^{b}	5.37 ± 0.12^{b}	3.08 ± 0.09^{c}	2.37 ± 0.26^{b}	1.45 ± 0.29^{c}

Different superscripts between values within the same column indicate significant differences (P<0.05).

Table (6): Total E. coli O111 count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

Treated groups / Durations	Control	T1	T2	T3	T4	T5
Zero day	5.67 ± 0.24^{b}	5.50 ± 0.25^{a}	5.47 ± 0.24^{a}	5.50 ± 0.24^{a}	5.73 ± 0.07^{a}	5.67 ± 0.03^{a}
24 hours	6.27 ± 0.14^{ab}	5.30 ± 0.12^{a}	5.30 ± 0.12^{a}	4.87 ± 0.13^{ab}	4.70 ± 0.21^{b}	4.27 ± 0.15^{b}
48 hours	6.77 ± 0.15^{a}	5.37 ± 0.19^{a}	5.07 ± 0.15^{a}	4.50 ± 0.45^{b}	4.70 ± 0.21^{b}	3.93 ± 0.24^{b}

Table (7): Total Salmonella Typhimurium count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

Treated groups / Durations	Control	T1	T2	T3	T4	T5
Zero day	6.23 ± 0.14^{b}	6.07 ± 0.12^{a}	5.63 ± 0.34^{a}	5.70 ± 0.30^{a}	5.60 ± 0.35^{a}	5.70 ± 0.30^{a}
24 hours	6.77 ± 0.20^{ab}	5.37 ± 0.26^{a}	4.77 ± 0.15^{b}	4.07 ± 0.37^{b}	3.37 ± 0.15^{b}	2.96 ± 0.32^{b}
48 hours	7.00 ± 0.15^{a}	5.50 ± 0.06^{a}	5.27 ± 0.07^{ab}	3.08 ± 0.22^{c}	2.91 ± 0.10^{b}	2.40 ± 0.21^{b}

Table (8): Total Vibrio parahaemolyticus count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

	Control	T1	T2	T3	T4	T5
Zero time	5.00 ± 0.01^{c}	4.99 ± 0.01^{b}	4.98 ± 0.01^{b}	4.99 ± 0.01^{a}	4.98 ± 0.01^{a}	4.99 ± 0.01^{a}
After 24 h	5.80 ± 0.11^{b}	5.00 ± 0.06^{b}	4.66 ± 0.24^{b}	4.03 ± 0.39^{b}	3.72 ± 0.14^{b}	2.90 ± 0.30^{b}
After 48 h	6.76 ± 0.14^{a}	5.63 ± 0.08^{a}	5.56 ± 0.20^{a}	3.16 ± 0.17^{c}	2.97 ± 0.13^{c}	2.18 ± 0.23^{c}

Table 9. Total A. flavus count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

Treated groups / Durations	Control	T1	T2	T3	T4	T5
Zero day	7.10 ± 0.10^{b}	7.10 ± 0.10^{b}	7.10 ± 0.10^{b}	7.10 ± 0.10^{a}	7.00 ± 0.01^{a}	7.00 ± 0.01^{a}
3rd day	7.95 ± 0.03^{a}	7.4 ± 0.23^{b}	6.89 ± 0.06^{b}	6.08 ± 0.11^{b}	5.03 ± 0.26^{b}	3.85 ± 0.34^{b}
6th day	R	8.09 ± 0.21^{a}	7.72 ± 0.21^{a}	5.00 ± 0.17^{c}	3.40 ± 0.46^{c}	ND
9th day	R	R	R	5.95 ± 0.05^{b}	ND	ND

Table (4): Total mould and yeast count (Mean ± SE log10 cfu/g) of treated and untreated groups of tilapia fish samples

Treated groups / Durations	Control		T1		T2		T3		T4		T5	
	Yeast	Mold	Yeast	Mold	Yeast	Mold	Yeast	Mold	Yeast	Mold	Yeast	Mold
1st day	2.95 ± 0.09^{b}	2.70 ± 0.7^{b}	2.91 ± 0.06^{b}	2.73 ± 0.07^{b}	2.83 ± 0.09^{b}	2.60 ± 0.02^{c}	2.93 ± 0.18^{bc}	2.70 ± 0.10^{c}	2.67 ± 0.17^{ab}	2.66 ± 0.02^{ab}	2.94 ± 0.2^{a}	2.63 ± 0.17^{a}
3rd day	3.37 ± 0.23^{ab}	3.21 ± 0.11^{b}	2.60 ± 0.10^{b}	2.77 ± 0.14^{b}	2.50 ± 0.20^{b}	2.56 ± 0.15^{c}	2.56 ± 0.26^{b}	2.38 ± 0.18^{c}	2.33 ± 0.10^{b}	2.43 ± 0.20^{b}	1.83 ± 0.01^{b}	1.98 ± 0.02^{b}
5th day	3.81 ± 0.12^{a}	3.94 ± 0.09^{a}	2.87 ± 0.12^{b}	2.87 ± 0.13^{b}	2.67 ±0.08^{b}	2.58 ± 0.02^{c}	2.83 ± 0.17^{bc}	2.49 ± 0.05^{c}	2.40 ± 0.04^{b}	2.30 ±0.30^{b}	1.91 ± 0.02^{b}	1.75 ±0.05^{b}
7th day	R		3.73 ± 0.27^{a}	4.10 ± 0.11^{a}	3.26 ± 0.03^{ab}	3.31 ± 0.07^{b}	2.99 ± 0.28^{bc}	2.82 ± 0.17^{bc}	2.50 ± 0.02^{b}	2.68 ± 0.36^{ab}	1.81 ± 0.11^{b}	1.93 ± 0.02^{b}
9th day	R		R		3.52 ± 0.26^{a}	3.98 ± 0.30^{a}	3.11 ± 0.06^{b}	3.40 ± 0.30^{b}	2.79 ± 0.32^{ab}	2.68 ± 0.39^{ab}	2.66 ± 0.17^{a}	2.62 ± 0.13^{a}
11th day	R		R		R		3.86 ± 0.20^{a}	4.03 ± 0.09^{a}	2.93 ± 0.15^{ab}	2.89 ± 0.12^{ab}	2.86 ± 0.09^{a}	2.79 ± 0.15^{a}
13th day	R		R		R		R		3.22 ± 0.09^{a}	3.0 ± 0.04^{a}	2.92 ± 0.01^{a}	2.90 ± 0.11^{a}
15th day	R		R		R		R		R		3.19 ± 0.18^{a}	3.27 ± 0.16^{a}

Different superscripts between values within the same column indicate significant differences (P<0.05).

R: Rejected