JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 8 (2021) 162-168

Biofilm Formation and Antibacterial Properties of *Lactobacillus* Isolated from Indigenous Dairy Products

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HIGHLIGHTS

- Probiotic biofilms of indigenous *Lactobacillus* strains were manufactured in culture medium.
- The probiotic biofilm was used to control of food pathogens.
- The probiotic biofilms showed excellent antibacterial activity.

Article type
Original article

Keywords
Biofilms
Lactobacillales
Lactobacillus
Probiotics
Anti-Infective Agents
Dairy Products

Article history
Received: 7 Dec 2020
Revised: 1 Aug 2021
Accepted: 23 Oct 2021

Acronyms and abbreviations
BHI=Brain Heart Infusion
CFS=Cell-Free Supernatant
CFU=Colony Forming Units
LAB=Lactic Acid Bacteria
MRS=De Man-Rogosa-Sharpe
NaCl=Sodium chloride
OD=Optical Density
SEM=Scanning Electron Microscope

ABSTRACT

Background: The health benefits of probiotic bacteria are not unknown to anyone. On the other hand, indigenous dairy sources are a potential source of native probiotics. This study aimed to describe the inhibitory activity of Cell-Free Supernatant (CFS), planktonic cells, and biofilm form of *Lactobacillus* strains isolated from native dairy sources on food pathogens.

Methods: Antibacterial activities of the CFS of *Lactobacillus* strains were assessed by the microplate method and via violet staining, and in planktonic cells, and biofilm forms were performed by the spread plate method.

Results: The results showed that despite the large differences in biofilm formation power among the strains, most of them can produce biofilm. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* subsp. *Lactis*, *Lactobacillus brevis*, and *Lactobacillus lactis* subsp. *lactis* formed the strongest biofilm, respectively. Planktonic states reduce the pathogens bacterial by about 1.43 log, but in biofilm forms, decreased *Listeria monocytogenes* by about 4.8 log compared to the control, and in the case of *Pseudomonas aeruginosa*, a growth reduction of about 2.8 logs was observed.

Conclusion: According to the study, biofilm produced by probiotic strains can be considered a new approach for biological control. Also, indigenous dairy sources can be considered by researchers to extract natural and beneficial probiotics.

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Introduction

Biofilm is composed of many surface-related microbial cells which are present in a matrix of extracellular polymeric substances (EPS) and contain materials such as proteins (1-2%) including enzymes, DNA (<1%), polysaccharides (1-2%), RNA (<1%), and water (nearly 97%) which builds the majority of the biofilm (Rezaei et al., 2021b). In this structure, there are intermediate spaces and water channels for the transport of oxygen and nutrients which help the cells existing in the biofilm grow (Koohestani et al., 2018). The polysaccharide structure acts as the main form of biofilm and provides a cohesive shelter for the bacteria living in the biofilm. It also plays

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To cite: Rezaei Z., Salari A., Khanzadi S. (2021). Biofilm formation and antibacterial properties of *Lactobacillus* isolated from indigenous dairy products. *Journal of Food Quality and Hazards Control*. 8: 162-168.

DOI: 10.18502/jfqhc.8.4.8257 Journal website: http://jfqhc.ssu.ac.ir

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[DOI: 10.18502/jfqhc.8.4.8257]

a significant role in the functioning of different biofilm communities. For instance, it prevents some antimicrobial drugs from entering the biofilm and limits the release of environmental compounds from entering into the biofilm (Rezaei et al., 2021a; Salas-Jara et al., 2016). To date, scientists have looked at bacterial biofilms as a serious dilemma, and biofilms have been a serious problem for researchers, industry, and health professionals around the world (Moori Bakhtiari and Javadmakoei, 2017). *Listeria monocytogenes* is a food-related pathogen that can cause serious infections in susceptible individuals. *L. monocytogenes* can form biofilms at the food processing tools, thereby transmitting contamination to food and threatening public health (Di Ciccio et al., 2012; Warke et al., 2017).

Pseudomonas aeruginosa is an opportunistic pathogen found in the soil and causes disease in humans, animals, and plants. This bacterium plays an important role in causing acute and chronic infections due to its capability to form biofilms (Kyere et al., 2020). On the other hand, dairy products are the best carriers of probiotics. If native probiotics can be identified successfully, they are beneficial for people in several ways because the isolated environment is compatible with the food environment of many industries, especially the dairy industry. Besides, food pathogens have caused the most problems in the dairy industry and are very common. Hence, the presence of probiotic bacteria of dairy origin turns the threat of biofilm to an opportunity (Furukawa, 2015; Guerrieri et al., 2009; Sadishkumar and Jeevaratnam, 2017)

The genetic resources in Iran's ecosystem are excellent sources for producing starters and probiotics. If new probiotics can be discovered from these sources, a unique property can be obtained which cannot be found in commercial strains. It is worth mentioning that most of the available commercial strains are genetically modified, which makes its usage a controversial issue. In this study, the strains were screened to select strains that could produce biofilms and have an antibacterial effect on pathogens in cases where biofilm problems of pathogens are considered a natural defense barrier and are used as a substitute for detergents and antibiotics (Khiralla et al., 2015; Ouali et al., 2014). The supernatant of the Lactic Acid Bacteria (LAB) also exhibits biofilm removal activity against food-borne pathogen (Aminnezhad and Kasra-Kermanshahi, 2014; Wang et al., 2013).

In this regard, the purpose of the present study was to evaluate antibacterial and biofilm removal activity of planktonic, biofilm, and cell-free supernatant (CFS) of *Lactobacillus* strain against *L. monocytogenes* and *P. aeruginosa*.

Materials and methods

Bacterial strains and growth conditions

Nineteen strains of LAB were isolated from indigenous dairy sources such as yogurt, milk, and cheese (Edalatian et al., 2012; Hajimohammadi Farimani et al., 2016) (Table 1*). Lactobacillus* strains were subcultured from a stock culture [De Man-Rogosa-Sharpe broth (MRS) (Merck, Germany) containing 20% glycerol (v/v)] on MRS agar medium (Merck, Germany) and incubated for 72 h at 37 ℃ under microaerophilic conditions by using an anaerobic jar and Gas Pack C (Merck, Germany) (Aoudia et al., 2016).

Biofilm assay

One ml of culture medium containing 1.5×10^8 Colony Forming Units (CFU)/ml from each strain was poured into each well and incubated at 30 °C for 48 h. After incubation, the culture medium was drained from the wells and washed twice with 0.5 ml of 150 mM sodium chloride (NaCl) solution. The microplate was then stained for 45 min with 1 ml of 0.05% (v/v) of crystalline violet solution and washed twice. One ml of 96% ethanol (v/v) was added to each well, and the optical density (OD) was determined at 430 and 595 nm (Aoudia et al., 2016). Adhesion rate was set to be B and can be calculated as followings: (Chen et al., 2017; Zhang et al., 2013)

$$B = \frac{OD_{430} - OD_C}{OD_{595} - OD_C}$$

OD_C refer to the optical density value in the Control.
No biofilm producer=B<0.1;
Weak biofilm producer=0.1≤B<0.5;
Moderate biofilm producer=0.1≤B<1;
Strong biofilm producer=B≥1.

Antibacterial activity

The antibacterial activity of probiotic bacteria on food pathogens was investigated in three models: biofilm, planktonic form, and CFS.

-Antibacterial effect of probiotic biofilm

The selected strain formed biofilm, which could produce strong biofilms biofilms *(Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*). After the incubation time, the culture was omitted from wells, and the microplates were washed twice with 500 ml of 150 mM NaCl solution. Then, 1 ml per well of fresh Brain Heart Infusion broth (BHI; Merck, Germany) was inoculated with 1.5×10^8 CFU/ml of each pathogenic

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bacteria (*L. monocytogenes* ATCC7644 as well as *P. aeruginosa* PTCC1074) was dispensed in a microplate with 24-well containing LAB biofilm and incubated for 48 h at 30 °C. After the incubation time, the medium was removed from each well, the microplates were washed twice with 500 ml of 150 mM NaCl solution. The number of *L. monocytogenes* and *P. aeruginosa* were counted by the spread plate method in selective media (Oxford-*Listeria*-Selective-Agar (Base) and *Pseudomonas* agar base, respectively). The control sample biofilm of the pathogen was formed similar to *Lactobacillus* biofilm (Aoudia et al., 2016).

-Antibacterial effect of planktonic cells of probiotics

One ml per well of fresh MRS broth culture was inoculated with 1.5×10^8 CFU/ml of *lactobacillus* strains (*L. plantarum* and *L. delbrueckii* subsp. *bulgaricus*) and was dispensed in a 24-wells microplate. Subsequently, 1 ml of it was added per well of fresh BHI broth inoculated with 1.5×10^8 CFU/ml of each pathogenic bacteria (*L. monocytogenes* ATCC7644 and *P. aeruginosa* PTCC 1074) and incubated for 48 h at 30 °C. After incubation time, the medium was removed from each well, and the microplates were washed twice with 500 ml of 150 mM NaCl solution. Evaluation of microorganisms was performed by the spread plate method. For each test, 1 ml of the samples was mixed with 9 ml of sterile peptone water.

After sequential dilutions, appropriate dilutions were plated on set Oxford-*Listeria*-Selective-Agar (Base (Merck)) for *L. monocytogenes* and *Pseudomonas* agar base (Merck) for *P. aeruginosa* and incubated at 37 °C for 72 h. The total counts of the viable bacteria were reported as log CFU/g. All the experiments were performed in triplicate, which means that each experiment was repeated at least three times (Aoudia et al., 2016).

-Antibacterial effects of cell-free supernatant of probiotics

To prepare the CFS of the selected LAB, 1.5×10^8 CFU/ml of each LAB was inoculated into sterile distilled water. The sample was subjected to ultrasonic vibration (60 Hz for 5 min) to fragment the membrane of cells and centrifuged (4,000 g, 10 min, 4 °C). Then, 1.5×10^8 CFU/ml of each pathogenic bacterium were inoculated into BHI broth and was poured into each well of a 24-well microplate and then, 0.1 ml of supernatant was added to each well. After incubation, washing and staining, OD was determined at 595 nm (Aoudia et al., 2016; Zamani et al., 2017).

Investigation of biofilm structure by Scanning Electron Microscope (SEM)

The probiotic bacterium *L. plantarum* isolated from cheese was selected. The biofilm was formed according to the instructions of the previous step. After washing with sterile distilled water, structure was examined under a SEM. Biofilm was fixed in 2.5% glutardialdehyde solution in 10 mM sodium cacodylate buffer for 24 h at 4 °C. Then, washed thrice for 15 min in 10 mM sodium cacodylate buffer by gentle mixing at room temperature, dehydrated in a graded ethanol series (50, 70, 80, 90, 95, and 100%). The samples were air-dried, placed on SEM stub, coated with gold/palladium by Sputter Coater device Model SC7620 (England), and investigated by a LEO 1450 VP SEM (Zeiss, Germany) with resolution 2.5 nm and maximum voltage 35 kv (Stefania et al., 2017).

Statistical analysis

The experiment was conducted according to a completely randomized factorial design with three replications. Analysis of variance (ANOVA) was carried out using Minitab software (Minitab Release 16, Minitab Inc., and (USA)). Significant differences in treatment means were compared using the Tukey method at 5% significance level.

Results

The strains were divided into four groups: strong, moderate, weak, and non-biofilm-producing according to biofilm formation capability. Five strains were able to form a strong biofilm. Eight strains were the able to produce moderate biofilms. Four strains formed poor biofilm and two strains did not have biofilm production. The strains that formed the strongest biofilm are *L. delbrueckii*, *L. plantarum*, *L. delbrueckii* subsp. *lactis*, *Lactobacillus brevis*, and *Lactobacillus Lactis* subsp *lactis.* The present study results revealed that the majority of strains could form biofilms, but the density and thickness of the biofilm formed can be slightly different depending on the species. For instance, *L. plantarum*, and *L. delbrueckii* subsp. *bulgaricus* formed a more cohesive and stronger biofilm and have been a residual OD>1 at a wavelength of 595 nm.

The antibacterial activity of *Lactobacillus* strains was examined in biofilm, planktonic, and CFS form on the growth of food pathogenic bacteria (*L. monocytogenes* ATCC7644 and *P. aeruginosa* PTCC1074). As shown in Table 2, in the presence of *L. delbrueckii* subsp. *Bulgaricus* and *L. plantarum* biofilm, the ability to produce biofilm by *L. monocytogenes* decreased about 5 and

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4.7 log compared to the control (*L. monocytogenes* biofilm), respectively. In the case of *P. aeruginosa*, a growth reduction of about 3 and 2.7 logs was observed in the presence of biofilms of *L. delbrueckii* subsp. *bulgaricus* and *L. Plantarum*, respectively. The results of the antibacterial activity of probiotics in the planktonic form on the growth of pathogens showed that these bacteria can also reduce the growth of pathogens in plankton form. For example, *L. delbrueckii* subsp. *bulgaricus*, in the planktonic form, reduced the growth of *L. monocytogenes* about 1.4 logs and *P. aeruginosa* about 1.47 logs. Also, the planktonic form of *L. Plantarum* decreased the growth of *L. monocytogenes* and *P. aeruginosa* about 1.19 and 0.87 logs, respectively. A comparison of antibacterial results in both biofilm and planktonic forms showed that the antibacterial effect in biofilm form was more stronger and intense. The results indicated that a significant effect of treatments on *L. monocytogenes* growth and biofilm was more effective but no difference was observed between *L. plantarum* and *L. bulgaricus* on *L. monocytogenes*. CFS of *L. delbrueckii* subsp. *bulgaricus* reduce OD of *L. monocytogenes* to 1.5. On the other hand, in the presence CFS of *L. plantarum*, a value of OD was obtained 1.8, while OD value of control sample was 2.8. The results revealed a significant effect of CFS on the growth rate of *P. aeruginosa* and *L. monocytogenes*, while there was no difference between CFS of *L. delbrueckii* subsp. *bulgaricus* and CFS of *L. plantarum* on the growth of *pseudomonas* spp.

CFS of *L. delbrueckii* subsp. *bulgaricus* reduced OD of *P. aeruginosa* to 1.4. On the other hand, in the presence CFS of *L. plantarum*, a value of OD was obtained 1.6, while OD value of control sample was 2.7. Figure 1 shows the biofilm-forming *L. plantarum.*

Table 1: *Lactobacillus* strains isolated from traditional dairy products

***Lactobacillus* strains**	**Incubation Temperature (℃)**	**Isolation Source**	**Reference**
Lactobacillus delbrueckii subsp. *lactis*	42	Yogurt Toomaq	
L. delbrueckii subsp. *lactis*	42	Yogurt Toomaq	
L. delbrueckii subsp. *bulgaricus*	42	Yogurt Hamzeh-khanloo	Hajimohammadi Farimani et al. (2016)
L. delbrueckii subsp. *lactis*	42	Hamzeh-khanloo	
L. delbrueckii subsp. *bulgaricus*	42	Yogurt Kenarkhaneh	
Lactobacillus plantarum	30	Cheese	
L. plantarum	30	Cheese	
Lactobacillus Lactis ssp *lactis*	37	Milk	
L. Lactis ssp *lactis*	42	Milk	
L. Lactis ssp *lactis*	42	Milk	
L. plantarum	30	Milk	
Lactobacillus brevis	30	Milk	
L. plantarum	37	Milk	
L. Lactis ssp *lactis*	37	Cheese	Edalatian et al. (2012)
L. plantarum	37	Cheese	
L. plantarum	30	Cheese	
L. plantarum	30	Cheese	
L. plantarum	30	Cheese	
L. plantarum	37	Cheese	

* Incubation time was 72 h for all strains.

Table 2: Antagonistic activity of *Lactobacillus* strains in biofilm and planktonic form on the growth of food pathogenic

Treatment		**Food pathogen**	
		Listeria monocytogenes	*Pseudomonas aeruginosa*
Biofilm of probiotics	*Lactobacillus delbrueckii* subsp. *bulgaricus*	3.3±0.1 ^{c}	5.3±0.11 ^{b}
	Lactobacillus plantarum	3.6±0.01 ^{c}	5.6±0.31 ^{b}
Planktonic of probiotics	*L. delbrueckii* subsp. *bulgaricus*	6.9±0.35 ^{b}	7.1±0.56 ^{a}
	L. plantarum	7.11±0.4 ^{b}	7.43±0.14 ^{a}
Control*		8.3±0.03 ^{a}	8.3±0.04 ^{a}

* Biofilm of *Listeria monocytognes* and *Pseudomonas aeruginosa*

Mean values in the same column followed by different superscript letters are significantly different (p <0.05)

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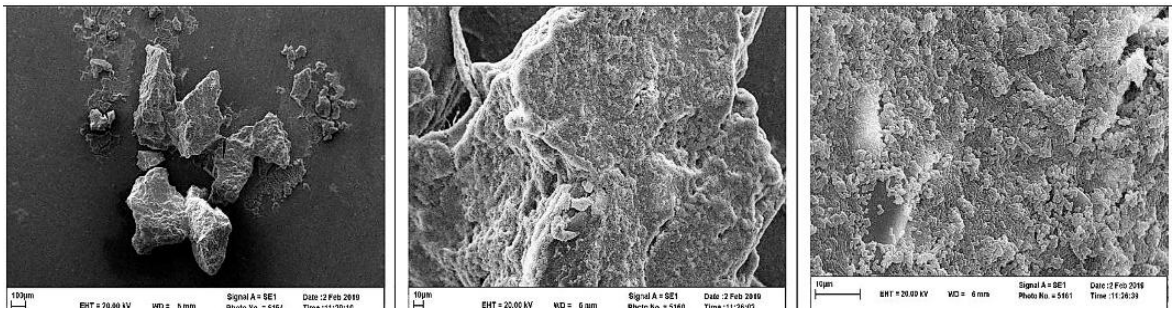


Figure 1: Scanning Electron Microscopy (SEM) images of biofilm-forming *lactobacillus plantarum* in De Man-Rogosa-Sharpe (MRS) agar medium in ×100 and ×10

Discussion

In recent years, several studies have investigated the ability of *Lactobacillus* strains biofilm formation ability and their antagonistic activity in different forms separately. In this study, the strains of LAB isolated from indigenous dairy products were examined, and their potential for biofilm production was measured. These strains were able to grow in the microplate and mature biofilm formation, and there was a slight difference in the biofilm density of the strains. Bujňáková and Kmeť (2012) reported that the four studied strains of *Lactobacillus fermentum* (*L. fermentum* 202, *Lactobacillus galinarum* 7001, *Lactobacillus rhamnosus* 183, and *L.plantarum* L2-1) in the planktonic state have tremendous potential for inhibiting pathogenic pathogens such as *Escherichia coli* and *Salmonella* species also showed strong biofilm formation capacity at the same time. Kaur et al. (2018) showed that all the seven isolates of *Lactobacillus* spp. used in the study inhibited the biofilm formation of *Vibrio cholerae* by more than 90%. Speranza et al. (2020) showed that pathogenic cell loads were always lower in presence of biofilm *bifidobacterium longum* subsp. *infantis* and *Lactobacillus reuteri* as (6.5–7 log CFU/cm^2). For *E. coli* O157:H7, a significant decrease (>1–2 log) was recorded; for *L. monocytogenes*, *Staphylococcus aureus*, and *Salmonella enterica*, cell load reductions ranged from 0.5 to 1.5 log (Speranza et al., 2020). Guerrieri et al. (2009) reported of the antilisterial activity in biofilms developed in a small-scale model by two LAB bacteriocin producers (*L. plantarum* 35d, *Enterococcus casseliflavus* IM 416K1) and by two non-producers (*L. plantarum* 396/1, *Enterococcus faecalis* JH2-2) against *L. monocytogenes* NCTC 10888. The LAB biofilms showed the capability to influence the survival and the multiplication of the pathogen with differences among the strains. *L. plantarum* 35d displayed the highest efficacy reducing *L. monocytogenes* by 5.4 log in the planktonic population (Guerrieri et al., 2009). Aoudia et al. (2016), reported biofilms of *L. plantarum* and *Lactobacillus fermentum* strains have high ability to control pathogens. However, in most cases, a simultaneous comparison has not been performed. Another aim of this study was to investigate the antibacterial properties of biofilm and compare it with planktonic and supernatant states. The result showed that both strains inhibited the growth of food pathogens, but this property and antibacterial effect were more stronger and more intense in the biofilm model. Guerrieri et al. (2009), reported the biofilm produced by different strains of *Lactobacillus* has the ability to reduce the survival and growth of *L. monocytogenes* and *P. aeruginosa* . One reason can be attributed to the strong and stable structure of the biofilm, in which probiotics are more viable and have a larger population, so they produce more lactic acid and have a stronger effect (Mirnejad et al., 2013). Recent studies showed that biofilm growth has an antibacterial effect against pathogens, even though this phenomenon is strain-specific. So, the mechanism of action may be different. Lactic acid, bacteriocin, and hydrogen peroxide may be involved independently or may create a cumulative effect (Pereira and Heman Castro Gómez, 2007). In the study of Guerrieri et al. (2009), the LAB biofilms showed the highest efficacy reducing *L. monocytogenes* and this effect can be partly related to bacteriocins. CFS also has a good inhibitory effect on inhibiting the growth of pathogens due to its high volume of bacteriocin compounds and lactic acid released from the body of probiotics (Leccese Terraf et al., 2012). According to the results, probiotic bacteria can be considered as a good candidate for inhibiting the growth of food pathogens (Kawarai et al., 2007).

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Numerous studies have been conducted to examine the antibacterial effects of probiotic bacteria in forming planktonic and biofilm on a variety of food pathogens, most of which point to the unparalleled power of probiotics in inhibiting pathogens. The results of Mirnejad et al. (2013) study showed strong antibacterial activity of *Lactobacillus casei* against *Shigella sonnei* and *Shigella flexneri*. Besides, the good antibacterial activity of *Lactobacillus* strains that isolated from local traditional fermented products was reported against *Bacillus cereus*. Consequently, another achievement of this study is the use of these identified bacteria as starters in the food industry and as anti-biofilm compounds in the places where food pathogens have problems with biofilm production, such as problems caused by *Pseudomonas* in water reservoirs (Hall-Stoodley et al., 2004).

Recently, one of the most important challenges in the probiotic manufacturing industry is protecting them against internal and external stresses and maintaining them. Thus, several techniques have been developed to protect them in recent years. Compared to existing conventional methods such as encapsulation, granulation, and trapping (Afzaal et al., 2019). Compared to existing conventional methods such as encapsulation, granulation, and trapping; the use of biofilm as a simple, inexpensive, natural, and ideal protection method can lead to a great change in the relevant industries. In this regard, paying attention to local and indigenous strains as genetic resources and considering their phylogenetic relationship with the microbiome of human communities should be considered as a priority (Iravani et al., 2015).

Conclusion

The present study had two main achievements; firstly, indigenous dairy was found to be a powerful source of biofilm-producing probiotic bacteria; secondly, these bacteria as *L. delbrueckii*, *L. plantarum*, *L. delbrueckii* subsp. *lactis*, *L. brevis*, and *L. Lactis* subsp *lactis* have strong antagonistic properties and can retain this property in biofilm form. So, they can be used for multiple purposes and can create the necessary protective effects through producing the biofilm. Finally, the product is preserved by producing bacteriocin compounds.

Author contributions

A.S. created the original idea; Z.R. and S.K. expanded the idea; Z.R. carried out the experiments; A.S. and S.K. directed the project. All authors analyzed and interpreted the data and contributed to the writing of the manuscript. All authors read and revised the final manuscript.

Conflicts of interest

All the authors declared that this is no conflict of interest in the study.

Acknowledgements

This research is the result of a Ph.D. thesis with code 44721, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. The authors express their gratitude.The authors would like to thank the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad for providing raw material and for financial support and providing the facilities that make this project possible**.**

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[DOI: 10.18502/jfqhc.8.4.8257]