

# Investigation of *BRUCELLA SPP* in milk and dairy products and lifespan of *BRUCELLA MELITENSIS* and *BRUCELLA ABORTUS* in kefir

## Süt ve süt ürünlerinde *BRUCELLA SPP*'nin araştırılması ve *BRUCELLA MELITENSIS* ile *BRUCELLA ABORTUS*'un kefirde yaşamı

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**Received/Accepted:** October 24, 2019 / June 27, 2020

**Conflict of interest:** There is not a conflict of interest.



### SUMMARY

Brucellosis is common disease in our country and it is an important zoonotic infection that is up to date in terms of public health, animal industry and economic losses.

The aim of this study was to determine *Brucella* IgG positivity of raw milk and dairy products purchased non-packed and non-labelled. In this study, a total of 450 samples including 250 raw milk, 100 white cheese, 50 butter and 50 yoghurt samples were investigated by ELISA method for *Brucella* IgG positivity. 85 (56.6 %) of 150 raw milk samples obtained from Sivas province center and surrounding villages, 32 (32 %) of 100 raw milk samples obtained from the milk deliverers of Tokat province center and Reşadiye and Pazar districts of Tokat were detected positive for *Brucella* spp. 2 (4 %) of 50 white cheese samples collected in Sivas province center and 9 (18 %) of 50 white cheese samples collected in Tokat province center were positive for *Brucella* spp. None of the yoghurt and butter samples were positive for *Brucella* IgG antibodies

Additionally, in this study, the survival time of *Brucella melitensis* and *Brucella abortus* bacteria prepared in different bacterial density in kefir obtained from 3 different sources (A, B brand and homemade) were investigated. Bacteria in A and B brand kefir lived between 0-72 hours, while homemade kefir lived between 0-24 hours. No reproduction was observed at the end of the period. This study investigated *Brucella* Ig G positivity in milk and dairy products in Sivas and Tokat provinces and milk and dairy products may pose a risk for Brucellosis. Additionally, it was observed that *Brucella* could survive up to 3 days in probiotic fermented milk drink Kefir and the kefir suppresses *Brucella* growth.

**Keywords:** *Brucella* spp, milk and dairy products, kefir

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### ÖZET

Bruselloz ülkemizde sık görülen bir hastalıktır ve halk sağlığı, hayvan endüstrisi ve ekonomik kayıplar yönünden güncelliğini koruyan önemli zoonotik bir enfeksiyondur.

Bu çalışmanın amacı, çiğ süt ile paketsiz ve etiketsiz süt ürünlerinin *Brucella* Ig G pozitifliğini belirlemektir. Bu çalışmada, 250 çiğ süt, 100 beyaz peynir, 50 tereyağı ve 50 yoğurt örneği içeren toplam 450 örnek, *Brucella* IgG pozitifliği yönünden

ELISA yöntemi ile incelendi. Sivas il merkezi ve çevre köylerinden elde edilen 150 çiğ süt örneğinin 85'i (% 56,6), Tokat il merkezindeki sütçülerden ve Tokat'ın Reşadiye ve Pazar ilçelerinden elde edilen 100 çiğ süt örneğinin 32'si (% 32) *Brucella* spp. yönünden pozitif bulunmuştur. Sivas il merkezinde toplanan 50 beyaz peynir örneğinden 2'si (% 4) ve Tokat il merkezinde toplanan 50 beyaz peynir örneğinden 9'u (% 18) *Brucella* spp. yönünden pozitif bulunmuştur. Yoğurt ve tereyağı numunelerinin hiçbirinde *Brucella* Ig G antikorları yönünden pozitiflik saptanamamıştır.

Ek olarak bu çalışmada 3 farklı kaynaktan (A, B markası ve ev yapımı) elde edilen kefirde farklı bakteri yoğunluğunda hazırlanan *Brucella melitensis* ve *Brucella abortus* bakterilerinin yaşam süreleri incelenmiştir. A ve B marka kefirlerde bakteriler 0-72 saat arasında yaşarken, ev yapımı kefir içerisinde 0-24 saat arasında yaşamıştır. Süre sonunda üreme gözlenmemiştir.

Bu çalışma Sivas ve Tokat illerinde süt ve süt ürünlerinde *Brucella* Ig G pozitifliği gösterilmiştir ve süt ve süt ürünleri Bruselloz için bir risk oluşturabilir. Ayrıca, probiyotik fermente süt içeceği kefirde *Brucella*'nın 3 güne kadar canlı kalabildiği ve kefirin *Brucella*'nın üremesini baskıladığı gösterilmiştir.

**Anahtar sözcükler:** *Brucella* spp, süt ve süt ürünleri, kefir

## INTRODUCTION

Brucellosis, a zoonotic disease, cause facultative Gram-negative, intracellular *Brucella* bacteria, animals such as cattle, sheep, goats, pigs and dogs to settle in their genital organs and udders, complications such as offspring, infertility, mastitis, orchitis and arthritis. Brucellosis is present in more than 160 countries around the world and approximately 500,000 new human cases are reported annually<sup>1-3</sup>.

*Brucella* spp. can be directly transmitted from infected animals and contaminated tissues to humans via inhalation or through skin lesions particularly in endemic regions<sup>4,5</sup>. However, the most common way to be infected is ingestion of contaminated raw milk and dairy products in the world and in our country<sup>6,7</sup>. The vast majority of milk and dairy products produced in our country in very large quantities are produced from raw milk in unhygienic conditions, with primitive methods. While there is a decrease in disease incidence, our country is still among the endemic countries in terms of brucellosis<sup>8,9</sup>.

*Brucella* are Gram-negative coccobacilli (short rods) measuring about 0.6 to 1.5 µm by 0.5-0.7 µm. They are non-sporing and non-encapsulated or flagella and, therefore, are non-motile<sup>10</sup>.

Brucellosis occurs as an acute and non-specific febrile disease. Classically, such symptoms may include fever, chills, generalized fatigue, headache, muscle aches (myalgias), loss of appetite, weight loss, night sweats, joint pain (arthralgia) and inflammation (arthritis), back pain, constipation. Because of the protean clinical signs of brucellosis, it is very important to take a detailed history and

pay attention to epidemiological information. Care should be taken to determine whether the person is in contact with infected animals or whether milk and dairy products obtained from them are consumed<sup>7,11</sup>.

Detection of *Brucella* bacteria in milk and dairy products is difficult and time consuming, therefore, currently the use of indirect methods has increased in diagnosis. In serological techniques, ELISA is widely used in various fields due to its high sensitivity and specificity, the ability to work with multiple samples at the same time, to obtain results in a short time and to be inexpensive<sup>12</sup>.

In this study, *Brucella* IgG antibodies were detected by ELISA method in raw milk and dairy products such as white cheese and butter and yoghurts in Sivas and some villages and in Tokat province center. Additionally, the lifespan of the *Brucella* bacterium was determined in the fermented milk drink kefir and the survival times of the two different brands of kefir offered for commercial consumption were compared with the home-made kefir.

## MATERIAL AND METHODS

In this study, a total of 450 different samples consist of 250 raw milk, 100 white cheese, 50 yoghurts and 50 butter were tested for *Brucella* IgG positivity by ELISA. Distribution of milk and dairy products examined is summarized in Table 1.

100 ml of milk samples, 20 gr of white cheese, butter and yogurt samples were taken into sterile containers and stored in the freezer at -20 °C until ELISA test.

**Table 1:** Distribution of milk and dairy products examined for *Brucella* IgG by ELISA

Locality	Raw Milk	White Cheese	Yoghurts	Butter	Total
Sivas Province	150	50	25	25	250
Tokat Province	26	32	25	25	108
Reşadiye	50	18	-	-	68
Pazar	24	-	-	-	24
<b>Total</b>	<b>250</b>	<b>100</b>	<b>50</b>	<b>50</b>	<b>450</b>

Pathogen bacteria species inoculated into kefir samples to investigate the survival of *Brucella* bacteria in kefir; *B. abortus* (RSCC 279) and *B. melitensis* (RSCC 310) strains obtained from Refik Saydam National Type Culture Collection

#### Preparation of samples for ELISA and conducting the experiment

The milk samples were processed directly and the yoghurt samples were homogenized in 10 g / 30 ml of sterile physiological saline. Samples of cheese and butter were homogenized with blender in 10 g / 200 ml sterile PBS (Phosphate Buffered Saline) and placed in eppendorf tubes. Institut Pourquier (France) brand *Brucella* IgG-ELISA Kit was used for the ELISA test. All samples were tested for *Brucella* IgG positivity according to the procedure indicated in the kit. The results were calculated according to the manufacturer's recommendations.

#### Investigation of Survival of *Brucella* Bacteria in Kefir

##### Preparation of Kefir Samples

In this research, three different kefir prepared from pasteurized milk were studied. One of the kefirs was produced from kefir grains, the other two were kefirs available for commercial consumption.

Pasteurized milk was taken into 1000 ml sterile container, 15-20 gr of kefir grains were added and mixed thoroughly and the lid of the container was closed and incubated for 24 hours at ambient temperature of 20-25 °C. The kefir was passed through a plastic sieve placed on a container to separate the grains. Prepared and commercially purchased kefir were distributed in 20 ml each in 100 ml sterile containers. No treatment was performed on 2 samples per 3 kefir and removed as

a control group. *B. melitensis* and *B. abortus* were used as pathogenic bacteria for inoculation.

#### Experimental Procedure

In this study, *B. melitensis* and *B. abortus* bacteria produced in Serum Dextrose Agar (SDA) were added to 3 different kefir samples of 20 ml each from suspensions of different bacterial concentrations to be 100 µl each. Then the samples were incubated at 37°C. At the end of 24, 48, 72, 96 and 120 hours, 50 µl of each kefir sample were inoculated SDA media and incubated at 37 °C.

At the end of this period, bacterial growth in SDA was studied individually. Bacterial morphology was evaluated by gram staining from each different colonies. Gram-negative, small coccobacilli, coccoid morphology colonies were identified with polyvalent antiserum specific to *Brucella* species (*B. abortus*, *B. suis*, *B. melitensis*).

Statistical analysis of the study was performed using IBM SPSS Statistic 22.0 (IBM Co., Armonk, NY, USA). Chi-square ( $\chi^2$ ) test was used to compare the data. Values of  $P \leq 0.05$  were considered statistically significant.

## RESULTS

### ELISA Test Results in Milk and Dairy Products

117 (46.8 %) of 250 raw milk samples were positive for *Brucella* spp. 85 (56.6 %) of 150 raw milk samples taken from Sivas province center and surrounding villages and 32 (32 %) of 100 raw milk samples obtained from Tokat province center milk deliverers and Reşadiye and Pazar districts were found to be positive for *Brucella* spp. (Table 1). The difference between provinces was statistically significant ( $p = 0.000$ ).

**Table 2:** Occurrence of *Brucella* spp. among raw milk samples by ELISA\*

Sivas Province and Villages	Total number (%)	Number of positive (%)
Sivas Province	13 (5,2)	11 (4,4)
Sivas Surrounding Villages	137 (54,8)	74 (29,6)
Tokat Province and Districts		
Tokat Province	26 (10,4)	7 (2,8)
Reşadiye	50 (20)	16 (6,4)
Pazar	24 (9,6)	9 (3,6)
<b>Total</b>	<b>250 (100)</b>	<b>117 (46,8)</b>

\*ELISA=Enzyme-linked immunosorbent assay

Additionally, 11 (11 %) of 100 cheese samples were positive for *Brucella* Ig G. *Brucella* seropositivity in white cheeses collected from Sivas, Tokat province and Reşadiye districts were 4 %, 12.5 % and 27.7 % respectively (Table 2). The

difference between the results obtained from Sivas, Tokat and Reşadiye was statistically significant ( $p = 0.02$ ). None of the yoghurt and butter samples were positive for *Brucella* IgG antibodies.

**Table 3:** Occurrence of *Brucella* spp among white cheese samples by ELISA

Locality	Total number (%)	Number of positive (%)
Sivas Province	50 (50)	2 (4)
Tokat Province	32 (32)	4 (12,5)
Reşadiye	18 (18)	5 (27,7)
<b>Total</b>	<b>100</b>	<b>11 (11)</b>

### ***B. melitensis* and *B. abortus* bacteria in different kefir samples to determine the survival of the results of the study**

As a result of this study; *B. abortus* (RSCC 279) and *B. melitensis* (RSCC 310) bacteria in A and B brand kefir samples purchased in markets,  $0.9 \times 10^7$ ,  $1.2 \times 10^7$  and  $1.5 \times 10^7$  bacteria densities while it can reproduce within of 24-72 hours, reproduction has not been observed at the end of 72 hours. In the homemade kefir, these three bacteria survived in the first 0-24 hours, but no growth was observed after 24 hours.

## **DISCUSSION**

Brucellosis remains the commonest and important zoonotic disease worldwide which is transmitted from animals to humans, has been a major health problem with more than 500 000 new cases annually. The main source of contamination for brucellosis is unpasteurized milk and dairy products (fresh cheese, cream, butter, etc.)

consumption. The majority of dairy products are produced from raw milk in unhygienic conditions, primitive methods, and thus the disease remains a serious health problem in developing countries and in our country<sup>13-14</sup>.

In our study, 117 (46.8 %) of 250 raw milk samples were positive for *Brucella* spp. 85 (56.6 %) of 150 raw milk samples taken from Sivas province center and surrounding villages and 32 (32 %) of 100 raw milk samples obtained from Tokat province center milk deliverers and Reşadiye and Pazar districts were found to be positive for *Brucella* spp. The difference between these provinces was statistically significant ( $p = 0.000$ ,  $p < 0.05$ ). It is thought that *Brucella* seropositivity is higher in the milk samples collected from Sivas province than Tokat, the distribution of samples is mostly belong to villages, it is thought that the animals in the villages may carry a higher risk of brucellosis than living in the province. One of the reasons for the occurrence of *Brucella* Ig G in nearly half of the

raw milk used in our study (46.8 %) may be due to the fact that the organism localizes in the supra-mammary lymph nodes and mammary glands in the of 80 % of the infected animals and pass the infection agent into the milk during lactation periodically or intermittently<sup>15</sup>. It has been shown in studies that 1 % and 40 % isolations of *Brucella* spp. were from raw milk in our country and other countries,<sup>16-19</sup>.

It has been shown in studies that 0 % and 20.5 % isolations of *Brucella* spp. were from cheese in our country<sup>20-24</sup>. In our study, 11 (11 %) of 100 cheese samples were positive for *Brucella* Ig G. *Brucella* seropositivity in white cheeses collected from Sivas, Tokat province and Reşadiye districts were 4 %, 12.5 % and 27.7 % respectively. The difference between the provinces was statistically significant ( $p = 0.02$ ,  $p < 0.05$ ). Cheese samples obtained from Tokat province showed higher positivity in terms of *Brucella* Ig G compared to Sivas due to the unpacked cheeses purchased in the markets and from the Reşadiye.

Fermented dairy products such as cheese made from raw milk because it is difficult and time consuming to make the cheese from the milk that has been processed such as boiling and pasteurization. It is thought that there will be a decrease in the amount and flavor of the cheese that will be obtained after boiling. The majority of the cheeses from Sivas were sourced from markets and delis. Cheeses purchased in such places are offered for sale with very different brands. These companies must comply with certain criteria in their production processes and meet the necessary requirements, especially in relation to pasteurization of milk. As a result, cheeses produced in accordance with quality standards reach the consumer in a healthy way. Therefore, this difference in the cheese population is reflected in the results.

None of the butter samples were positive for *Brucella* IgG. The reason is that the butter is not prepared from the milk of the infected animals, boiling the milk, pasteurizing or churning the yogurt to obtain butter. However, considering the fact that butter can be produced from raw and inadequately treated milk, the possibility of a health risk for brucellosis should always be considered. In the study of Taşçı et al. (2009) In butter, cream and whipped cream cakes offered for consumption in Ankara. They did not isolate the *Brucella* spp. in any of the 135 samples examined.

None of the yoghurt samples were positive for *Brucella* IgG. The reason is that lactic acid bacteria in yogurt, which is fermented food, inhibit the

growth of many pathogenic microorganisms. Lactic acid produced by lactic acid bacteria against many acid-sensitive bacteria, *Brucella* spp. These bacteria also have show bacteriostatic effect. Additionally, heat treatment of milk in the production process of yogurt is thought to prevent the growth of these bacteria.

Additionally, in this study, the survival time of *B.melitensis* and *B.abortus* bacteria in kefir, a valuable, complex probiotic fermented dairy product, was determined. Bacteria survive between 0- 72 hour in A and B brand kefir, and they cannot grow as from 72 hour. Bacteria in homemade kefir survive between 0-24 hour and cannot grow as from 24 hours. No significant difference was observed between the two bacterial species in terms of survival. In homemade kefir, microflora is the dominant species *Candida* spp. whereas lactic acid bacteria predominate in other kefir. Differences in the survival of pathogens in homemade kefir and kefir belonging to two different brands were determined by lactic acid bacteria and *Candida* spp. may be due to the difference in metabolic products. The metabolic products exhibits antimicrobial activity by inhibiting the growth of patogen bacteria. Kefir antimicrobial activity is associated with the production of organic acids, peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol and diacetyl. Ito et al. (2003) in their study *Lactobacillus lactis* subsp. *lactis* with H<sub>2</sub>O<sub>2</sub> formation *Escherichia coli*, *Yersinia enterocolitica*, *Listeria* that the inhibitory effect on species. They also found that pH plays a negative role in the development of the species mentioned<sup>26</sup>.

Chifiriuc et al. (2011) indicated that all milk fermented with kefir grains had antimicrobial effect against *Bacillus subtilis*, *Staphylococcus aureus*, *E. coli*, *Enterococcus faecalis* and *Salmonella enteritidis*. Rodrigues et al. (2005) reported the potential antimicrobial activity of the polysaccharide kefiran against bacteria and *C. albicans*<sup>27,28</sup>.

## CONCLUSION

As a result, in the present study was showed *Brucella* Ig G positivity in milk and dairy products in Sivas and Tokat provinces and raw milk and dairy products may pose a risk for Brucellosis. Furthermore, it was observed that kefir suppresses *Brucella* bacteria growth.

Brucellosis, which is one of the important zoonoses, is very common among sensitive animals and humans in Turkey. Thus successful, meticulous and continuous implementation of

disease control programs should be carried out in the eradication of brucellosis. Further research focusing on the isolation of *Brucella* is required to determine regional disease rates and to obtain a comprehensive understanding of the *Brucella* spp.

Kefir, which is another research topic we have studied, has been shown many important properties such as antimutagenic, antioxidant, antimicrobial in animal studies. Considering the increasing consumption of kefir in our country, studies on this subject will provide valuable data to the literature as well as guiding for industrial production.

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