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Research Article / Araştırma Makalesi

Influence of Milk Fat on Listeria monocytogenes Viability Under Simulated Gastrointestinal Conditions and on the Viable But Not Countable State

Simule Edilmiş Gastrointestinal Koşullar Altında Süt Yağının Listeria monocytogenes Canlılığına ve Canlı Ancak Sayılmayan Durumu Üzerine Etkisi

Jerina RUGİİ[1], Elif ÖZGÜR[1], Zühal ÇALIŞKAN[1], Ahmet Hulusi DİNÇOĞLU[2*], İldeniz YILDIRIM[2]

[1]Burdur Mehmet Akif Esoy University, Institute of Health Sciences, Department of Food Hygiene and Technology, Burdur, Türkiye
[2]Burdur Mehmet Akif Esoy University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Burdur, Türkiye

Abstract: Along with the high nutritional value, milk represents an excellent medium for the growth of certain microorganisms, some of which can be life threatening. Milk fat has been found to affect the survival of *L. monocytogenes* in milk. The present study aimed to evaluate the effect of milk fat in the survival of *L. monocytogenes* in milk under simulated gastrointestinal conditions. Four compartments (saliva, gastric, small intestine and large intestine) mimicking the human physiological conditions were established to evaluate the viability of *L. monocytogenes* inoculated in milk. Given that milk is generally consumed as a breakfast meal, the evaluation was done in the fasted state of the gastrointestinal system. A decrease to 5 $\log_{10}$ CFU/ mL was determined in saliva compartment, in the evaluation after 48 h of cold storage. In the viable but not countable evaluation, *L. monocytogenes* counts were determined to be 8 $\log_{10}$ CFU/ mL for skim milk and semi-skim milk, and 9 $\log_{10}$ CFU/ mL for whole fat milk in the saliva compartment. Regardless the fat content, *L. monocytogenes* was not detected in any of the milk groups in the lower parts of the simulated gastrointestinal compartments.

Keywords: *Listeria monocytogenes*, Milk, Milk Fat, Simulated Gastrointestinal System.

Öz: Süt, yüksek besin değeriyle birlikte, yaşamı tehdit edebilen belirli mikroorganizmaların büyümesi için de mükemmel bir ortamdır. Süt yağının, sütte bulunan *L. monocytogenes*'in canlılığına etki ettiği görülmüştür. Bu çalışmada, simüle edilmiş gastrointestinal koşullar altında sütte *L. monocytogenes*'in hayatta kalmasında süt yağının etkisinin değerlendirilmesi amaçlanmıştır. Süte aşılanan *L. monocytogenes*'in canlılığını değerlendirmek için insan fizyolojik koşullarını taklit eden dört farklı ortam (saliva, mide, ince bağırsak ve kalın bağırsak) oluşturulmuştur. Sütün genellikle kahvaltı öğünü olarak tüketildiği göz önüne alındığında, değerlendirme gastrointestinal sistemin aç olduğu durumda yapılmıştır. 48 saat soğukta saklama sonrası yapılan değerlendirmede saliva ortamında 5 $\log_{10}$ CFU/ mL'ye kadar düşüş tespit edilmiştir. Canlı ancak sayılamayan değerlendirmede saliva ortamında *L. monocytogenes* sayısı yağsız ve yarım yağlı süt için 8 $\log_{10}$ CFU/ mL, tam yağlı süt için 9 $\log_{10}$ CFU/ mL olarak belirlenmiştir. Simüle edilmiş gastrointestinal ortamların alt kısımlarında incelenen farklı yağ içeriğine sahip olan süt gruplarında *ise L. monocytogenes* tespit edilmemiştir.

Anahtar Kelimeler: *Listeria monocytogenes*, Süt, Süt Yağı, Simüle Edilmiş Gastrointestinal Sistem.

*Corresponding author : Ahmet Hulusi DİNÇOĞLU e-mail : adincoglu@mehmetakif.edu.tr
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Introduction

Milk and dairy products represent an important fraction of the human diet, providing a supply of proteins, vitamins, and minerals (Bianchi et al., 2013; Huth et al., 2006). There is a wide range of dairy accessible, the majority of which is produced from pasteurized milk. Although, a rising consumer demand for raw milk is present alongside this developed market (Latorre et al., 2009). In addition to their rich composition (Bianchi et al., 2013) dairy products can support the growth of various foodborne pathogens, such as *Listeria monocytogenes* (Arqués et al., 2015; Lee et al., 2019).

L. monocytogenes is a ubiquitous pathogen that is able to remain its viability even under unfavourable conditions such as refrigeration temperatures, high salt concentration and low pH (Dalzini et al., 2016). Despite that mortality rate from listeriosis is between 20 and 30%, categories such as new-borns, pregnant women, immunocompromised individuals, or the elderly, may anticipate far worse outcomes (Eicher et al., 2020; Erol and Taşçı, 2021). Due to its psychrophilic properties, even very low counts can result in an increase of the population of *L. monocytogenes* in the milk stored in refrigeration temperatures (Mortazavi and Aliakbarlu, 2019). Because contaminated milk can be consumed directly or used to make dairy products, there is an ongoing need to develop ways for managing and monitoring *L. monocytogenes* (Chen and Zhong, 2017). The presence of *L. monocytogenes* in milk as a result of pasteurization defects or post-pasteurization contamination remains a point of concern (Lee et al., 2019). It has been determined that fat plays a pivotal role in the protection of *L. monocytogenes* from thermal damage (Verheyen et al., 2020, Özkale and Kahraman, 2022). *L. monocytogenes*, under adverse conditions, is be able to enter a "viable but non-culturable" (VBNC) form, which might help it propagate up the food chain (de Medeiros Barbosa et al., 2020; Overney et al., 2017).

To the knowledge of authors, no studies have been conducted to evaluate the effect of milk fat on the viability of *L. monocytogenes* in different compartments of the simulated gastrointestinal system simultaneously. The present research aimed to evaluate the effect of the milk fat in the viability of *L. monocytogenes* in the vegetative and viable but not countable state under simulated gastrointestinal conditions.

Material and Method

The UHT milk used in the study was purchased commercially. The viability of *L. monocytogenes* was evaluated using standardized skimmed (0.1%), semi-skimmed (1.5%), and full-fat milk (3%).

L. monocytogenes Isolation

Isolation of *L. monocytogenes* from milk (presence/absence test) was performed according to EN ISO 11290-1:2017 standard. Milk samples were plated onto ALOA (*Merck,1.00427.0500*) supplemented with 2 supplements (*Merck, 100432 and Merck, 100439*) and incubated at 37 °C for 48 h prior to inoculation. Blue/ green colonies surrounded by a halo were evaluated as *L. monocytogenes*.

Preparation of the Bacterial Inoculum

L. monocytogenes (ATCC 13932, serovar 4b) was initially inoculated into 10 mL of Tryptic soy broth (*Merck, Millipore*) and incubated for 24 hours at 30 ºC. After incubation, the pellet was centrifuged at 4200 rpm for 5 minutes (*Eppendorf Centrifuge 5810 R*). Afterwards, the supernatant was removed and 10 mL of 0.9% sterile physiological saline was added to the pellets. This centrifugation process was repeated twice. The pathogen concentration was adjusted to 10^9 CFU/ mL and confirmed by microbiological cultivation.

Sample Preparation

A total of six UHT milk samples were acquired from a local market in 200 mL aseptic tetra pack containers. The whole fat milk (WFM), semi-

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skimmed (SSM) and skimmed (SM) UHT milk were maintained at +4 °C until the trials were performed. *L. monocytogenes* was inoculated at a concentration of 10^9 CFU/mL. After each inoculation of the stock samples, by spreading the dilutions on plates, the final concentration of *L. monocytogenes* cells in each milk sample was found to be around 10^7 CFU/mL. The inoculated stock samples were stored at +4 °C and analysed on the 24 and 48th day.

Establishment of the in vitro System

A simulated gastrointestinal system was established to evaluate the survival of *L. monocytogenes* in three types of milk. Four consecutive compartments, each mimicking the saliva, gastric fluid, small and large intestines in a fasting state, were prepared according to the method described by Rugji and Dinçoğlu, (2022). Parameters such as transition time, pH and overall temperature of each compartment were selected based on physiological conditions of healthy individuals (Prezzi et al., 2020).

Evaluation of *L. monocytogenes* in UHT Milk to Determine Viability in the Simulated Gastrointestinal Tract

Initially, 1 mL of each milk sample was individually transferred to saliva compartment (10 mL) and incubated at 37 °C for 5 min/ 50 rpm. At the end of the incubation, respective samples were manually transferred to the other compartments and incubated 2 h in the gastric fluid, 2 h in the small intestine compartment and 2 h in the large intestine compartment at 37 °C/50 rpm. At the termination of incubation for each compartment 1 mL was sampled from each compartment. Samples were serially diluted and respective dilutions were pour-plated on ALOA (EN ISO 11290-2:2017). To determine the VBN counts, parallelly samples were pour plated on ALOA-Nutrient agar (NA) medium using thin agar layer (TAL) method as described by Evert-Arriagada et al. (2018) with minor modifications. The purposely inoculated milk samples were preserved at +4 °C. The same procedures were repeated 24 and 48 hours later.

Statistical Evaluation

The effect of the fat on the viability of *L. monocytogenes* under simulated gastrointestinal conditions were determined by analysis of variance (ANOVA), followed by Tukey test. The factors were fat and simulated gastrointestinal compartments: skimmed (group A), semi-skimmed (group B), and full-fat milk (group C). All determinations were carried out in triple. The data are evidenced as the mean ± standard deviation.

Results

Table 1 exhibits *L. monocytogenes* counts in three different fat level milk groups during a 48-h evaluation under simulated gastrointestinal conditions. The counts in all groups were determined at a 6-log level. In the initial evaluation, no differences were observed between groups in terms of bacterial counts in the simulated saliva fluid. *L. monocytogenes* was not detected in the lower parts of the simulated gastrointestinal fluid in the initial evaluation. In the following evaluation (24 and 48 h later) a decrease in *L. monocytogenes* counts was determined (Fig. 1). For both evaluations counts were determined at a 5-log level at the saliva fluid. Similar to the initial evaluation, *L. monocytogenes* counts were not detected in the lower parts of the simulated gastrointestinal fluid.

Table 2 displays *L. monocytogenes* viable but not countable counts in three different fat level milk groups during a 48-h evaluation under simulated gastrointestinal conditions. The results of the initial evaluation (0-h) are similar to the results obtained from the standard count evaluation in the saliva fluid (6-log for all groups). An increase in *L. monocytogenes* counts was determined in the following determinations. For both evaluations, *L. monocytogenes* counts were determined to be 8-log for skim milk and semi-skim milk, and 9-log for whole fat milk (Fig. 2). *L. monocytogenes* was not

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detected in the lower parts of the simulated gastrointestinal fluids in any of the evaluation days.

Table 1. The survival of *Listeria monocytogenes* in milk samples under simulated gastrointestinal system on ALOA agar (log_{10} CFU/mL)

	0 h			24 h			48 h		
	SM	**SSM**	**WFM**	**SM**	**SSM**	**WFM**	**SM**	**SSM**	**WFM**
SF	6.0±0.01	6.0±0.01	6.0±0.01	5.0±0.01	5.0±0.01	5.0±0.01	5.0±0.01	5.0±0.01	5.0±0.01
GF	ND	ND	ND	ND	ND	ND	ND	ND	ND
SIF	ND	ND	ND	ND	ND	ND	ND	ND	ND
LIF	ND	ND	ND	ND	ND	ND	ND	ND	ND

SF- Saliva fluid, GF- gastric fluid, SIF- small intestine fluid, LIF- large intestine fluid
SM- Skimmed milk, SSM- semi skimmed milk, WFM- whole fat milk
A-B Differences between groups with different superscripts in the same line are important (P<0.05)
ND- Not Detected (< 1 log_{10} CFU/g)

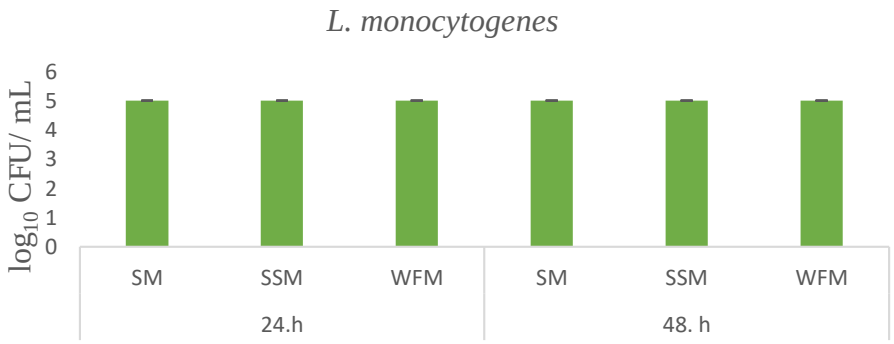

Figure 1. Evaluation of *L. monocytogenes* on saliva fluid

Discussion

L. monocytogenes is a ubiquitous microorganism well-known for its resistance to adverse ambient factors such as low pH. The acid tolerance response (ATR) of *L. monocytogenes* is thought to be critical for its survival, resulting in the pathogen's persistence in food-processing conditions. Temperature and pH are major triggers of the ATR state of *L. monocytogenes*. The pH range that triggers *L. monocytogenes* ATR is 4.5-6.0 at 30 °C or 37 °C (Liu et al., 2020). Considering these properties, Listeriosis an important concern in terms of food safety (Lee et al., 2019). Due to its high nutritional value, pH balance, and high-water activity, milk is an ideal growing habitat for a variety of bacteria (Bando et al., 2009; Geigl et al., 2008; Lee et al., 2019). The use of heat treatments such as pasteurization and sterilization has lowered the occurrence of milk-borne illnesses (Headrick et al., 1998; Martin et al., 1997). Nonetheless, the rising trend of raw milk

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consumption necessitates the monitoring of microbiological health hazards associated with raw milk consumption (Claeys et al., 2013; Perkiomäki et al., 2012; Castro et al., 2017). The current investigation outline that *L. monocytogenes* counts were higher in the WFM sample in ALOA-NA medium after 24 hours of inoculation. A similar trend was determined in the evaluation after 48h in ALOA-NA medium. Thin agar layer method is utilized to determine the counts of bacteria that may be damaged or in the sublethal form (Evert-Arrigada et al., 2018). The TAL technique has been established in 1998 for the enumeration of heat-damaged foodborne pathogens by Kang and Fung. In this technique, a nonselective medium is added to a pathogen-specific selective medium that has already been pre-poured (Wu et al., 2001). As the fat content in milk increased, so did the viability of *L. monocytogenes*. Similar to the present study, Özkale and Kahraman, (2022) evaluated the effect of the milk fat in the viability of *L. monocytogenes* during ohmic treatment. It was determined that the increased fat content had an important inhibitory effect on the pathogen inactivation. Studies on various food matrices have been conducted to evaluate the resistance of *L. monocytogenes* under simulated gastrointestinal conditions. Vieira et al. (2019) assessed the outcome of edible coating with essential oils on apples on the survival of *L. monocytogenes*. Contrary to the present research, the exposure to saliva, gastric and gut fluid caused a significant reduction in the bacterial counts. Dong et al. (2020) determined a decrease in *L. monocytogenes* counts inoculated in cabbage exposed to simulated gastrointestinal system. Akritidou et al. (2022) evaluated the effect of gastric pH and bile acids on the survival of *Listeria monocytogenes* during simulated gastrointestinal digestion. It was determined that *L. monocytogenes* presented high gastric acid tolerance, but increased bile sensitivity during in vitro digestion. When exposed to stressful factors such as cleaning and sanitation and lack of nutrients, some pathogens transit to a metabolic state known as viable but not culturable (Kumar et al., 2019; Zhao et al., 2017). Noll et al. (2020) investigated the ability of benzalkonium chloride effect on the VBNC state of *L. monocytogenes*. Similarly, Truchado et al. (2021) determined that peroxyacetic acid and chlorine dioxide unlike chlorine induce the VBNC stage of *Listeria monocytogenes*. The presence of various disinfectants and acidic conditions, chlorine, electrolyzed water have been found to induce the VBNC state of *L. monocytogenes* (Afari et al., 2019; Arvaniti et al., 2021; Highmore et al., 2018).

Table 2. The survival of *Listeria monocytogenes* in milk samples under simulated gastrointestinal system on ALOA-NA (log_{10} CFU/mL)

	0 h			**24 h**			**48 h**		
	SM	**SSM**	**WFM**	**SM**	**SSM**	**WFM**	**SM**	**SSM**	**WFM**
SF	6.0±0.01	6.0±0.01	6.0±0.01	8.0±0.01B	8.0±0.01B	9.0±0.01A	8.0±0.01B	8.0±0.01B	9.0±0.01A
GF	ND	ND	ND	ND	ND	ND	ND	ND	ND
SIF	ND	ND	ND	ND	ND	ND	ND	ND	ND
LIF	ND	ND	ND	ND	ND	ND	ND	ND	ND

SF- Saliva fluid, GF- gastric fluid, SIF- small intestine fluid, LIF- large intestine fluid
SM- Skimmed milk, SSM- semi skimmed milk, WFM- whole fat milk
A-B Differences between groups with different superscripts in the same line are important ($P<0.05$)
ND- Not Detected (< 1 log_{10} CFU/g)

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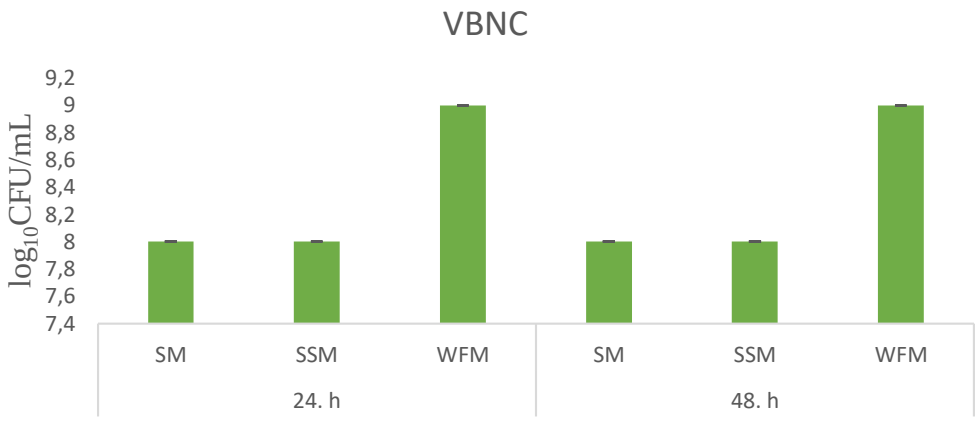

Figure 2. Evaluation of VBNC state of *L. monocytogenes* on saliva fluid

Despite its well-known adaptability and resistance toward low acidity, in the present research *L. monocytogenes* was not detected in the simulated gastric fluid. In the same manner counts were not detected in the simulated small and large intestinal fluids. Authors claim that the lack of detection of *L. monocytogenes* in the aforementioned sections may be associated with several factors including the degree of sublethal injury caused by the pH shock, lack of techniques to recover viable cells from the samples, lack in the sensitivity of the method used for detection or low inoculation levels. In light of this, investigations to a greater extent need to be done to evaluate the activity of *L. monocytogenes* toward different ambient stressor.

Conclusion

Due to its rich constitution, milk is regarded as one of the most comprehensive providers of nutrients. However, raw milk also fosters the growth of a number of potentially dangerous microorganisms. *L. monocytogenes* due to its ability to preserve the viability under adverse conditions, viable but not countable state and occurrence in the post-pasteurization contamination represents an important pathogen in terms of public health. The rise in consumption of minimally processed products has led to an increase in the consumption of raw milk. Parallelly to this trend, increases the need for proper monitoring of health hazards related to raw milk consumption. The present study revealed that the milk fat had a protective effect in the viability of *L. monocytogenes*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper**.**

Authorship Contributions

During the study's preparation, all authors contributed equally.

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