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Behavior of Shiga-toxin-producing *Escherichia coli* in ewe milk stored at different temperatures and during the manufacture and ripening of a raw milk sheep cheese (Zamorano style)

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ABSTRACT

This study was conducted to assess the survival of 2 wild Shiga toxin-producing *Escherichia coli* strains (one serotype O157:H7 and one non-O157:H7) in ewe milk stored at different conditions and to examine the fate of the O157 strain during the manufacture and ripening of a Spanish sheep hard variety of raw milk cheese (Zamorano). The strains were selected among a population of 50 isolates, which we obtained from ewe milk, because of their high resistance to 0.3% lactic acid. Both strains were inoculated (approximately 2 $\log_{10}$ cfu/mL) in raw and heat-treated (low-temperature holding, LTH; 63°C/30 min) ewe milk and stored for 5 d at 6, 8, and 10°C and also according to a simulation approach for assessing the effects of failures in the cold chain. The minimum growth temperature for the O157:H7 strain in LTH and raw ewe milk was 8°C. For the non-O157:H7 strain, the lowest temperature showing bacterial growth in LTH ewe milk was 6°C, but it did not grow at any of the tested conditions in raw milk. It appears that the O157 strain was more susceptible to cold stress but was likely a better competitor than the non-O157 strain against the milk autochthonous microbiota. For manufacture of Zamorano cheese, raw milk was inoculated with approximately 3 $\log_{10}$ cfu/mL, and after 2 mo of ripening at 10 to 12°C, the cheeses showed the expected general characteristics for this variety. The O157:H7 strain increased 0.9 $\log_{10}$ cfu/g after whey drainage and during ripening and storage decreased by 2.9 $\log_{10}$ cfu/g. Nevertheless, its detectable level (estimated at 6.2 cfu/g) after 2 mo of ripening suggests that Zamorano cheese manufactured from raw ewe milk contaminated with *E. coli* O157:H7 could represent a public health concern.

Key words: non-O157, O157:H7, sheep milk, Zamorano cheese, *E. coli* behavior

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (**STEC**) is a pathotype of diarrheagenic *E. coli* frequently involved in foodborne infections worldwide. According to data reported by the European Centre for Disease Prevention and Control (ECDC, 2020), during the 2014 to 2018 period, the incidence of STEC infections in the European Union (**EU**) showed an increasing trend that peaked in 2018.

A considerable number of STEC serotypes can cause human illness, including hemorrhagic colitis, which may progress to severe complications such as hemolytic uremic syndrome, particularly in young children and the elderly, and thrombotic thrombocytopenic purpura. Among the STEC serotypes, O157:H7 has been the cause of many major outbreaks and sporadic cases, but strains belonging to non-O157 serogroups have been increasingly associated with cases of hemolytic uremic syndrome (Gonzalez-Escalona et al., 2019). In the EU, non-O157 serogroups are responsible for more than one-half of STEC illness (ECDC, 2018).

Ruminants are an important reservoir for STEC strains, which can contaminate milk from dairy animals (Gyles, 2007; Farrokh et al., 2013). The presence of these bacteria in milk is likely to arise from contamination by fecal material during the milking process.

Concern over the safety of milk (both raw and pasteurized) and raw milk cheeses has prompted research on the survival of STEC into these products, with most studies focusing on cow milk (Wang et al., 1997; Massa et al., 1999; Alhelfi et al., 2012) and also on how these bacteria behave during cow milk cheese manufacture and subsequent ripening and storage periods (Montet et al., 2009; Miszczycha et al., 2013; Peng et al., 2013; Bellio et al., 2018). Although raw sheep milk and sheep cheese made from unpasteurized milk can also be a vehicle for the transmission of *E. coli* O157:H7 and non-O157 STEC strains to humans (Caro et al., 2006; Rey et al., 2006; Caro and García-Armesto, 2007; Otero et al., 2017), the number of studies concerning the behavior of wild STEC isolates in sheep milk and

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sheep milk cheeses is limited (Ramsaran et al., 1998; Govaris et al., 2002).

In 2019, Spain accounted for 25% of the sheep population in the EU (Eurostat, 2020). Therefore, ewe breeding is an important activity that is concentrated particularly in the inner regions. Almost all of the sheep milk produced is used for cheesemaking (Ministry of Agriculture, Fisheries, and Food, 2019). Zamorano cheese is one of the most appreciated sheep cheeses in Spain. This is a variety made from raw milk of Churra and Castellana sheep breeds in the province of Zamora, in the autonomous community of Castilla y León. The minimum ripening period is 60 d for cheeses under 1.5 kg.

This study was undertaken to assess (1) the survival of wild-type STEC strains in sheep milk stored at different conditions (temperature and heat treatment), and (2) the *E. coli* O157:H7 behavior during the manufacturing and ripening of a Spanish hard sheep cheese (Zamorano style).

MATERIALS AND METHODS

No Institutional Animal Care and Use Committee (IACUC) approval by the University of León (Spain) was necessary, as sampling consisted only of acquiring bulk-tank raw milk from a farm, which follows EU hygiene requirements. This provision did not require contact with animals. Therefore, no animals were utilized for experimentation.

Strains Used for Inoculation Experiments

A collection of 50 STEC strains isolated in a previous work (Otero et al., 2017) from bulk-tank ewe milk collected from 388 farms was tested for 0.3% lactic acid resistance. Each strain was plated separately on Sorbitol MacConkey Agar, supplemented with Cefixime Tellurite Selective Supplement (Oxoid), and incubated at 37°C for 24 h. Then, one colony was inoculated into 10 mL of Tryptone Soya Broth (**TSB**; Oxoid) containing 0.3% lactic acid (Panreac Química, S.L.U.). The TSB with only lactic acid and the TSB with only the bacteria served as the controls. Cultures and controls were incubated at 37°C. After 3, 6, 9, and 24 h of incubation, a 200-μL aliquot of each culture was transferred to microplate wells and the optical density (**OD**) at 620 nm was measured using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific Inc.). All determinations were performed in duplicate. For inoculation experiments, strains were cultured overnight in TSB at 37°C and 10-fold serially diluted with 0.1% peptone (Oxoid) solution to obtain the required concentration.

Collection and Transport of the Ewe Milk

The raw ewe milk used was in accordance with the requirements established by the European Commission Regulation No. 853 of 2004 (European Parliament and Council, 2004) for milk from species other than cows intended for the manufacture of products made with raw milk. The milk was provided by the farm of the University of León, just milked and transferred to sterile bottles, which were kept in an isothermal box until the arrival at the laboratory (within an hour). Enriched milk samples were tested for the presence of *stx* genes (Otero et al., 2017) and stored under refrigeration conditions (4°C) until it was used.

Bacterial Counts in Raw and Pasteurized Ewe Milk Stored at Different Temperatures

Aerobic plate counts and lactic acid bacteria (**LAB**) were enumerated at each sampling day. Total counts were determined by plating 10-fold dilutions (1 mL) on Plate Count Agar (Oxoid) and incubating at 30°C for 48 h. The LAB were counted on overlaid plates of de Man, Rogosa, and Sharpe agar (Oxoid), incubated aerobically at 30°C for up to 72 h.

Behavior of STEC Strains in Raw and Pasteurized Ewe Milk Stored at Different Temperatures

Before inoculation with the strains M240VO or M294aVO, milk (4 L) was dispensed into sterile beakers. One-half was treated at 63°C for 30 min (low-temperature holding; **LTH**) in a water bath and the other half remained untreated and refrigerated.

Samples of raw and LTH milk were inoculated with the appropriate dilution of the tested strains (approximately 2 $\log_{10}$ cfu/mL). Six trials using fresh and LTH ewe milk were conducted: (1) raw milk inoculated with strain M240VO, (2) LTH milk inoculated with strain M240VO, (3) raw milk inoculated with strain M294aVO, (4) LTH milk inoculated with strain M294aVO, (5) noninoculated raw milk, and (6) noninoculated LTH milk.

For each trial, triplicate 20-mL samples of milk were held for 5 d at 3 specific temperatures and bacterial determination were carried out at each sample by duplicate. These requirements were established in the Regulation (EC) No 853/2004 (European Parliament and Council, 2004) in the following way: 6°C (maximum temperature for on-farm cooled milk if collection is not daily), 8°C (maximum temperature for on-farm cooled milk in the case of daily collection), and 10°C (maximum temperature on arrival at the establishment

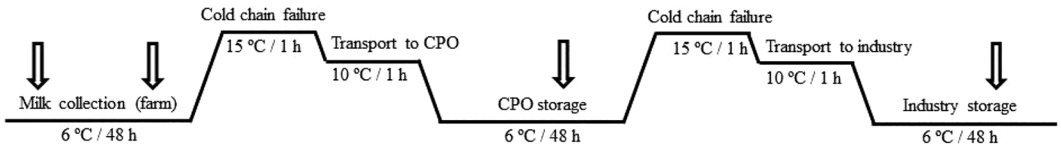

Figure 1. Flowchart of the cold chain break scenario. CPO = dairy cooperative society (Consorcio de Promoción del Ovino). Arrows indicate sampling points.

of destination). In addition, a simulation approach for assessing the effect of failures in the cold chain (**FCC**) was used based on data provided by a dairy cooperative society (Consorcio de Promoción del Ovino; **CPO**). The thermal profile (times and temperatures) is shown in Figure 1. Simulated failures were included during tank loading for transport from the farm to the CPO, and during milk loading for its transport from the CPO to a raw ewe milk cheese factory.

Temperatures were monitored using a Testo175-T2 (Instrumentos Testo S.A.) data logger with internal sensor and external probe with an accuracy of ±0.2°C, which was programmed to read every 10 min. Data were downloaded to a computer and exported to a spreadsheet for further analysis.

Noninoculated and inoculated raw and LTH milk samples (100 µL for each sampling) were serially diluted (1:10) in Peptone (Oxoid) solution and tested for STEC levels. Counts were carried out on SMAC agar (Oxoid) and CHROMagar STEC (Scharlab) for pasteurized milk samples and on CHROMagar O157 and CHROM agar STEC (Scharlab) for raw milk samples. On both chromogenic media, STEC strains grew in a mauve colony color. Plates were incubated at 37°C for 24 to 48 h. For LTH and raw milk kept at 6, 8, and 10°C and for the FCC simulating approach, samples were taken after 0, 2, 4, and 5 d of storage.

Manufacture and Sampling of Raw Milk Sheep Cheese (Zamorano Style)

Zamorano style cheeses from raw ewe milk obtained at the farm of University of León, as described above, were manufactured according to specifications of the Regulatory Council Queso Zamorano Protected Designation of Origin (Government of Spain, 1993). Three cheeses by each condition (spiked milk and noninoculated milk, respectively) were produced. A typical Zamorano cheese shows the following characteristics: fat content not less than 45% of the DM, minimum DM of 55%, minimum total protein of 25%, and pH value ranging from 5.1 to 5.8.

All cheeses were made on the same day and from the same batch of milk. The pH values were measured with a standard pH meter (Testo 205; Instrumentos Testo S.A.). An appropriate volume of cells of strain M240VO (serotype O157:H7) in Buffered Peptone Water (Oxoid) were added to pre-warmed (30°C) ewe milk and kept under constant gentle agitation to provide approximately 3 $\log_{10}$ cfu/mL. The inoculum was chosen as representative of the maximum level of *E. coli* O157:H7 cells that would be expected in fresh milk and ground beef patties (ICMSF, 2002; Schlesser et al., 2006).

Calcium chloride (Laboratorios Arroyo) was added to obtain a final concentration of 0.2 g/L. A commercial starter culture, containing *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp., *lactis* biovar *diacetylactis*, and *Streptococcus thermophilus* (Danisco Choozit LYO 05DCU MA4001) was prepared by incubating overnight in sterile 12% skim milk (Oxoid) at 32°C. The starter was added at the 1% level and the inoculated milk was kept for 30 min until the pH decreased by 0.1 units. Then a commercial rennet solution (Laboratorios Arroyo) was added (1:10,000) and coagulation took place in 40 min at 30 to 32°C. The curd was cut until it formed grains of 5 to 10 mm and was stirred for 30 min while gradually increasing the temperature up to 38°C. Afterward, the whey was drained and the curd was transferred to 3 cylindrical plastic molds. The cheese blocks were pressed (3.5 kg/cm^2) for 10 h until the pH reached approximately 5.5 and were salted by immersion in a 20% NaCl (wt/vol) solution for 4.5 h at 8 to 10°C. After salting, cheeses were left to dry for 24 h at room temperature. The cheeses were aged for 2 mo, in controlled chambers, at 10 to 12°C and 85 to 90% relative humidity. During ripening, cheeses were regularly turned. The same number of cheeses without added *E. coli* O157:H7 were also manufactured as controls.

For each cheese, the following samples, in duplicate, were taken: (1) from milk; (2) from curd after 40 min of pressing; (3) from the cheese after salting (4.5 h); (4) from the cheese after draining (1 d); and (5) throughout ripening on d 1, 7, 30, and 60.

Analysis of Cheese Samples

For each parameter and treatment, analyses were performed by duplicate from each cheese (n = 2 × 3).

Enumeration of E. coli O157:H7 and LAB. For enumeration of the *E. coli* O157:H7 strain in spiked ewe milk dedicated to cheesemaking, 100-μL aliquots of milk and decimal dilutions (1:10) in Peptone Water (Oxoid) were plated on CHROM agar O157 (Scharlab) and incubated at 37°C for 24 h. For curd and cheese, 10-g samples were blended in a BagMixer-400 blender (Interscience) with 40 mL of TSB containing 0.6% yeast extract (TSBYE, Oxoid), serially diluted (1:10) in 0.1% Peptone (Oxoid), and 400-μL aliquots streaked onto two 150-mm plates containing the same selective and differential medium (bacterial detection limit being estimated at 6.25 cfu/g). Plates were also incubated at 37°C for 24 h. The LAB counts were carried out as described above.

Physicochemical Determinations. Curd and cheese pH values were measured using a penetration electrode (Testo 205; Instrumentos Testo S.A.). The NaCl content was determined according to the Volhard method through the AOAC Official Method 935.43 (Bradley, 2006). The values of water activity ($\mathbf{a_w}$) were measured with an Aqualab water activity meter (model series CX2 T, Decagon Devices).

Statistical Analysis

Bacterial counts were transformed and expressed as $\log_{10}$ colony-forming units per milliliter or $\log_{10}$ colony-forming units per gram. Basic descriptive statistics of each parameter (mean and SD) were calculated and linear regression analysis was used to determine the relationship between parameters. The ANOVA test was employed to determine the effects of several factors in each experimental design: (1) strain, incubation time, and OD values (lactic acid resistance); (2) strain, milk treatment, temperature, storage time, and STEC counts (inoculated milk); and (3) pH values, a_w, and salt content, and LAB and *E. coli* O157:H7 counts (cheesemaking). Subsequently, post hoc pairwise comparisons were performed through Fisher's least significant difference test. All statistical analyses were performed using Statistica version 14.0 for Windows (StatSoft Europe).

RESULTS

Strains Used for Inoculation Experiments

When testing for lactic acid resistance, 2 STEC strains (M240VO and M294aVO) were selected based on their ability to grow in the presence of 0.3% lactic acid as shown by their significant photometric measures ($P < 0.05$). Strain M240VO belonged to serotype O157:H7 and carried the *stx2*, *hlya*, and *eae* genes.

Table 1. Behavior of 2 Shiga-toxin-producing *Escherichia coli* (STEC) strains in pasteurized (LTH) and raw ewe milk stored at different temperatures and during simulation of cold chain failure (FCC)[1]

		Strain M240VO (*E. coli* O157:H7)				Strain M294bVO (*E. coli* STEC non-O157)			
Milk	ST[2]	0 d	2 d	4 d	5 d	0 d	2 d	4 d	5 d
LTH[3]	6°C	2.13 ± 0.09^{a}	$<1.00^{a}$	$<1.00^{a}$	$<1.00^{a}$	2.17 ± 0.17^{a}	1.90 ± 0.35^{a}	2.38 ± 0.15^{b}	2.94 ± 0.07^{c}
	8°C	2.14 ± 0.12^{a}	2.33 ± 0.21^{a}	2.45 ± 0.38^{a}	2.57 ± 0.30^{a}	2.10 ± 0.15^{a}	2.62 ± 0.16^{b}	3.18 ± 0.20^{c}	3.43 ± 0.05^{c}
	10°C	2.08 ± 0.16^{a}	2.90 ± 0.25^{b}	3.31 ± 0.44^{b}	4.37 ± 0.17^{c}	1.97 ± 0.14^{a}	3.25 ± 0.25^{b}	2.40 ± 0.42^{c}	3.24 ± 0.15^{b}
	FCC	2.06 ± 0.07^{a}	1.28 ± 0.70^{b}	2.06 ± 0.54^{c}	2.42 ± 0.27^{c}	1.96 ± 0.21^{a}	2.26 ± 0.20^{a}	3.04 ± 0.06^{b}	3.29 ± 0.08^{b}
Raw	6°C	1.82 ± 0.26^{a}	$<1.00^{a}$	1.38 ± 0.33^{b}	$<1.00^{a}$	1.92 ± 0.12^{a}	$<1.00^{a}$	$<1.00^{a}$	$<1.00^{a}$
	8°C	1.65 ± 0.19^{a}	1.88 ± 0.15^{a}	2.20 ± 0.24^{a}	2.49 ± 0.06^{b}	1.92 ± 0.17^{a}	1.82 ± 0.34^{a}	1.87 ± 0.21^{a}	1.95 ± 0.24^{a}
	10°C	1.94 ± 0.15^{a}	2.66 ± 0.10^{b}	1.80 ± 0.25^{a}	1.93 ± 0.17^{a}	1.81 ± 0.18^{a}	1.44 ± 0.35^{a}	$<1.00^{a}$	$<1.00^{a}$
	FCC	2.04 ± 0.08^{a}	$<1.00^{b}$	1.88 ± 0.20^{b}	1.52 ± 0.81^{b}	1.82 ± 0.11^{a}	$<1.00^{a}$	$<1.00^{a}$	$<1.00^{a}$

[a–c]For each strain, mean values within a row with different superscripts are significantly different ($P < 0.05$).
[1]Mean ± SD, average of 6 determinations ($\log_{10}$ cfu/mL). Means ranged between 1 and 2 $\log_{10}$/mL and must be considered as estimated counts (under the range of 10–250 colonies per plate). Lower limit of enumeration: 1 $\log_{10}$ cfu/mL.
[2]ST = storage temperature.
[3]LTH = low-temperature holding (30 min/63°C).

Strain M294aVO was STEC non-O157 and bore the *stx1* and *hlya* genes (Otero et al., 2017).

Detection of STEC (stx Genes) in Raw Milk

All the tested enriched samples were negative for the presence of *stx* genes.

Behavior of Strains M240VO and M294aVO in Raw and Pasteurized Ewe Milk Stored at Different Temperatures

The behavior of the STEC O157:H7 and non-O157 *E. coli* selected strains in LTH and raw ewe milk stored at different temperatures and during FCC simulation is shown in Table 1. Counts of both strains were significantly ($P < 0.05$) affected by milk treatment (LTH or fresh), storage conditions (temperature and FCC), and storage time.

For strain M240VO (serotype O157:H7) in LTH ewe milk, numbers after 5 d of storage significantly ($P < 0.05$) decreased at 6°C and significantly ($P < 0.05$) increased at 10°C and during FCC simulation. At 8°C, there was a nonsignificant increase ($P > 0.05$). For strain M294aVO (non-O157) in LTH ewe milk, counts after 5 d of storage significantly ($P < 0.05$) increased under all storage conditions.

In raw ewe milk, counts of strain M240VO (serotype O157:H7) after 5 d of storage decreased (6°C and FCC simulation), increased (8°C), or remained constant (10°C). For strain M294aVO (non-O157), cell numbers significantly ($P < 0.05$) decreased at 6°C, 10°C, and during FCC simulation. When held at 8°C, there was a nonsignificant increase ($P > 0.05$) in cell numbers.

The numbers of aerobic mesophilic microbiota and LAB in ewe milk at the different tested conditions are shown in Table 2. Total counts only significantly decreased during the storage of LTH milk at 6°C and 8°C. Milk had 1.21 ± 0.12 $\log_{10}$ cfu/mL LAB after the pasteurization treatment, whereas LAB reached 3.81 ± 0.07 $\log_{10}$ cfu/mL in raw milk. This bacterial population significantly ($P < 0.05$) increased in all studied conditions after 5 d of storage except at 6°C and 8°C in LTH milk.

Behavior of Strain M240VO (Serotype O157:H7) During Manufacture and Ripening of a Raw Milk Sheep Cheese (Zamorano Style)

Changes in numbers of *E. coli* strain M240VO (O157:H7 serotype) during the manufacture and ripening of a Zamorano style cheese, as well as the evolution of LAB counts, pH and a_w values, and NaCl contents are given in Table 3.

Table 2. Changes in aerobic plate counts and lactic acid bacteria (LAB) numbers in noninoculated pasteurized (LTH) and raw ewe milk stored at different temperatures and during simulation of cold chain failure (FCC)[1]

Milk	ST[2]	Aerobic plate count				LAB			
		0 d	2 d	4 d	5 d	0 d	2 d	4 d	5 d
LTH[3]	6°C	2.53 ± 0.14^{a}	1.46 ± 0.07^{b}	1.50 ± 0.12^{b}	1.87 ± 0.19^{c}	1.21 ± 0.12^{a}	1.40 ± 0.06^{b}	1.45 ± 0.05^{b}	1.25 ± 0.30^{a}
	8°C	2.53 ± 0.14^{a}	1.56 ± 0.16^{b}	1.22 ± 0.27^{b}	1.62 ± 0.13^{b}	1.21 ± 0.12^{a}	1.58 ± 0.08^{b}	1.44 ± 0.06^{c}	1.10 ± 0.16^{a}
	10°C	2.53 ± 0.14^{a}	2.47 ± 0.19^{a}	2.77 ± 0.08^{b}	3.83 ± 0.14^{c}	1.21 ± 0.12^{a}	2.65 ± 0.02^{b}	2.89 ± 0.06^{c}	3.36 ± 0.09^{d}
	FCC	2.53 ± 0.14^{a}	1.46 ± 0.32^{b}	1.26 ± 0.31^{b}	3.11 ± 0.06^{c}	1.21 ± 0.12^{a}	1.55 ± 0.07^{b}	1.45 ± 0.08^{c}	1.35 ± 0.23^{d}
Raw	6°C	4.85 ± 0.08^{a}	6.01 ± 0.41^{b}	7.21 ± 0.12^{c}	7.67 ± 0.06^{d}	3.81 ± 0.07^{a}	4.65 ± 0.08^{b}	5.08 ± 0.05^{c}	5.76 ± 0.09^{d}
	8°C	4.85 ± 0.08^{a}	6.26 ± 0.02^{b}	7.86 ± 0.08^{c}	8.17 ± 0.03^{d}	3.81 ± 0.07^{a}	4.87 ± 0.41^{b}	5.46 ± 0.03^{c}	6.04 ± 0.07^{d}
	10°C	4.85 ± 0.08^{a}	7.56 ± 0.07^{b}	7.90 ± 0.13^{c}	8.33 ± 0.04^{d}	3.81 ± 0.07^{a}	7.23 ± 0.07^{b}	7.82 ± 0.03^{c}	8.10 ± 0.06^{d}
	FCC	4.85 ± 0.08^{a}	6.30 ± 0.13^{b}	7.24 ± 0.21^{c}	7.76 ± 0.04^{d}	3.81 ± 0.07^{a}	4.46 ± 0.07^{b}	4.82 ± 0.27^{c}	6.08 ± 0.05^{d}

$^{a-d}$For each bacterial count, mean values within a row with different superscripts are significantly different ($P < 0.05$).
[1]Mean ± SD, average of 6 determinations ($\log_{10}$ cfu/mL). Lower limit of enumeration: 1 $\log_{10}$ cfu/mL.
[2]ST = storage temperature.
[3]LTH = low-temperature holding (30 min/63°C).

Table 3. Behavior of *Escherichia coli* O157:H7 strain M240VO during the manufacture and ripening of a raw milk sheep cheese (Zamorano style) and development of lactic acid bacteria (LAB), pH, water activity (a_w), and NaCl content[1]

Sampling	pH	a_w	NaCl	LAB (log_{10} cfu/g)	*E. coli* O157:H7[2] (log_{10} cfu/g)
Spiked milk	6.51 ± 0.01[a]	1.000 ± 0.001[a]	ND[3]	ND	2.79 ± 0.09[a]
Curd	6.41 ± 0.01[b]	0.996 ± 0.001[b]	ND	8.17 ± 0.18[a]	3.43 ± 0.21[b]
After salting	5.41 ± 0.02[c]	0.985 ± 0.003[c]	1.73% ± 0.01[a]	9.40 ± 0.06[b]	3.72 ± 0.05[c]
After drying	5.38 ± 0.03[d]	0.980 ± 0.003[d]	1.71% ± 0.04[a]	9.38 ± 0.16[b]	3.73 ± 0.05[c]
1 d of ripening	5.34 ± 0.01[e]	0.976 ± 0.001[e]	1.60% ± 0.04[b]	9.36 ± 0.07[b]	3.17 ± 0.26[d]
7 d of ripening	5.35 ± 0.03[e]	0.967 ± 0.002[f]	1.67% ± 0.10[ab]	9.17 ± 0.09[c]	2.95 ± 0.27[d]
30 d of ripening	5.32 ± 0.01[f]	0.962 ± 0.002[g]	1.70% ± 0.05[a]	9.34 ± 0.02[d]	0.98 ± 0.32[e]
60 d of ripening	5.30 ± 0.01[g]	0.955 ± 0.001[h]	1.94% ± 0.04[c]	9.27 ± 0.10[d]	0.80 ± 0.01[e]

[a–h]Mean values within a column with different superscripts are significantly different ($P < 0.05$).

[1]Mean ± SD, average of 6 determinations. For *E. coli* O157:H7 counts, means ranging from 0.80 to 1.10 log_{10}/g must be considered as estimated counts (under the range 10–250 colonies per plate). Estimated lower limit of detection: 0.80 log_{10} cfu/g.

[2]*E. coli* O157:H7 strain M240VO.

[3]ND = not determined.

After ripening for 2 mo, physicochemical parameters complied with specifications of the Regulatory Council, Queso Zamorano PDO (Government of Spain, 1993). A negative correlation ($P < 0.05$) was found between the NaCl content and both pH ($r = -0.99$) and a_w ($r = -0.82$) values.

The LAB counts significantly increased ($P < 0.05$) after salting and then decreased by 0.13 log_{10} units at the end of ripening. A significant ($P < 0.05$) increase of *E. coli* strain M240VO (O157:H7 serotype) also occurred during the time between inoculation and after drying. Afterward, mean numbers decreased to an estimated concentration of 0.80 ± 0.01 log_{10} cfu/g after 2 mo of ripening. Significant ($P < 0.05$) relationships between data were only found for levels of *E. coli* strain M240VO and a_w values.

DISCUSSION

Behavior of Strains M240VO and M294aVO in Raw and Pasteurized Ewe Milk Stored at Different Temperatures

Storage temperature is an important extrinsic factor that influences the survival and growth of pathogenic and nonpathogenic *E. coli* strains in milk and other substrates (ICMSF, 1996; Alhelfi et al., 2012). Several authors (Kauppi et al., 1996; Palumbo et al., 1997; Wang et al., 1997; Massa et al., 1999; Alhelfi et al., 2012) have studied the behavior of *E. coli* O157:H7 strains in sterilized, UHT, pasteurized, and unpasteurized cow milk at different temperatures, but little is known about the growth and survival of O157 and non-O157 strains in milk from other species.

Previous studies carried out in pasteurized and UHT cow milk have shown the ability of some strains of *E. coli* O157:H7 to grow or maintain their numbers at temperatures ranging between 8°C and 12 to 15°C and to a lesser extent in raw milk (Kauppi et al., 1996; Palumbo et al., 1997; Wang et al., 1997; Massa et al., 1999). At 5°C, the bacterium did not grow and the population decreased; therefore, holding milk at ≤5°C has been recommended to prevent the growth of this pathogen in cow milk. The significant differences of the behavior of *E. coli* O157:H7 in raw and heat-treated cow milk have been attributed to the antagonistic activity of the larger population of background flora of the raw milk and, perhaps, to the natural antimicrobials compounds such as the lactoperoxidase system also present in raw milk (Wang et al., 1997; Alhelfi et al., 2012).

Results obtained in our study for strain M240VO show the ability of a wild *E. coli* O157:H7 strain to a significant increase by 0.84 log_{10} units after 5 d of storage in raw ewe milk at 8°C, which is the legal maximum temperature allowed in the EU for raw milk in the case of daily collection. A nonsignificant increase of 0.43 log_{10} units in LTH milk was also observed at the same temperature. In both LTH and raw ewe milk, the M240VO strain decreased at 6°C. At 10°C and during the FCC simulation, the response of the strain was likely related to the antagonistic activity of the background microbiota (Tables 1 and 2).

In LTH ewe milk, strain M294aVO (non-O157) grew at 6°C and above, as well as during the FCC simulation. It has been reported that 13 STEC strains (O157 and non-O157) grew at 6.5°C in cow milk autoclaved at 121°C for 15 min (Kauppi et al., 1996). Our data are in accordance with those from the latter authors, who concluded that the minimum temperature of growth for STEC strains in milk is between 5.5°C and 6.5°C. It has been shown that some STEC strains have genetic mechanisms that allow their growth at low temperatures (Vidovic et al., 2011). In raw ewe milk, numbers of this strain remained constant at 8°C but significantly

decreased at 6°C, 10°C, and during FCC simulation, although viable cells were detected at the end of the storage. It appears that the M240VO (O157) strain was more susceptible to cold stress than the STEC non-O157 strain, but the former was a better competitor with the microbial population of the unpasteurized ewe milk. The transcription of the *rpoS* gene is a key mechanism of the general stress response in *E. coli*. This response may be different among STEC strains, even more variation in behavior in cheese matrix, but more scientific evidences should be provided (Peng et al., 2011).

In conclusion, the minimum growth temperature in both LTH and raw ewe milk for the O157 *E. coli* strain M240VO was 8°C. For the non-O157 *E. coli* STEC strain M294aVO, the minimum growth temperature in LTH ewe milk was 6°C, but in raw ewe milk it did not grow at any of the tested conditions.

Behavior of Strain M240VO (Serotype O157:H7) During Manufacture and Ripening of a Raw Milk Sheep Cheese (Zamorano Style)

The presence of *E. coli* O157:H7 in raw ewe milk (Caro et al., 2006; Rey et al., 2006; Otero et al., 2017) raises the possibility of its survival in raw sheep milk cheeses (Caro and García-Armesto, 2007; Baylis, 2009). Cheesemaking involves several different steps such as curd formation, draining, salting, and ripening, although the exact process depends on the cheese variety. Most authors have reported that STEC counts generally increase during the initial phase of cheesemaking (due to growth, cell concentration, or both, during curd formation) and decrease during ripening (Peng et al., 2011; Farrokh et al., 2013). This is in accordance with our results because after whey drainage, there was an increase of 0.94 $\log_{10}$ cfu/g and, afterward, the numbers decreased during ripening by 2.93 $\log_{10}$ cfu/g.

Data on the growth and survival of pathogenic bacterial species in raw milk cheeses vary greatly depending on the processing conditions, stress (acidic, osmotic, and so on) response abilities of the tested strains, and the analytical methods used (Peng et al., 2011). Therefore, it is difficult to compare results obtained from different studies. The viability of *E. coli* O157:H7 during manufacture, ripening, and storage of different cheese varieties was reviewed extensively by Farrokh et al. (2013). Most studies show a decrease of this bacterium during ripening and storage, although the rate of this decline will vary. It should be noted that some works reported that this pathogen survived for long time and even increased in Camembert and Feta cheeses (Ramsaran et al., 1998) or in Pecorino cheese (Centorotola et al., 2021). In contrast, other data show that the bacterium was absent or only detectable after enrichment in French raw milk cheeses (Vernozy-Rozand et al., 2005) or in Gouda and Cheddar cheeses (D'Amico et al., 2010). Farrokh et al. (2013) concluded that the rate of inactivation of *E. coli* O157:H7 during cheese ripening and storage mainly depends on the strain and cheese type.

The growth and survival of pathogenic *E. coli* in cheese and other foods depend on several intrinsic and extrinsic factors (ICMSF, 1996). Table 3 shows that in Zamorano cheese, the physicochemical parameters and the levels of LAB followed the expected pattern during the manufacture and ripening of a hard cheese variety; additionally, after 2 mo of ripening at 10 to 12°C, numbers of the spiked *E. coli* O157:H7 strain had declined from approximately 3 $\log_{10}$ cfu/g to an estimated concentration of 0.8 $\log_{10}$ cfu/g. Our data show that after draining, LAB numbers were always higher than 9 $\log_{10}$ cfu/g, and after ripening, pH and a_w values significantly decreased and NaCl content significantly increased (Table 3). It is well known that the survival of STEC strains during ripening and storage of raw milk cheeses may be affected by different microbial hurdle factors such as low pH and a_w values, NaCl concentration and antimicrobial agents produced by the milk, autochthonous microbiota, and starter culture organisms (Farrokh et al., 2013).

The estimated infectious dose of *E. coli* O157:H7 causing human illness ranges from <10 to 100 bacterial cells. This low infectious dose and the severity of the O157:H7-associated disease makes this enteric pathogen among the most hazardous foodborne bacteria (Gonzalez-Escalona et al., 2019).

In this study, after a ripening period of 2 mo at 10 to 12°C, numbers of *E. coli* O157:H7 found in Zamorano cheese manufactured with inoculated raw ewe milk (approximately 10^3 cfu/mL) were estimated at 6.25 cfu/g. If we consider consumer consumption of at least 1 cheese slice (12 g), this would result in the ingestion of 75 cfu of *E. coli* O157:H7, which is in the range of the minimum infectious dose. The concept of infectious dose can be generally understood as the number of individual cells below which the probability of infection is insignificant. However, this explanation cannot be suitable for *E. coli* O157:H7 as its infectivity appears to be negligible on exposure to a simple bacterial cell. This statement is supported by the low levels of the pathogen reported in a variety of outbreaks (FAO and WHO, 2018; Gonzalez-Escalona et al., 2019).

In our experience, it is unlikely that ewe milk contains approximately 3 $\log_{10}$ cfu/mL of *E. coli* O157:H7 (Otero et al., 2017), but this inoculum level was chosen on the basis of data regarding the worst-case scenario in minced meat and milk (ICMSF, 2002; Schlesser et

al., 2006). Nevertheless, considering the low infectious dose, strategies for reducing human exposure to *E. coli* O157:H7 from consumption of raw milk cheeses include targeted interventions at the farm and the cheese industry.

In summary, our results show that although STEC O157 levels decrease during 2 mo of ripening, the Zamorano cheese manufactured from raw ewe milk contaminated with approximately 3 $\log_{10}$ cfu/mL of *E. coli* O157: H7 is likely to contain the bacterium at levels that are known to cause illness.

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