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Farm management factors with potential effect on culturable microbiota in bulk tank milk from sheep farms

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Introduction: Ensuring high microbiological quality in sheep milk is crucial for public health, product quality, and the sustainability of traditional dairy systems. This study investigated the association between the bacteriological quality of bulk tank milk and key management and productive factors.

Methods: A total of 308 sheep's milk samples from 77 farms were analyzed for major microbial groups using culture methods, and a farm survey was conducted to characterize management practices. Factor analysis identified four distinct groups of culturable bacteria that collectively explained 64.9% of the total variance in the model. These groups were characterized as: (i) environmental and hygienic bacterial counts, (ii) thermotolerant bacterial counts, (iii) bacterial counts associated with udder health, and (iv) spore-forming bacteria counts.

Results: The first factor, which accounted for the largest proportion of explained variance, showed strong positive associations among lactic acid bacteria, Gram-positive cocci, *Pseudomonas* spp., *Escherichia coli*, coagulase-negative staphylococci, and coliforms counts. The study also revealed that flock and seasonal variations are statistically associated with the distribution of groups of culturable bacteria, highlighting their potential use in the qualitative monitoring of bulk tank milk for cheesemaking.

Conclusion: Higher culturable bacteria prevalence was observed in smaller, low-producing flocks, particularly those exposed to environmental contamination or suboptimal hygiene. Microbial counts decreased with regular replacement of milking filters. Feeding practices were also associated with culturable bacteria, with higher counts in hay-fed flocks and greater contamination linked to the use of wet by-products. These findings highlight how seasonality and farm management practices shape the microbial quality of milk intended for cheesemaking.

KEYWORDS

bulk tank milk, culture methods, differential microbiology, factor analysis, farm management factors, sheep

1 Introduction

Sheep milk has a high content of fat, protein, lactose, minerals, and vitamins, as well as a high-water activity and near-neutral pH (Balthazar et al., 2017; Flis et al., 2025), and therefore serves as a valuable source of nutrients for both humans and animals. Across all dairy species, milk composition and yield are primarily influenced by genetic background, environmental conditions, and dietary factors (Legarra et al., 2014; Renes et al., 2020).

Milk produced by a healthy sheep's mammary gland typically contains a low microbial load (Quigley et al., 2013). Nevertheless, contamination can occur during and after the milking process, as milk may be exposed to a wide range of microorganisms present on the teat surface, air, feed, milking equipment, and during on-farm management practices (Tonamo et al., 2009; Quigley et al., 2013; Gonzalo, 2017; Gonzalo et al., 2019; Quintana et al., 2020), leading to undesirable changes in both raw milk and its dairy products. Microbial contamination significantly compromises the quality, and technological suitability of milk for further processing.

The implications of microbial contamination in sheep milk extend beyond food safety and quality, particularly in regions where dairy sheep farming constitutes a cornerstone of the local economy and culture. This is especially relevant in Mediterranean and Black Sea areas, where sheep milk and its derivatives represent a staple in the diet and a key economic resource (Ripoll-Bosch et al., 2012; Ramón et al., 2016; Mohamed-Brahmi et al., 2024; Gonzalez-Ronquillo et al., 2025). Within this context, countries such as Greece, Italy, and Spain are especially prominent, being collectively responsible for approximately 60% of global sheep cheese production (FAO, 2024). This broader regional importance of sheep dairy production is reflected at a national level in Spain, where the breed Manchega plays a central role in the dairy sector and the adaptive evolution of production systems in response to economic and technological advancements. In recent years, production systems have undergone significant evolution, characterized by an increase in average farm size and the incorporation of animals genetically selected for milk production. These changes have led to modifications in the production system, particularly in critical aspects such as feeding, hygienic management of milk collection and preservation, and udder health, among others (Rivas et al., 2015; Morantes et al., 2022).

Ensuring high microbiological quality of sheep milk is essential not only for protecting public health, but also to maintain the viability of traditional production systems and to support sustainability of rural communities that depend on them. The socio-economic relevance and nutritional value of sheep milk products are closely tied to their hygienic quality, making the control of microbial contamination a critical aspect of dairy production (Boyazoglu and Morand-Fehr, 2001; Ahmad et al., 2025). Therefore, a comprehensive assessment of microbial risks throughout the milk production chain is fundamental to ensure product quality and preserve the safety and integrity of both raw milk and dairy products (Quigley et al., 2013; Yang et al., 2025). At this respect, Regulation (CE) No. 854/2004 (European Union, 2004) identifies the total mesophilic bacterial count (SPC), measured at 30°C, as a key parameter for assessing microbiological quality of

sheep milk. However, several authors argue that this parameter lacks specificity, as evidenced by various studies focused on differential microbial counts in cow (Jayarao et al., 2004; Elmoslemany et al., 2009; Vacheyrou et al., 2011; O'Grady et al., 2020) and sheep bulk tank milk (De Garnica et al., 2011; De Garnica et al., 2013; Arias et al., 2024).

There is a limited number of studies exploring the relationship between microbial contamination of bulk tank sheep milk and farm management practices (Gonzalo et al., 2019; Poveda et al., 2020; Quintana et al., 2020; Caraba and Caraba, 2023). Therefore, this study aims to investigate the association between the bacteriological quality of bulk tank sheep milk in Manchega flocks and key productive and management factors at the farm level.

2 Material and methods

2.1 Study design and sample determination

The research was conducted in sheep farms located in the region of Castilla-La Mancha, Spain (38°–41°N, 1°–5°W), a predominantly flat region in central Spain that covers approximately 800,000 hectares. Land use is mostly agricultural, with 70% dedicated to arable farming, primarily rainfed crops, while forests and natural pastures account for 20% and 10%, respectively. The region has a continental Mediterranean climate, characterized by dry winters, hot summers, and irregular rainfall concentrated in autumn and spring. Annual rainfall ranges from 400 to 1,000 mm, with average temperatures of 5°C in winter and 25°C in summer (Caballero and Fernández-Santos, 2009).

A total of 308 milk samples were collected from 77 Manchega sheep farms were analyzed for major microbial groups by using culture methods, which were certified under the Protected Designation of Origin (PDO) "Queso Manchego" (PDO Queso Manchego, 2023). These farms represent approximately a 15% of those officially registered under this PDO. The average flock size was 1,144 ewes, with farms ranging from 150 to 5,500 animals. Mean annual milk yield per ewe was 143 kg, with values ranging from 91 to 200 kg, over an average lactation period of 121 days (ranging from 100 to 150 days).

All dairy farmers were asked about their willingness to participate in the study and were surveyed to obtain comprehensive data on their management systems, including the number of ewes, milk yield, health and sanitary status of the animals, feeding regimes, and routine maintenance practices. Flocks were visited quarterly (once per season), for the collection of 308 bulk tank milk samples. Each sample comprised milk from the morning milking on the day of the visit, and that from the evening milking of the previous day. Samples were collected in hermetically sealed sterilized containers and maintained at 4°C during transport to the Experimental Dairy Science and Cheese Laboratory at the Regional Centre for Animal Selection and Reproduction (CERSYRA-IRIAF, Valdepeñas, Ciudad Real, Spain). Upon arrival, samples were aliquoted and distributed to specialized laboratories for subsequent analyses.

2.2 Laboratory analysis

Microbiological analyses were conducted at CERSYRA-IRIAF within 24 hours of milk sampling to ensure data reliability. The procedures employed adhered to standardized methods, previously established and published by our research group (Jiménez et al., 2024).

Serially diluted samples into sterile physiological saline serum were inoculated onto different culture media for specific bacterial counts. Total mesophilic bacterial count (SPC) was assessed on PCA (Plate Count agar) media (Panreac, Barcelona, Spain) following incubation under aerobic conditions at 30°C for 72 hours (ISO 4833-1:2013, 2013). Thermophilic bacteria (THERMO) were counted on the same medium and under identical incubation conditions as SPC, but milk samples had been previously pasteurized at 62.8°C for 30 minutes (ISO 4833-2:2013, 2013). Psychrotrophic bacteria (PSYCHRO) were counted on PCA plates and incubated at 6.5°C for a period of 10 days (ISO 17410:2019, 2019). For *Pseudomonas* spp. (PSEUDO), counts on Cetrimide agar (Panreac, Spain) plates were used and were incubated at 35°C for 48 hours (Dore et al., 2007). *Escherichia coli* (ECOLI) and other coliforms (COLI) were quantified using CromoID™ Coli medium (bioMérieux, Madrid, Spain) with incubation at 37°C for 24 hours (ISO 16649-2:2001, 2001). Enumeration of Gram-positive catalase-negative cocci (GPCNC) was carried out on modified Edwards medium supplemented with colistin and oxolinic acid (Oxoid, Basingstoke, Hampshire, UK) and incubated at 35°C for 48 hours (De Garnica et al., 2013). Lactic acid bacteria (LAB) were cultivated on MRS agar (Panreac, Barcelona, Spain), acidified to pH 5.7, and plates incubated at 30°C for 72 hours (ISO 15214:1998). The count of lactate-fermenting *Clostridium* spores (BAB) (Oxoid, Basingstoke, Hampshire, UK) was carried out using the most probable number (MPN) method in Bryant and Burkey Broth (BBB, Merck, Darmstadt, Germany) (USDA. United States Department of Agriculture, 2003). For the quantification of coagulase-positive (CPS) and coagulase-negative staphylococci (CNS), Baird–Parker RPF agar (bioMérieux, Madrid, Spain) was employed, with incubation at 37°C for 24 hours (Jayarao et al., 2004). Counts were expressed as colony-forming units per milliliter of milk (CFU/mL), except for the *Clostridium* spores that were reported as number of spores/mL. All microbial counts underwent logarithmic transformation (\log_{10}) to normalize the data distribution.

2.3 Statistical analysis

Factor analysis (FA) was used to identify patterns of common variation in the counts of microorganisms in milk and synthesize the information contained in the set of variables observed, called latent common factors (Garzón et al., 2021). Principal components analysis was used as an extraction method to calculate the factorial scores of each sample of milk, and varimax rotation was applied to maintain orthogonality of the extracted factors (Rivas et al., 2015). The factors scores were calculated using the coefficients from the rotated factor matrix as follows:

$$F_{ij} = P_{i1} X_{1j} + P_{i2} X_{2j} + \dots + P_{in} X_{nj}$$

where F_{ij} is the factor score of the sample j in the factor i , P_{in} is the factor ponderation of the variable n in the factor i , and X_{nj} is the score of the sample j in the original variable n . Following the Kaiser criterion, only factors with eigenvalues greater than one were extracted (Giorgis et al., 2011). The communality of each variable was also calculated as the proportion of the variance that can be explained by the factorial model obtained. The analysis was interpreted from a biological point of view, observing the saturation coefficients of the original variables with the extracted factors rotated. A loading threshold of $|0.40|$ was used to determine whether a variable contributed meaningfully to a given factor.

The individual factor scores calculated for each milk sample were analyzed using the following linear mixed model (Gonzalo et al., 2019):

$$y_{ij} = \beta_0 + \text{season}_j + u_i + e_{ij}$$

where y_{ij} is the factor score, season is a fixed effect with four levels, u_i is the random effect of flock ($u_i \sim N(0, \sigma_{\text{flock}}^2)$), and e_{ij} is the residual error ($e_{ij} \sim N(0, \sigma_e^2)$). Variance components were estimated using REML, and model assumptions were checked using the Shapiro-Wilk test for residual normality and the Breusch-Pagan test for homoscedasticity. Interaction terms between flock and season were not included because the data structure did not provide replication within flock-season combination (Cipolat-Gotet et al., 2018).

Finally, the relationships between the primary flock characteristics and the common latent factors defining the patterns of variation in microorganism counts were examined using ANOVA, or Student's t-tests for variables with two levels. Tukey's HSD test was applied to identify homogeneous groups. Previously, the quantitative variables were categorized into four-level ordinal variables based on quantiles of 25% (Table 1).

Due to missing values in certain variables, the effective sample size decreased from 308 to 245 complete observations. All statistical analyses were performed using the software Statgraphics Centurion

TABLE 1 Description of flock characteristics used in ANOVA, comprising original qualitative and categorized quantitative variables.

Variable	Categories
Milk (L/year)	< 55,000; 55,000-100,000; 100,000-190,000; > 190,000
Number of ewes	< 460; 461-713; 714-1,250; > 1,251
Density (ewes/m ²)	< 0.53; 0.54-0.67; 0.68-0.85; > 0.85
Calving interval (days)	< 292; 293-343; 344-383; > 384
Replacement rate (%)	< 24.95; 24.95-25.64; 25.65-30.33; > 30.34
Number of milking places	≤ 19; 20-26; 27-35; ≥ 36
Milk pipeline height	high line; low line
Milk filter change frequency	after each milking; daily
Automatic cluster removal	yes; no
Vacuum cut clusters	yes; no
Use of acid to clean the milking machine	dairy; no dairy

XV (StatPoint Technologies Inc., Warrenton, VA, USA). Figure 1 was generated with R 4.4.1 (ggplot2, ggrepel, dplyr, stringr packages).

3 Results and discussion

3.1 Differential microbiological characterization of bulk tank milk

Table 2 shows the descriptive statistics for the different microbial counts analyzed in bulk tank milk.

Mean value for SPC was $5.68 \pm 0.77 \log_{10}$ CFU/mL, consistent with findings from other studies on sheep milk (De Garnica et al., 2013). This enumeration is routinely performed using automated equipment as part of standard quality control protocols. It serves as a key indicator of hygienic conditions during on-farm milk collection. In addition, it shows a lower coefficient of variation (CV) than both psychrotrophic and thermophilic bacterial counts.

The PSYCRO group encompasses a diverse array of bacterial genera with significant technological relevance in the dairy industry, primarily due to their lipolytic and proteolytic enzymatic activities (Poveda et al., 2004; Decimo et al., 2014), as well as of sanitary concern because of the potential presence of *Pseudomonas* spp (de Almeida et al., 2017).; SPC and PSEUDO have similar coefficients of variation (Table 2). The mean concentration of PSYCRO was $5.25 \pm 1.08 \log_{10}$ CFU/mL, a value lower than those documented in previous studies of dairy sheep milk (De Garnica et al., 2011; Fotou et al., 2011). These microorganisms thrive under refrigeration conditions, which can lead to their proliferation during prolonged storage of milk. In addition, the likelihood of growth of these bacteria is further increased when hygienic practices during milk collection are suboptimal (Oh and Lee, 2024; Yalew et al., 2024).

The count for PSEUDO was $3.14 \pm 0.84 \log_{10}$ CFU/mL, comparable to findings in other studies on sheep milk in Greece and Italy (Kondyli et al., 2012; Faccia et al., 2015), though lower than those reported in studies on cow's milk ($4.47 \log_{10}$ CFU/mL

(Pinto CL de et al., 2006). These bacteria are able to form biofilm, allowing them to persist for extended periods throughout the milking system (including milking machines, pipelines, and tanks), and to proliferate rapidly in the tank due to their high physiological and genetic adaptability. This results in a reduction in shelf-life and changes in coagulation properties. It can also lead to the development of bitter or rancid flavors in milk. These effects are mainly due to the bacteria's ability to produce proteolytic and lipolytic enzymes (Jayarao and Wang, 1999; Spiers et al., 2000; Richter and Vedamuthu, 2001; De Jonghe et al., 2011; Martin et al., 2021).

The count of THERMO was $3.25 \pm 0.86 \log_{10}$ CFU/mL, which is higher than that reported in a previous study on dairy sheep milk (De Garnica et al., 2011). The presence of THERMO has been associated with the hygienic condition of the milking equipment and the milking parlor. These microorganisms, which are capable of withstanding high temperatures, are of technological relevance in cheese production (Bhanduriya et al., 2022).

Several environmentally derived bacterial groups identified in milk—namely, LAB, GPCNC, and BAB—demonstrated comparable coefficients of variation (Table 2). The LAB count was $4.90 \pm 0.72 \log_{10}$ CFU/mL, similar to that reported by Onofre e Silva et al (Onofre e Silva et al., 2020), and higher than that reported by Centi et al (Centi et al., 2017). LAB is the most important bacterial group in the milk of healthy sheep (Toquet et al., 2021). These microorganisms play a crucial role in cheese-making technology, being essential for the cheese fermentation process and the development of the typical flavors and aromas (Montel et al., 2014). However, negative effects have also been reported, such as the production of biogenic amines (Quigley et al., 2011; Renes et al., 2019).

The count of GPCNC was $4.16 \pm 0.81 \log_{10}$ CFU/mL, similar to that reported by Alexopoulos et al. (2011) ($4.95 \log_{10}$ CFU/mL), and higher than that reported by De Garnica et al. (2013) ($2.95 \log_{10}$ CFU/mL). This broad group of microorganisms includes species of hygienic concern (e.g., enterococci and streptococci), which are associated with intramammary infections in small ruminants (Bergonier et al., 2003; Marogna et al., 2010). Additionally, it comprises bacteria of technological importance in milk

TABLE 2 Descriptive statistics of microbiological quality variables, expressed as \log_{10} CFU/mL, for bulk tank milk samples (n = 245) from sheep.

Variable	Description	Mean	SD	CV	P25	P75
SPC	Standard plate count	5.68	0.77	13.56	5.19	6.17
PSYCRO	Psychrotrophs	5.25	1.08	20.57	4.57	5.95
THERMO	Thermophilic	3.25	0.86	26.46	2.73	3.72
LAB	Lactic acid bacteria	4.90	0.72	14.69	4.37	5.36
GPCNC	Gram-positive catalase-negative cocci	4.16	0.81	19.47	3.61	4.80
PSEUDO	<i>Pseudomonas</i> spp.	3.14	0.84	26.75	2.65	3.77
ECOLI	<i>Escherichia coli</i>	1.68	1.14	67.86	0.00	2.31
COLI	Coliforms others than <i>Escherichia coli</i>	2.93	1.17	39.93	2.44	3.67
CNS	Coagulase-negative staphylococci	4.32	0.55	12.73	4.10	4.53
CPS	Coagulase-positive staphylococci	2.50	1.37	54.80	1.95	3.47
BAB	Lactate-fermenting <i>Clostridium</i> spores	3.41	0.62	18.18	2.96	3.63

Descriptive statistics: SD, standard deviation; CV, coefficient of variation; P25, 25th percentile; P75, 75th percentile.

coagulation and characteristic cheese aroma development (Franz, 2003).

BAB represent spore-forming bacteria that constitute a main technological challenge for the cheese industry, the so-called “late blowing” especially in pressed cheeses (Visser et al., 2006). Risk factors for milk contamination include the use of silage and moist by-products in the diet feeding strategy of dairy sheep, as well as inadequate hygiene in the milking parlor. In our study, the BAB count was $3.41 \pm 0.62 \log_{10}$ spores/mL, which is consistent with results previously reported by our group in milk from sheep of the same breed under comparable feeding conditions (Arias et al., 2013).

The *Escherichia coli* and coliforms other than *Escherichia coli* counts were $1.68 \pm 1.14 \log_{10}$ CFU/mL, and $2.93 \pm 1.17 \log_{10}$ CFU/mL, respectively, with both of them being lower than those reported in previous studies (De Garnica et al., 2011). The coefficient of variation for ECOLI (CV = 67.86%) was higher than for the remaining microorganisms analyzed, highlighting that the presence of more specific pathogens depends on particular sources of contamination rather than the general microbial background. Coliforms in milk are considered a reliable indicator of on-farm hygiene conditions (e.g., bedding, flooring, contaminated water, and inadequate cleaning and disinfection systems) (Elmoslemany et al., 2009). Total coliform counts are currently being used in quality control systems for sheep milk in certain regions of the European Union (e.g., Roquefort and the Pyrénées-Atlantiques), where milk is scored or classified according to different coliform count thresholds (Pirisi et al., 2007).

Lastly, the CPS count was $2.50 \pm 1.37 \log_{10}$ CFU/mL, which is consistent with values reported in other studies on dairy sheep milk (De Garnica et al., 2011). These bacteria are responsible for clinical mastitis in dairy sheep. However, their incidence is lower than that of coagulase-negative staphylococci (CNS). CNS are the main pathogens associated with subclinical mastitis in sheep flocks (Gonzalo et al., 2002). In fact, the value for CNS count was higher than that for CPS ($4.32 \pm 0.55 \log_{10}$ CFU/mL), with their respective coefficients of variation reaching values of $CV_{CPS} = 54.80\%$ and $CV_{CNS} = 12.73\%$. Both clinical and subclinical mastitis cause significant economic losses for sheep farms. These losses result from reduced milk yield, higher replacement rates, and increased veterinary costs. Mastitis also leads to a deterioration in milk quality. This is largely due to the release of enzymes such as the plasmin-plasminogen complex, which destabilize and degrade caseins, ultimately impairing milk coagulation (Caballero-Villalobos et al., 2018).

3.2 Factor analysis

Bartlett’s test of sphericity was significant ($P < 0.001$), and the KMO (Kaiser-Meyer-Olkin) value was 0.79, indicating that the database is suitable for FA (Köbrich et al., 2003). FA extracted four factors with eigenvalues greater than one, which accounted for 64.9% of the total variance (Table 3). The variance explained by FA exceeded 70% in four bacterial count groups (4 out of 11), ranged between 60% and 70% in two (2 out of 11), between 50% and 60% in three (3 out of 11), and between 40% and 50% in two (2 out of 11).

TABLE 3 Factors extracted and saturation coefficients with the original variables after varimax rotation ($n = 245$).

Variable	F ₁	F ₂	F ₃	F ₄	Commonality
LAB	0.73	0.36	0.07	0.11	0.68
GPCNC	0.69	0.29	0.13	-0.11	0.59
PSEUDO	0.66	0.17	0.29	-0.18	0.58
ECOLI	0.65	-0.04	-0.18	-0.03	0.46
CNS	0.61	-0.08	-0.30	0.22	0.52
COLI	0.55	0.22	0.34	-0.02	0.46
SPC	0.30	0.83	0.04	0.00	0.79
PSYCRO	0.21	0.82	0.17	-0.07	0.75
THERMO	0.20	0.75	-0.23	0.13	0.66
CPS	0.11	-0.02	0.84	0.09	0.72
BAB	-0.04	0.05	0.07	0.96	0.93
Variance (%)	34.03	11.62	9.83	9.42	-
Eigenvalue	3.75	1.28	1.08	1.03	-

LAB, lactic acid bacteria; GPCNC, gram-positive catalase-negative cocci; PSEUDO, *Pseudomonas* spp.; ECOLI, *Escherichia coli*; CNS, coagulase-negative staphylococci; COLI, coliforms other than *Escherichia coli*; SPC, total mesophilic bacteria (standard plate count); PSYCHRO, psychrotrophs; THERMO, *thermodurics*; CPS, coagulase-positive staphylococci; BAB, lactate-fermenting *Clostridium* spores. The factors represent the following bacterial count groups: F₁, environmental and hygienic bacterial counts; F₂, thermotolerant bacterial counts; F₃, bacterial counts associated with udder health; and F₄, spore-forming bacterial counts.

The bold values indicate the percentage of variance explained by each bacterial count group within each of the four factors.

The lowest proportions of variance explained by the factor model corresponded to ECOLI and COLI, with a common variance of 46%.

The first factor (F₁) accounted for the largest proportion of variance (34.03%) and revealed a positive relationship among the counts of LAB, GPCNC, PSEUDO, ECOLI, CNS, and COLI, constituting the group of bacterial counts indicative of hygiene and environmental conditions (Group EH₁; environmental and hygienic bacterial counts). A previous study performed by our research team (Jiménez et al., 2018) also reported positive and significant correlations among these bacterial count groups ($r = 0.31$ – 0.51). This heterogeneous group of microorganisms has different sources of contamination, but they exhibit a common statistical covariation pattern that explains their behavior in raw milk, as reported in other studies. For example, *Escherichia coli* serves as an indicator of fecal contamination, whereas LAB and GPCNC are primarily environmental (Jayarao et al., 2004; De Garnica and Gonzalo, 2013). These findings demonstrate the intricate association among bacterial counts originating from both intra-mammary and environmental sources post-milking. Certain groups, such as lactic acid bacteria and specific Gram-positive cocci not classified as enterococci, are considered beneficial in the context of cheesemaking. Conversely, the presence of microorganisms like coliforms and *Pseudomonas* spp. is notable for raising significant hygienic and sanitary concerns due to their association with potential spoilage and reduced milk quality (Ávila et al., 2025).

The second factor (F₂) explained 11.62% of the total variance and was positively correlated with the counts of SPC, THERMO, and PSYCRO bacteria, constituting a broad group differentiated by their optimal growth temperature (Group TT₁; thermotolerant bacterial count). This finding offers valuable insight into the

resistance and resilience characteristics of the microorganisms present in bulk tank milk. Samples exhibiting higher scores for this factor contained greater counts of these microbial groups, with significant and positive correlations observed among them ($r = 0.41\text{--}0.64$) (Jiménez et al., 2018). These results further corroborate the relationships previously reported in bovine bulk tank milk (Jayarao et al., 2004).

The third factor (F_3) explained 9.83% of the total variance and was strongly correlated only with CPS. This group is associated with specific and virulent bacterial species characterized by their ability to produce the coagulase enzyme, among which *Staphylococcus aureus* is the most representative (Roşu et al., 2025). The fourth factor (F_4) explained 9.42% of the total variance. It was strongly and exclusively related to BAB, an indicator associated with a bacterial group characterized by its ability to form spores and subsequently germinate into vegetative, gas-producing cells during cheese production. Both types of bacteria (CPS and BAB) are distinguished from the others by the specific issues they cause, which are frequently linked to particular farm management practices. Previous works from our group demonstrated that environmental and hygienic bacterial groups (SPC, LAB, COLI, CGPCN) are interrelated, but not with CPS or BAB (Jiménez et al., 2018). This indicates that routine total germ counts at 30°C, commonly used in bulk tank milk quality control, fail to inform about farm conditions related to microbial groups linked to clinical mastitis or 'late blowing'.

3.3 Variation of bacteria in bulk tank milk

A linear mixed-effects model was applied to evaluate the influence of flock and season on the common latent factors identified through factor analysis (FA). Residual normality and homoscedasticity for all four mixed models were supported by the Shapiro–Wilk and Breusch–Pagan tests, respectively. Variance component estimates indicated that flock contributed notably to the variability of the latent factors. The proportion of variance attributable to flock was 41% for F_1 ($\sigma^2 = 3.01$), 46% for F_2 ($\sigma^2 = 2.96$), 29% for F_3 ($\sigma^2 = 0.40$), and 28% for F_4 ($\sigma^2 = 0.30$), with the remaining variance corresponding to within-flock residual variability. Results are presented in Table 4. Overall, the model demonstrated an acceptable and statistically significant fit ($p < 0.001$), with adjusted coefficients of determination ranging from 51.5% for F_1 to 31.5% for F_4 . Figure 1 presents the seasonal variations observed for the different factors.

Group EH_b (F_1) was significantly affected by flock and season. Season was the most important factor in this relationship. Values tend to increase in summer and decrease in winter–spring, a circumstance that many studies have attributed to greater microbial growth under poor hygienic conditions during the summer months (Elmoslemany et al., 2010; Zucali et al., 2011). In this regard, the high counts of PSEUDO, ECOLI, and COLI in summer are likely related to hygienic deficiencies in the management and cleaning of the milking parlor and bulk milk tank, as well as to an increased risk of contamination from water or soil during this season (De Jonghe et al., 2011; Ribeiro Júnior et al.,

TABLE 4 Mixed models result for effects of flock and season on the extracted factors ($F_1\text{--}F_4$; $n = 245$).

Factor	Flock	Season	Model
F_1			
<i>F-value</i>	3.20	30.98	4.28
<i>P-value</i>	< 0.001	< 0.001	< 0.001
<i>Adjusted R²</i>			51.50
F_2			
<i>F-value</i>	3.74	2.98	3.66
<i>P-value</i>	< 0.001	0.03	< 0.001
<i>Adjusted R²</i>			46.30
F_3			
<i>F-value</i>	2.32	5.34	2.47
<i>P-value</i>	< 0.001	0.002	< 0.001
<i>Adjusted R²</i>			32.25
F_4			
<i>F-value</i>	5.65	2.23	2.42
<i>P-value</i>	0.001	< 0.001	< 0.001
<i>Adjusted R²</i>			31.52

The factors represent the following bacterial count groups: F_1 , environmental and hygienic bacterial counts; F_2 , thermotolerant bacterial counts; F_3 , bacterial counts associated with udder health; and F_4 , spore-forming bacterial counts.

2018). BAL also shows significantly higher counts in summer, consistent with the findings of other authors (Nalepa et al., 2018).

Group TTb (F_2) was significantly affected, in decreasing order, by flock and season, with values tending to decline in spring and to rise in summer–autumn. Several studies have attributed this pattern to milk storage time and refrigeration temperature (Jayarao and Wolfgang, 2003; de Oliveira et al., 2015). In general, milk collection from farms is carried out more frequently during periods of higher milk production, which typically occurs in spring.

Group CPS (F_3) was significantly affected by season and flock, in that order. Values were significantly higher in spring–summer than in autumn–winter. The prevalence of these bacteria is related to the lactation status of the animals (Gonzalo et al., 2002); therefore, the distribution of lactations throughout the year and the mixing of milk from ewes at different stages of lactation in the bulk tank may condition the seasonality of their counts. In any case, some studies performed in dairy goats indicate that summer temperature and humidity conditions favor the increase of staphylococci (Tombarkiewicz et al., 2009). Moreover, farming practices exert a significant influence on CPS occurrence. Management measures such as the removal of infected animals from the flock, implementation of an appropriate milking order, maintenance of proper milking hygiene, adequate post-milking teat disinfection, and the application of dry-off therapy have been demonstrated to effectively prevent the onset of new intramammary infections (Contreras et al., 2007).

Group BAB (F_4) was significantly affected by flock and season (in this order of importance). Values were significantly higher in spring–summer than in autumn–winter. Some authors attribute

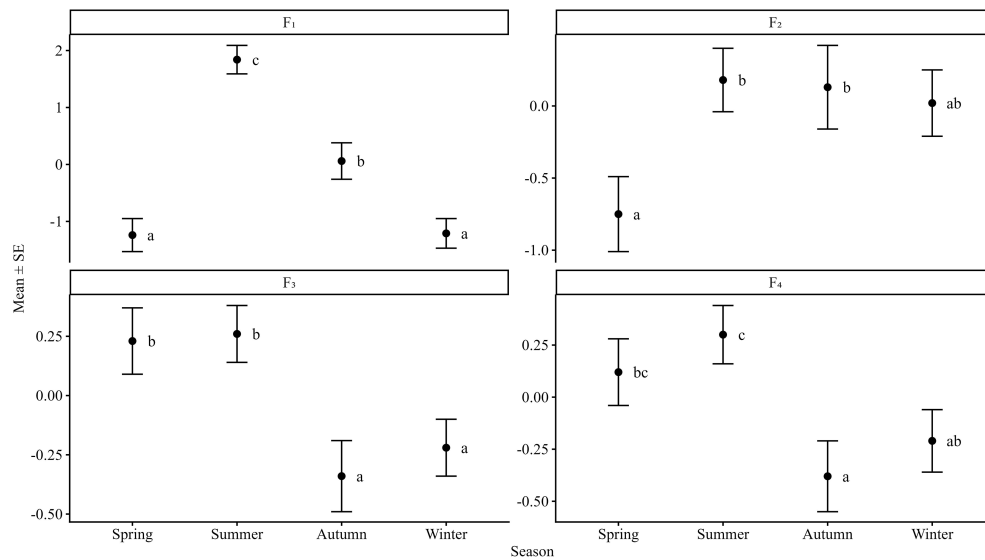


FIGURE 1

Least squares means and standard errors (SE) of the extracted factors (F₁–F₄) for different seasons estimated in the mixed models (n = 245). The factors represent the following bacterial count groups: F₁, environmental and hygienic bacterial counts; F₂, thermotolerant bacterial counts; F₃, bacterial counts associated with udder health; and F₄, spore-forming bacterial counts. Different superscripts (a–c) within each quadrant of the graph indicate significant differences (P < 0.05).

variations in spore counts to differences in dairy sheep diet, particularly increased silage and moist by-products. Feed ingredient choices vary substantially based on price fluctuations (Colombari et al., 2005). This could explain the discrepancies with some previous studies (Arias et al., 2013) that did not observe significant seasonal differences in these counts in bulk milk from Manchega sheep.

3.4 Associations between flock characteristics and the variation of bacterial counts in bulk tank milk

Flock had a significant effect on all extracted factors (Table 4) in relation to the culturable microbial community present in the bulk tank milk of Manchega sheep. Different production systems and management-related factors influence the microbial composition of bulk tank milk in cattle, goats, and other dairy sheep breeds (Quigley et al., 2013). Table 5 shows flock characteristics—production level, size, and management practices—significantly associated with extracted factors linked to bulk tank milk's main microbial groups.

Milk production level and flock size showed an association with TTb (F₂), which groups bacterial counts according to their thermal tolerance (SPC, THERMO, and PSYCRO). These serve as indicators of bacteria more prevalent in smaller flocks with lower production levels. In flocks producing less than 100,000 liters/year, the counts of these bacterial groups were higher than those with greater production levels. However, other studies found no significant differences between microbial counts and production level, which was attributed to the management conditions of the different production systems studied (Caraba and Caraba, 2023). Similarly, higher counts were observed in flocks with fewer than 460 ewes compared to larger flocks. A study performed on sheep also

reported a decrease in total microbial counts in larger flocks, indicating that those producers had more likely implemented safer practices and greater professionalism (Alexopoulos et al., 2011). This might be related to an enhanced milk cooling capacity or to improved hygienic management of animal housing, as previously reported in dairy cows (Hayes et al., 2001; Jayarao and Wolfgang, 2003; Driehuis, 2013).

Conversely, ewe housing density in milking facilities exhibited a significant association with CPS (F₃), with flocks maintained at lower densities (<0.67 ewes/m²) showing reduced counts of coagulase-positive staphylococci. These results and their implications should be interpreted through the lens of udder health in dairy sheep farming, distinguishing between flock size (total number of ewes) and housing density (ewes/m²). Some previous works also noted the risk that high animal densities, often associated with inadequate ventilation and high relative humidity, may lead to an increase in staphylococcal counts (Bergonier et al., 2003).

The frequency of milking filter replacement significantly affected bacterial counts in three of the groups considered, Ehb (F₁), TTb (F₂), and BAB (F₄). Most of these counts indicate environmental bacteria associated with inadequate hygiene, with levels decreasing upon milking filter replacement after each session. This finding is consistent with previous studies in dairy cattle, which have associated hygienic management of milking equipment with an increase in the microorganisms grouped under Ehb (F₁) (SPC (Hayes et al., 2001; Jayarao and Wolfgang, 2003; Wallace, 2008; Driehuis, 2013; Twomey et al., 2025); TTb (F₂) (COLI (Jayarao et al., 2004); PSEUDO (Fagundes et al., 2006); and BAB (F₄) (Vissers and Driehuis, 2009). Other authors have also reported that milking parlor filters harbor high numbers of microorganisms, some of which are of major concern for food safety, such as *Listeria monocytogenes* (Latorre et al., 2009; Pantoja et al., 2012). Therefore, their replacement after each milking constitutes good practice to

TABLE 5 Means and standard deviations of the extracted factors (F₁-F₄; n=245) according to flock characteristics showing significant effects based on ANOVA or student's t-tests (only significant results are shown).

Categorical factors	F ₁	F ₂	F ₃	F ₄
Milk (liters/year)				
< 55,000	ns	0.26 ± 0.30 ^a	ns	ns
55,000-100,000	ns	0.55 ± 0.34 ^a	ns	ns
100,000-190,000	ns	-0.47 ± 0.26 ^b	ns	ns
> 190,000	ns	-0.41 ± 0.38 ^b	ns	ns
Number of ewes				
< 460	ns	0.89 ± 0.36 ^a	ns	ns
461-713	ns	-0.06 ± 0.32 ^b	ns	ns
714-1,250	ns	-0.48 ± 0.26 ^b	ns	ns
> 1,251	ns	-0.22 ± 0.35 ^b	ns	ns
Density (ewes/m²)				
< 0.53	ns	ns	-0.31 ± 0.17 ^b	ns
0.54-0.67	ns	ns	-0.39 ± 0.12 ^b	ns
0.68-0.85	ns	ns	0.01 ± 0.15 ^a	ns
> 0.85	ns	ns	0.06 ± 0.17 ^a	ns
Milking filter replacement				
After each milking	-0.23 ± 0.20	-0.36 ± 0.17	ns	-0.04 ± 0.17
Daily	1.08 ± 0.73	1.40 ± 0.44	ns	0.48 ± 0.19
Vacuum shut-off				
Yes	ns	ns	ns	-0.06 ± 0.08
No	ns	ns	ns	0.43 ± 0.15
Post-milking teat disinfection				
Yes	ns	-0.32 ± 0.20	ns	-0.12 ± 0.09
No	ns	0.44 ± 0.28	ns	0.22 ± 0.11
Dry-off sheep treatment				
No	0.35 ± 0.27	0.36 ± 0.21	ns	0.11 ± 0.09
Yes	-0.79 ± 0.27	-0.86 ± 0.24	ns	-0.20 ± 0.10
Type of forage				
Hay	ns	0.12 ± 0.17	ns	ns
Silage	ns	-1.15 ± 0.53	ns	ns
Feeding management by production groups				
No	0.38 ± 0.27	0.54 ± 0.22	0.14 ± 0.10	0.12 ± 0.09
Yes	-0.41 ± 0.28	-0.65 ± 0.23	-0.18 ± 0.12	-0.12 ± 0.11
Feeding wet by-products				
No	-0.28 ± 0.25	-0.29 ± 0.22	ns	-0.35 ± 0.09
Yes	0.46 ± 0.32	0.48 ± 0.16	ns	0.47 ± 0.09

The factors represent the following bacterial count groups: F₁, environmental and hygienic bacterial counts; F₂, thermotolerant bacterial counts; F₃, bacterial counts associated with udder health; and F₄, spore-forming bacterial counts. a, b: means with different letters differ significantly ($p < 0.05$).

safeguard milk quality, particularly in sheep milking, where pre-dipping is not performed.

Similarly, shutting off the vacuum before liner removal showed a significant association with BAB (F₄). Butyric acid bacteria increased in bulk tank milk when vacuum shut-off was not performed, a finding that has also been reported in previous studies on the Manchega breed (Arias et al., 2013; Quintana,

2021). Other authors found that the use of a specific type of commercial liners with automatic valves, without applying vacuum shut-off before detaching the milking unit, increased somatic cell counts and slightly elevated the risk of infection, particularly at the beginning of lactation (Manzur et al., 2018).

Post-milking teat disinfection proved significant for TTb (F₂) and BAB (F₄). Culturable bacteria associated with TTb (F₂) (SPC,

THERMO, and PSYCHRO) were lower when this practice was routinely implemented. In dairy cattle, bacterial counts in milk increase due to dirty teats and udders (Hayes et al., 2001; Jayarao and Wolfgang, 2003), and it has been reported that post-milking disinfection reduces the bacterial counts on the surrounding skin (Bramley et al., 1996). Along these lines, the reduction of BAB (F_4) associated with this practice can be explained, both in sheep (Arias et al., 2013) and in dairy cattle (Visser and Driehuis, 2009), has been indicated that all aspects related to farm hygiene are linked to the decrease in butyric spores. In cattle, the reduction in teat microbial counts associated with teat hygiene practices is even more evident (Gleeson et al., 2018), since in this species pre-dipping cleaning of teats is routinely carried out, unlike in small ruminants (Gelasakis et al., 2015). However, in the present study, no significant association was found between post-milking teat disinfection and the CPS factor (F_3) related to coagulase-positive staphylococci, as reported in other studies on Manchega sheep (Gómez et al., 1997). This could be attributed to the fact that CPS are characterized by dynamic fluctuations and cyclical shedding in milk (Katsafadou et al., 2019).

The application of dry-off treatment in ewes was significant for the variation of EHb (F_1), TTb (F_2), and BAB (F_4). The use of intramammary dry-off treatments at the end of lactation is associated with lower counts of these groups of bacteria, which are mostly of environmental and hygienic origin (De Garnica and Gonzalo, 2013). Currently, European Union legislation (European Union, 2019) imposes restrictions on the use of antibiotic treatments in animal production. Nevertheless, the findings of this study demonstrate that the implementation of dry-off therapy in ewes effectively reduces bacterial counts in bulk tank milk. These results underscore the need to establish stringent protocols for this preventive practice, incorporating continuous monitoring of subclinical mastitis status throughout lactation (e.g., somatic cell counts and microbiological analyses), as well as the development of safe and effective pharmaceutical agents for its application. Such measures are essential to mitigate a challenge with implications for both animal health and public health.

The type and management of feeding in dairy ewes also influenced the microbiological quality of bulk tank milk. The use of hay or silage proved significant for the variation of TTb (F_2), with higher counts observed in hay compared to silage for this group, which includes MESO, THERMO, and PSYCHRO bacteria. Similarly, studies performed on cow milk (Vacheyrou et al., 2011) reported that hay harbors a specific profile of these microbial counts, particularly *Eurotium* spp., mesophilic and thermophilic *Actinomycetaceae*, as well as Gram-positive (*Curtobacterium* spp., *Bacillus* and *Paenibacillus* spp.) and Gram-negative rods (*Pantoea* and *Pseudomonas* spp.). By contrast, silage feeding has generally been associated with increased bacterial counts in milk, particularly lactic acid bacteria (LAB), because of the fermentative nature of silage and the direct transfer of microorganisms from feed to milk. Nevertheless, the specific bacterial groups observed and their impact on milk quality depend on multiple factors, including the type of silage, the use of inoculants, and seasonal conditions, which may modulate microbial dynamics in bulk tank milk (Kennang Ouamba et al., 2022).

Feeding management of dairy ewes by milk production groups showed a significant association with all extracted factors related to

bacterial counts (EHb [F_1], TTb group [F_2], CPS [F_3], and BAB [F_4]). All of them decreased with the implementation of this practice, with the variation being greater in the EHb and TTb groups than in the CPS and BAB groups. Group-based feeding according to milk yield in dairy ewes requires highly controlled herd management, a practice generally found in more specialized and larger-scale farms, as highlighted by several studies in small ruminants (Milán et al., 2011; Louta et al., 2023; Gonzalez-Ronquillo et al., 2025).

The use of wet by-products in the feeding of dairy ewes was significant for EHb (F_1), TTb (F_2), and BAB (F_4). Culturable bacteria from these groups increase in bulk tank milk when wet by-products are used. These feedstuffs, generally derived from the food industry (e.g., brewers' grains, citrus pulp), are inexpensive but highly susceptible to microbial contamination due to their high water and nutrient content (Moura da Silva et al., 2024). Several studies across various species and breeds have reported the influence of diet type on milk's microbiological quality (Quigley et al., 2013; Skeie et al., 2019). These investigations particularly highlight the increased prevalence of butyric acid bacteria (BAB) spores in farms utilizing wet by-products, which are the primary cause of late blowing defects in pressed cheeses (Arias et al., 2016).

Other latent factors related to flock management were not significant in this study regarding the variation in bacterial group counts in bulk tank sheep milk. These include calving interval, replacement rate, number of milking places, milk pipeline height, automatic cluster removal, use of acid for cleaning the milking machine, and vaccination against gangrenous mastitis or contagious agalactia.

This study has some methodological limitations. The categorization of continuous farm variables, commonly used in milk-quality and udder-health research to facilitate comparisons and avoid assuming linear relationships, may reduce statistical power and should be considered exploratory (Pegolo et al., 2022). Additionally, multiple statistical tests were performed without formal corrections for multiple comparisons, increasing the risk of false-positive results. The sampling design, based on a single observation per flock-season combination, limits the ability to reliably estimate interactions between season and management factors. Culture-based methods may also underestimate microbial diversity by missing viable but non-culturable organisms, highlighting the potential value of complementary metagenomic approaches. Consequently, the associations reported here must be interpreted in this context, and future studies with larger sample sizes, repeated seasonal sampling, and refined statistical approaches are warranted.

4 Conclusions

This study confirms that the combined evaluation of total and differential microbiological counts, as well as bulk tank somatic cell counts, is an effective approach for characterizing the hygienic and sanitary quality of raw ewe's milk. The results reveal complex associations between beneficial microbiota relevant to

cheesemaking and contamination-associated bacterial groups that may compromise both milk safety and technological performance. Notably, routine total germ counts at 30°C, widely used in milk quality control programs, do not adequately reflect farm conditions related to microbial groups associated with clinical mastitis or late blowing defects, such as coagulase-positive staphylococci (CPS) and butyric acid bacteria (BAB).

Seasonal variation and flock-specific characteristics significantly influenced factors that group microbial counts, reinforcing their potential value as indicators for the qualitative monitoring of bulk tank milk. Elevated bacterial counts were consistently observed in smaller, low-producing flocks, particularly in farms facing greater environmental contamination or inadequate hygiene conditions. Conversely, regular replacement of milking filters was associated with lower microbial counts, emphasizing the relevance of effective milking system management. Furthermore, feeding practices contributed to differences in microbial composition, with higher bacterial counts detected in hay-based systems and increased contamination associated with the use of wet by-products. Overall, these findings underscore the critical role of farm management and seasonality in determining the microbiological quality of milk intended for cheesemaking and provide a scientific basis for developing targeted strategies to improve milk hygiene and technological suitability, despite the limitations arising from the use of culture-based methods. Therefore, it would be interesting to use this study as a basis and complement it with future metagenomic studies to draw more accurate conclusions about the microbiology of bulk tank milk.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements it did not involve experimental procedures on live animals. Milk samples from sheep were collected as part of routine farm management and standard milking practices, with no additional handling, intervention, or distress imposed on the animals beyond normal husbandry procedures. Therefore, according to institutional and national regulations on animal welfare, this research falls outside the scope of studies requiring ethical committee approval.

Author contributions

LJ: Conceptualization, Data Curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing –

original draft, Writing – review & editing; BO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing; MLJP: Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing; JPM: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review & editing; SS: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – review & editing; JCV: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing; JMP: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing; AG: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing; RA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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