Microbial wash water quality on dairy farms from Galicia (NW Spain)
F. J. Saavedra, E. Yus and F. J. Diéguez

ABSTRACT
This study evaluated the microbiological quality of wash water used in dairy farms from Galicia (NW Spain) based on the total coliform, faecal coliform and faecal streptococci counts. The paper aimed to identify geographical areas that are at a high risk for bacterial contamination of wash water and to study the temporal distribution of positive samples. Water samples from 120 farms, whose water supply came from a private well, were tested for the presence of the three mentioned bacterial groups by the membrane filtration method. To investigate whether the presence of positive herds for a particular bacterial group in water samples was higher in some areas or during a specific time period, data were tested using the Bernoulli model. In farms without a purification system, the prevalence of positive samples for total coliform, faecal coliform and faecal streptococci was 36.7%, 13.3% and 24.5%, respectively. In farms using hydrogen peroxide, these figures were 15.4%, 7.7% and 7.7%, respectively. In farms using chlorine or ultraviolet radiation all the samples were negative for the three bacterial groups. The spatial analysis identified one statistically significant spatial cluster of herds with positive samples of faecal streptococci in an area characterized by a high cattle density. Moreover, one most likely temporal cluster was identified for each of the three groups between July and August 2017, which were the driest months in the studied area.

Key words | dairy cows, faecal coliform, faecal streptococci, spatial and temporal clustering, total coliforms, wash water

INTRODUCTION
Microbiological quality of water is often assessed using indicator organisms whose concentration or density is usually associated with health risks. The World Health Organization recommends *Escherichia coli* (*E. coli*) as an ‘essential parameter’ of minimum water monitoring (WHO 2011). Quantifying faecal indicators plays a significant role in informing regulators and environmental managers of the levels of faecal microbial contamination and the hygienic status of water resources (Muirhead et al. 2011; Oliver et al. 2016). Thereby, the bacterial contamination of runoff water has traditionally been assessed using counts of selected bacterial groups, such as total coliforms (TC), faecal coliforms (FC), faecal streptococci (FS) or intestinal enterococci (*Hooda et al. 2000*).

In the European Union, legislation on microbiological water quality does not indicate specific parameters for water used in farms. In Spain, water for farming activities should have quality standards equivalent to drinking water and should contain 0 colony forming units (UFC) of coliforms, *E. coli* and enterococci in 100 ml of water (BOE 2003). On the other hand, people living in rural areas, where farming activities are settled, mainly depend on groundwater, often untreated, for their drinking water (*Krolik et al. 2013; Won et al. 2013; Krolik et al. 2014; Invik et al. 2017*).
As regards bacterial contamination of bulk tank milk, and despite growing knowledge about food safety, only a few studies have focused on determining the risk factors that can affect it (Cerva et al. 2014). The most consistent sources of bacterial contamination of raw milk seem to be inadequate pre-milking udder preparation, milk cooling and storage protocols, and improperly cleaned milking and cooling equipment surfaces (Elmoslemany et al. 2009a, 2009b, 2010; Perkins et al. 2009; Bava et al. 2011; Cerva et al. 2014). Inadequate surface disinfection could occur as a result of using contaminated water for washing (Perkins et al. 2009). In addition, water hardness minerals can react with cleaning agents and reduce their cleaning efficiency (Elmoslemany et al. 2009c).

Galicia, where the present study was carried out, is the main dairy cattle area of Spain with 55% of the farms and 38% of the milk production. The mean herd size is 42 cows, being lower than the national average (which is 59.3 cows) and still with family owned and managed herds prevailing (MAPAMA 2018). To date, no previous studies have explored the microbiological quality of water used in dairy farms either in Galicia or in other Spanish regions.

The first objective of the present study was to evaluate the microbiological quality of wash water used in dairy farms from Galicia (NW Spain) based on the TC, FC, and FS counts and to determine whether there exists an association between this microbiological quality and the bulk tank milk bacterial counts. Second, the study aimed to identify geographical areas that are at a high risk for bacterial contamination of wash water in Galicia and to study the temporal distribution of positive samples.

**METHODS**

**Study area and herds surveyed**

The present study was carried out in Galicia (NW Spain) throughout 2017. A total of 148 dairy farms were involved. This sample was convenient and included all farms that were enrolled in a food safety programme developed by a particular dairy industry. They were geographically distributed throughout the main milk production areas of the region. The mean herd size of these herds was 41 cows (SD = 38.30), with a minimum of nine cows and a maximum of 245.

**Sampling and testing**

Initially, the 148 farms involved were categorized based on the origin of their water sources: public water supply or water from a private well (and in this case, whether the farm had a water purification system). On those farms whose water supply came from a private well, a water sample was collected from the taps of the milk houses and subsequently tested for the presence of TC, FC, and FS. Point-of-use sampling was used to simulate the actual water-use practices on the farms, and no sterilization of the taps was performed.

A minimum of 100 mL of water samples was collected in sterile capped plastic containers, following strict aseptic procedures. The water samples were kept in an ice-box and transported to the analysis laboratory within 6 h of collection for bacteriological analysis.

Detection and enumeration of TC, FC, and FS were determined by the membrane filtration method (PNT/001 and PNT/003) (UNE EN ISO 7899/9 2000; UNE EN ISO 9508-1 2014). Results were expressed as colony forming units/mL (CFU/100 mL). For each bacterial group, samples with 0 CFU/100 mL were defined as negative, and all others were defined as positive.

In addition, for each farm, the total bulk tank milk bacterial count (BC) was tested using flow cytometry on the BactoScan FC (Foss, DK) at the Galician Laboratory of Milk Analysis (LIGAL). These data represent the average BC for the month in which the water sample was collected. Results were also expressed as CFU/100 mL.

**Statistical analysis**

Initially, Fisher exact tests were performed (using SPSS 15.0) to compare the prevalence of positive samples for TC, FC, and FS in farms whose water supply came from a private well, depending on the use of a water purification system.

Spearman’s rank correlation coefficients (ρ) were also generated to assess associations with the CFU counts of the three different bacterial groups in the water samples and the milk BC. For this analysis, the results obtained for each of the bacterial groups were divided into five categories: one for absence (0 CFU/100 mL) and the other four based on the quartiles of the CFU/100 mL obtained from positive
samples. Likewise, for the BC, another five categories were created based on percentiles: 20%, 40%, 60% and 80% distribution, since for the BC there was no category ‘0 CFU/100 mL’. These analyses were also performed in SPSS 15.0.

Using only data from those farms whose water supply came from a private well without a purification system, spatial, temporal and space-time analyses were performed. To assess the risk of positive samples in each Galician council (administrative division in Spain) from which samples were collected, excess risk maps were produced using GeoDa. The excess risk represents the ratio of the observed prevalence (% positive samples) at each council over the average prevalence of all councils. Additionally, a histogram was created showing the distribution of positive and negative samples throughout the year. Afterwards, to investigate whether the presence of positive herds for a particular bacterial group in the water samples was significantly higher in some areas or during a specific time period, data were tested using the Bernoulli model, as implemented in SaTScan 9.5 software. For each analysis, a herd positive for one of the bacterial groups was defined as the case, and the remaining herds were defined as non-cases. This widely applied method moves a circular or elliptic scanning window (across time and/or space) and compares observed and expected numbers of cases inside and outside this window in order to detect clusters. The scanning window was a circle or an ellipse over the study area (in space), an interval over the study time (in time), or a cylinder with a circular or elliptic base (in space-time). Likewise, it produces relative risk for the different clusters (as the ratio of observed/expected cases within the cluster, divided by the ratio of observed/expected outside the cluster), and a corresponding p-value for each cluster, based on the Monte-Carlo simulations. The maximum spatial cluster size and temporal cluster size varied from zero up to 50% of the population size (default value in SaTScan). SaTScan creates a shape file that includes cluster locations. This file was opened in Quantum GIS 2.18 to visualize clusters on the map.

**RESULTS AND DISCUSSION**

This is the first study to assess the microbiological quality of water used in dairy cattle farms in Spain. The studied herds could be considered a representative sample of the standard herds in Galicia, where the mean herd size is 42 cows (compared with 41 cows in the studied group) (MAPAMA 2018).

Twenty-eight out of 148 (18.9%) farms had a public water supply, whereas 120 (81.1%) used water from their own well on the farm; of these, 98 (66.2%) did not purify the water while 13 (8.8%) used hydrogen peroxide, eight (5.4%) used chlorine and one (0.7%) used ultraviolet (UV) radiation. Therefore, most farms in Galicia use well water as their main water source for dairy operations, without having a purification system.

In the 98 farms without a purification system, the prevalence of positive samples for TC, FC, and FS was 36.7% (36/98), 13.3% (13/98) and 24.5% (24/98), respectively. In the 13 farms using hydrogen peroxide, these figures were 15.4% (2/13), 7.7% (1/13) and 7.7% (1/13), respectively. In the eight farms using chlorine and the one using ultraviolet radiation all the samples were negative for the three bacterial groups. These data, along with the means and medians in CFU/100 mL for the positive samples, are summarized in Table 1. Although the prevalence seemed to be more favourable in farms that used some purification system, no statistically significant differences could be demonstrated when compared with those that did not use it (Fisher’s exact tests: $p$ (bilateral) = 0.491, $p$ (bilateral) = 0.858 and $p$ (bilateral) = 0.725 for TC, FC, and FS, respectively).

Table 1 | Number and percentage of positive water samples to total coliform (TC), faecal coliform (FC), and faecal streptococci (FS) samples in 120 farms in Galicia (NW Spain) whose water supply came from a private well (by a water purification system) and descriptive statistics for CFU/100 mL for the positive samples

<table>
<thead>
<tr>
<th>Purification system (n)</th>
<th>N positive samples (%)</th>
<th>Mean (SD)/median CFU/100 mL for the positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>36 (36.7%)</td>
<td>70.4 (27.6)/80</td>
</tr>
<tr>
<td>FC</td>
<td>13 (13.3%)</td>
<td>26.5 (22.3)/20</td>
</tr>
<tr>
<td>FS</td>
<td>24 (24.5%)</td>
<td>44.5 (23.6)/37</td>
</tr>
<tr>
<td>Hydrogen peroxide (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>2 (15.4%)</td>
<td>90.0 (14.1)/90</td>
</tr>
<tr>
<td>FC</td>
<td>1 (7.7%)</td>
<td>100 (0)/100</td>
</tr>
<tr>
<td>FS</td>
<td>1 (7.7%)</td>
<td>38 (0)/38</td>
</tr>
<tr>
<td>Chlorine (8)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ultraviolet radiation (1)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*Number of positive samples = 0 for the three bacterial groups.
respectively). This may be related to the small sample size of the group that used purification systems. In any case, both chlorination and ultraviolet light have been previously described as the most effective processes for small-scale rural water treatment (Corkal et al. 2004).

There are a few studies that have information on water contamination by specific pathogens on dairy farms. In the present study, TC was found in 36.7% of water samples from wells without purification systems, a smaller prevalence than previously reported: 45.5% in private rural wells in north-eastern Ohio (Won et al. 2015), 53.7% in water samples collected from a tap or water hose in the milk house from dairy farms in Ontario (Perkins et al. 2009), and 100% in supply water and wastewater from dairy farms in Antioquia, Colombia (Rodríguez et al. 2012).

However, in private and small public wells in Alberta, the prevalence of positive samples to TC was 14.6% (Invik et al. 2017). As regards FC (especially E. coli), Perkins et al. (2009) reported similar figures to those observed in the present paper (13.5% prevalence rate for E. coli). Lower rates were reported in other studies, i.e. 8.9% and 1.5% in Ohio and Alberta, respectively (Won et al. 2015; Invik et al. 2017), but higher in other populations: 20% in well water from Córdoba, Argentina (Bettera et al. 2011), 39.2% of water samples collected from pipes or mouthed washing containers in smallholder dairy farms in Ethiopia (Amenu et al. 2016), 43.3% in samples from dairy wastewater in Idaho (Dungan et al. 2014), 49% in private well waters in Ontario (Krolik et al. 2014), 50% in surface water and groundwater (well) samples in two dairies in northern California (Li et al. 2014), and again 100% in Antioquia, Colombia (Rodríguez et al. 2012).

The quantitative results of the microbiological analyses reported, on average, 70 CFU/100 mL of TC and 26.5 CFU/100 mL FC from positive water samples of private wells on 98 dairy farms without purification systems. Rodríguez et al. (2012) observed that the largest population of TC detected in water sources was $1.2 \times 10^5$ CFU/100 mL E. coli from supply water, and $1.5 \times 10^{10}$ CFU/100 mL from wastewater on 20 dairy farms from Antioquia, Colombia. In two dairy operations in northern California, concentrations of E. coli in groundwater ranged from 0 to 352.7 CFU/100 mL, much lower than that in surface water samples (Li et al. 2014).

Variation in results between different studies may reflect differences among regions, among sampling procedures or sample types (supply water, wastewater ponds, well water, and groundwater), among analytical methods, as well as different climatic conditions in the different areas or even different hydrogeological models. In this regard, the climate in Galicia is mild oceanic with abundant rainfall and moderate temperatures. In this region, some studies have been carried out on groundwater resources and their quality, taking into account that most of the rocks that exist in this region are igneous (granitic) and metamorphic (quartzite). Along with these geological characteristics, the high precipitation and the dense hydrological network of Galicia make the dominant hydrogeological model that of free surface aquifers (Juncosa et al. 2012).

Water quality is diminished primarily because of faecal contamination and rarely because of putrefaction in the water distribution systems. E. coli and total coliforms have been accepted as contamination indicator bacteria in treated, untreated and wastewater (Rodríguez et al. 2012). The presence of coliforms generally suggests contamination by groundwater through filtration of sewage and/or animal faecal matter. A previous study, also carried out in Galicia, indicated that the main source of water pollution is the infiltration of slurry and deficiencies in septic tanks that are used by most of the rural homes (where livestock activity takes place) due to the lack of a sewerage network (Moliner Huget et al. 1999). Thus, focused on the dairy cattle sector, an adequate maintenance and isolation of slurry tanks and manure management could contribute to reduce water contamination (which is especially important in high-density cattle areas). There are no previous publications on the characteristics of wells used as water sources in dairy farms in Galicia. However, according to what was observed during data collection, many farms have artisan-dug wells (approximately 1 m in diameter and 12 m deep) near the farm buildings. Drilled wells would present lower bacterial contamination basically because they can get water from a much deeper level, increasing the possibility of retention and extinction of bacteria from the surface to the collection point (López et al. 1994). Thus, it also seems important to address an upgrading of existing facilities.

Reductions in the bacterial concentrations from surface sources may result from reducing or preventing groundwater
Table 2 | Correlation matrix between the levels (CFU/100 mL) of total coliforms (TC), faecal coliforms (FC), and faecal streptococci (FS) in well water samples and the bulk tank milk bacterial count (BC) in 120 farms in Galicia (NW Spain)

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th>FC</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCb</td>
<td>ρc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSd</td>
<td>ρ</td>
<td>0.561</td>
<td>0.581</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BCe</td>
<td>ρ</td>
<td>-0.076</td>
<td>0.168</td>
</tr>
<tr>
<td></td>
<td>0.435</td>
<td>0.098</td>
<td>0.879</td>
</tr>
</tbody>
</table>

aTotal coliforms.
bFaecal coliforms.
cSpearman’s rank correlation coefficients.
dFaecal streptococci.
eBacterial count.

contamination by control of fertilizer and control of bacterial organisms in runoff (Hooda et al. 2000; Li et al. 2014). Safer dairy water storage and treatment is recommended to prevent post-collection contamination, together with point-of-use water quality monitoring. The establishment of best management practices on the farms in this study, including microbial water quality on the dairy farms, would be beneficial (Hooda et al. 2000).

Mean milk BC in the 120 farms whose water supply came from a private well was 24,770 CFU/100 mL (median = 21,000 CFU/100 mL). For the correlation analysis, the highest correlation was observed between the levels of TC and FS (ρ = 0.793; p < 0.001) and to a lesser extent between FC and FS (ρ = 0.581; p < 0.001) and between TC and FC (ρ = 0.561; p < 0.001). However, no correlation was found between the BC and TC, FC or FS levels (Table 2). Thereby, our results showed that the presence of TC, FC, and FS in the water on dairy farms was not related to an increase in microbial counts of raw milk. Previously, Perkins et al. (2009) indicated that the quality of the wash water used to clean the milking equipment had a small but significant effect on the quality of the raw milk produced. This discrepancy could be related to differences in other risk factors that affect milk BC. Thereby, herds with medium or high water hardness scores were more likely to have high bacterial counts in BTM than herds with lower hardness scores. Hard water can reduce the effectiveness of cleaning chemicals and may lead to formation of films or deposits in the milking system (Elmoslemany et al. 2009b). In Galicia, water is characterized by a very low hardness score (Xunta de Galicia 2010), which would favour the cleaning of milking and cooling equipment surfaces.

Regarding the possible impact of the results obtained in this paper on public health, the available data on zoonosis from Spain do not indicate a link between the results obtained and the occurrence of disease in humans (EFSA 2016). Excess risk maps for each of the three bacterial groups are presented in Figure 1. These maps are useful as a guide for further investigations into identifying hot spots of microbial contamination in groundwater and as a geographic baseline for future surveillance (Invik et al. 2017). The spatial cluster analysis identified one statistically significant spatial cluster of herds with positive samples for FS. The spatial cluster, located in the centre of the region (centroid: 42.88 N, 8.24 W), had a radius of 1.84 km. Within this cluster, the

Figure 1 | Excess risk maps of (a) total coliform, (b) faecal coliform and (c) faecal streptococci-contaminated water on dairy farms in Galicia (NW Spain). A significant spatial cluster of positive samples for faecal streptococci appears as a white circle.
RR for a herd to have a positive sample for FS was 5.52 times higher than expected \((p = 0.039)\) (Table 3; Figure 1). In the same area, there also seemed to be a higher risk (3.41 times higher) of having samples positive for TC; the RR was not significant at the 0.05 level but was close to the margin of statistical significance \((p = 0.070)\) (Table 3).

The statistically significant spatial cluster of herds with FS-positive samples found in this study could be linked to a greater cattle density and might indicate an underlying problem in the groundwater system. In this area, the number of livestock units per hectare was 0.98 (one of the highest of the Galicia region, where the average is 0.32) (IGE 2018). Previously, Díaz-Fierros & Núñez (1995) presented results obtained in the water analysis of 14 control points of a high-density cattle area also in Galicia. Most of the samples did not comply, due to the levels of bacteria, with standards for drinking water. Further investigations of the farm clusters identified in the present study for the presence of wildlife, migratory birds, underlying rock formations, and pollution of the ecosystem may also be worthwhile to identify possible spatial relationships (Perkins et al. 2009).

Distribution of positive and negative samples throughout the year for each of the three bacterial groups is presented in Figure 2. Temporal cluster analysis of TC, FC- and FS-positive cases showed that they were not distributed randomly in time. One most likely temporal cluster was identified for each of the three groups between July and August 2017 (Table 4). The overall RRs within the clusters were 2.47 \((p = 0.042)\), 6.06 \((p = 0.011)\) and 4.26 \((p = 0.004)\) for TC, FC and FS, respectively. In the study farms, as happens in most farms in Galicia (Benavides et al. 2018), water samples in dairy farms are tested once a year. The results obtained imply that the moment at which the sample is collected may have an important influence on the results obtained, which could be applied to other populations with similar climatic characteristics.

The highest prevalence rates of bacteria were found in the driest seasons of the year, similar to other studies for \(E.\ coli\) in dairy wastewater ponds and rural well water (Dungan et al. 2012; Li et al. 2014; Invik et al. 2017) and for TC and \(E.\ coli\) in supply water and wastewater (Rodríguez et al. 2017).

### Table 3
SaTScan statistics for spatial clusters with higher incidence of contaminated water samples in 98 farms without a water purification system in Galicia (NW Spain)

<table>
<thead>
<tr>
<th>Centroid</th>
<th>Cases</th>
<th>Expected cases</th>
<th>Relative risk</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC(^a)</td>
<td>4</td>
<td>1.27</td>
<td>3.41</td>
<td>0.070</td>
</tr>
<tr>
<td>FS(^b)</td>
<td>4</td>
<td>0.83</td>
<td>5.52</td>
<td>0.039</td>
</tr>
</tbody>
</table>

\(^a\)Total coliforms.
\(^b\)Faecal streptococci.

### Table 4
SaTScan statistics for temporal clusters with significantly higher incidence of contaminated water samples in 98 farms without a water purification system in Galicia (NW Spain)

<table>
<thead>
<tr>
<th>Time frame</th>
<th>Cases</th>
<th>Expected cases</th>
<th>Relative risk</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC(^a)</td>
<td>11</td>
<td>5.38</td>
<td>2.47</td>
<td>0.042</td>
</tr>
<tr>
<td>FC(^b)</td>
<td>7</td>
<td>1.98</td>
<td>6.06</td>
<td>0.011</td>
</tr>
<tr>
<td>FS(^c)</td>
<td>9</td>
<td>2.92</td>
<td>4.26</td>
<td>0.004</td>
</tr>
</tbody>
</table>

\(^a\)Total coliforms.
\(^b\)Faecal coliforms.
\(^c\)Faecal streptococci.

**Figure 2** Distribution of (a) total coliform, (b) faecal coliform and (c) faecal streptococci positive and negative samples throughout the year.
et al. 2012). This finding could be caused by the dilution effect of rainwater. The hydrodynamic model in Galicia, already described, makes the region very sensitive to periods of drought (Xunta de Galicia 2010). In addition, it is possible that elevated environmental temperatures favour multiplication of faecal bacteria in the farm environment and subsequent contamination of groundwater with bacteria from surface sources (faecal matter and slurry). Many sources of E. coli on dairy farms occur sporadically and therefore have little effect on the expected median concentrations in wells (Muirhead et al. 2011).

In the space–time assessment, no significant spatiotemporal clusters were identified. An explanation for this could be that there may be areas where sample collection was not distributed homogeneously throughout the year. In addition, study samples correspond, as mentioned, to a single year and to a single sample per farm.

**CONCLUSIONS**

In the studied population, 66.2% of dairy farms used water from wells without having a purification system. Of these, nearly 40% did not meet the requirements of zero coliforms, E. coli and enterococci per 100 mL. However, the microbiological quality of wash water did not have an effect on bulk tank milk BC in the study population. Spatial analysis identified one statistically significant spatial cluster of herds with positive samples of faecal streptococci in an area characterized by a high cattle density. One most likely temporal cluster was also identified for each of the three groups during the season of lower rainfall.

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