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# Aetiology and environmental factors of the Watery Mouth Disease associated with neonatal diarrhoea in lambs

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ABSTRACT

Watery Mouth Disease is the main disease in neonatal lambs, causing great economic losses. Despite this, the cause of the condition remains poorly understood. Therefore, we have analysed the main bacteria found in sick animals, their intestinal and temporal distribution, as well as the main sources of contamination. Twelve different farms were sampled, from which 331 samples were taken in total. From these samples, 184 environments were analyzed using *16S rRNA* amplicon sequencing, 164 isolates were identified by whole genome sequencing and 35 bacterial counts were performed. The dominant bacterial groups at the rectal level were *Escherichia-Shigella* (36 %) and *Clostridium* (29 %), with a homogeneous distribution along the digestive tract and a maximum abundance ranging between 12 and 24 hours of lamb life. Within *Escherichia-Shigella*: *Escherichia coli* and *Escherichia fergusonii* and within *Clostridium*: *Clostridium perfringens*, *Clostridium cadaveris*, *Clostridium tertium and Clostridium paraputricum* were identified as the main isolates present in sick animals. The high presence of *Clostridium* strains, especially potentially pathogenic species like *C. perfringens* in sick animals, point out *Clostridium* as a new important protagonist of watery mouth disease and the need of their inclusion in future studies. In particular, bedding was established as the main microbial contaminating factor, reaching the highest increase 48 hours after removal and cleaning of the lambing area (i.e. 8.03 $\times 10^8$, 1.88 $\times 10^6$, 3.88 $\times 10^6$, 4.85 $\times 10^7$ and 4.00 $\times 10^5$ CFU/g for mesophilic aerobes, coliforms, *E. coli*, mesophilic anaerobes and sporulates, respectively). These results highlight the need to increase cleanliness in bedding to reduce the presence of these bacteria.

## 1. Introduction

Diarrhoeal diseases represent a significant problem in intensive lamb production, causing substantial economic losses in livestock farming (Zhang et al., 2021). The main diarrhoeal condition in neonatal lambs is known as Watery Mouth Disease. This condition affects 25 % of lambs worldwide, with mortality rates reaching as high as 80 % (Zhang et al., 2021). Cases have been reported in most countries where intensive lamb production takes place, with reports from France (Savey, 1986), Spain (Garcia de Jalon, 1990), New Zealand (Sargison et al., 1997) and UK (Collins et al., 1985).

The first systematic study of watery mouth disease was carried out by Collins et al., (Collins et al., 1985). No hematological or biochemical abnormalities have been observed in clinical records (Eales et al., 1986). The diagnosis of the disease is based on symptomatology and post-mortem observations (Amanda, 2019). Symptoms of the condition appear within 6–12 hours after birth, and the affected lambs show depression, loss of appetite and diarrhoea, followed by a strong salivation that moistens the contour of the lips even to the point of dripping. Necropsy and anatomopathological studies have found no lesions in the intestinal villi, only a distended abomasum with gas, saliva and meconium retention (Collins and Carson, 2022). Despite its relevance, only a few studies have dealt with Watery Mouth Disease. Most of these studies have associated the symptoms with endotoxemia caused by an unknown

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pathotype of *E. coli*, previously thought to be non-pathogenic (Collins and Carson, 2022).

The aetiology of the Watery Mouth disease is not yet clear, although we have evidence of several points. In the first hours of the lamb's life, before the first colostrum intake, it has a relatively neutral abomasal pH, which facilitates the passage of bacteria into the intestine (Eales et al., 1986). In addition, during the initial 24 hours of life, the lamb exhibits reduced intestinal motility and an active pinocytosis mechanism (Hodgson et al., 1989). This mechanism facilitates the entry of a substantial number of beneficial molecules, such as maternal immunoglobulins, into the systemic circulation. Also would be used by *E. coli* to generate bacteremia, followed by a massive death of these bacteria and massive release of their endotoxins, thereby causing endotoxemia (Hodgson et al., 1989).

Although some authors have linked this to the unhygienic conditions on farms (Collins et al., 1985; Eales et al., 1986), the specific hotspots of contamination are still unknown. In the present article we have attempted to use a holistic approach to the investigation of Watery Mouth Disease. This was achieved through the use of next-generation sequencing techniques, which enabled the identification of the main bacterial groups present in sick animals and the location of the main sources of contamination which these animals are exposed to in livestock farms. Combining bacterial genus identification by *16S rRNA* amplicon sequencing with viable bacterial species identification by whole genome sequencing of isolates of the most common genera provides both an ecological and clinical approach to the disease.

## 2. Materials and methods

### 2.1. Description of the lambing system

The study was conducted in collaboration with 12 sheep farms, all of which employed a similar management system. The production process is divided into two sheds, an area for lambing and an area for colostrum feedings. Each area is further subdivided into enclosures that are watertight, thereby facilitating the separation of the gestation, lambing, and colostrum intake phases. All enclosures are covered with a 2 cm thick layer of straw. The ewes are relocated to the lambing area two months prior to the expected date of lambing. One week prior to the expected date of lambing, the straw covering is removed, the manure is swept and removed from the floor, and new straw is placed on the floor. Upon detection of the lamb's birth, the lamb is relocated to another shed. This is divided into a series of plots, the number of which varies according to the farm. The lambs are moved between the plots according to the number of hours they have been alive until the colostrum feedings are completed. The lambs in poorer health are moved to a pen where they are kept until they recover. Finally, the lambs that have completed the colostrum intake phase are grouped together in a separate enclosure, where they are fattened until they are slaughtered.

### 2.2. Sampling

A total of 331 samples were taken from 12 farms collaborating with the study. They were divided into 167 rectal and buccal swabs from lambs 85 samples of intestinal contents, 4 abomasal samples from live lambs, 25 beddings, 23 prepartum maternal faeces, 8 placentas, 9 colostrum, 5 buccal swabs from prepartum mothers and 5 vaginal swabs from prepartum mothers. Abomasal samples were collected from live lambs using an oral gastric tube. The date of collection and the analytical techniques used are detailed in Supplementary Table 1.

Animal welfare considerations were rigorously addressed in accordance with established ethical guidelines and institutional protocols. Prior to the commencement of the study, farm owners were provided with comprehensive information regarding the study's objectives, procedures, and any potential risks. Informed consent was obtained to ensure voluntary participation and compliance with ethical research standards.

### 2.3. Microbiological enumeration

Due to the different nature of the samples, a differential pretreatment was performed according to the type of microorganism and sample. Samples of beddings, placentas and maternal faeces were diluted by a factor of 1/10 in saline peptone (1 g/L Oxoid Bacteriological peptone-LP0037B with 8.5 g/L NaCl), using an AES Chemunex Dilumat 4 automatic diluter and shaken for 2 min in a Pulsifier II automatic shaker (Microgen) at maximum speed. The methodology for enumerating sporulating cells was described by Watterson et al (Watterson et al., 2014). All samples were subjected to serial dilutions. The incubation times, specific media and count process are described in Supplementary Methods.

### 2.4. Bacterial isolation

In order to isolate *E. coli*, seeding was performed on MC medium (Oxoid). In the case of solid matrices, such as placentas, maternal faeces or bedding, 250 mg of matrix was diluted in 1 ml of PBS and 100 µl per plate was seeded. Bacteria were incubated at 37°C for 24 hours. For the isolation of *Clostridioides difficile* and *Clostridium perfringens*, samples were processed in accordance with the protocols of Blanco et al., (Blanco et al., 2013) and Álvarez-Pérez et al., (Álvarez-Pérez et al., 2017), respectively. A more general isolation for other *Clostridium* and *Clostridioides* species was introduced using the *C. perfringens* protocol but changing the incubation temperature to 37ºC and using RCM (Oxoid) as solid medium for isolation.

### 2.5. DNA extraction and library preparation

For direct DNA extraction from the environmental samples, the QIAamp Fast DNA Stool Mini Kit (QIAGEN) was used following the manufacturer's instructions. In the case of DNA extraction for whole genome analysis, the DNeasy Blood and Tissue kit (QIAGEN) was used. DNA concentration was performed with the Qubit fluorometer 4 (Fisher Scientific), and DNA was stored at −20ºC until the preparation of libraries.

In order to study the relative abundance of bacteria, the V3-V4 region of the *16S rRNA* coding gene was amplified using the primers and PCR conditions specified by Klindworth et al., (Klindworth et al., 2013). For whole genome studies, shotgun sequencing was performed using the Illumina DNA Prep Reference Guide protocol (Illumina). In both cases, sequencing was performed by paired-end synthesis using a 2 × 300 V3 Illumina Kit (Illumina) on a MiSeq sequencer (Illumina). Specific details of the library preparation are described in Supplementary Methods.

### 2.6. Bioinformatics processing

*16S rRNA* amplicon sequences were filtered using the Sickle tool (Joshi and Fass, 2014). For taxonomic assignment, the QIIME 2 open-source bioinformatics pipeline (Bolyen et al., 2019) was used with its default values, using SILVA v138 as database. The pipeline output provides a table of Amplicon Sequence Variants (ASVs), from which Chloroplasts, Mitochondria and Archaea were removed for subsequent relative abundance analyses. Genome analysis was performed using the TORMES pipeline (Quijada et al., 2019) with its default values.

### 2.7. Statistical analysis

Principal component analysis (PCOA) was calculated using Bray-Curtis distances based on data subsampled to the minimum depth found, which was 6444 reads. The significance of these distances was calculated using the Adonis function as a multivariate permutational analysis of variance. This was all integrated in the R package MicrobeR

(Bisanz, 2018).

The Tidyverse package (Wickham et al., 2019) was used to study the relative abundances from the ASV tables. This package contains all the necessary libraries for analysis and for obtaining graphs. This tool was also used to calculate the mean and standard deviation of the microbiological counts.

The relative abundance of each sample was calculated by dividing specific taxon reads/total reads. The mean of the relative abundances was then calculated for each group examined (Estaki et al., 2020). To facilitate the interpretation of the data, the *Clostridium_sensu_stricto* taxa have been grouped under the name *Clostridium* without number extensión on the relative abundance table and figures.

## 3. Results

### 3.1. Patterns of bacterial composition present in watery mouth disease

To identify the main bacterial taxa, present in the watery mouth compared to healthy lambs or recovered lambs, rectal swabs were taken from 46 lambs displaying characteristic symptoms of Watery Mouth Disease (diarrhoea, general weakness and drooling), 5 healthy lambs and 11 recovered lambs with past disease from 12 farms. They were then analysed by *16S rRNA* amplicon sequencing for rectal bacterial composition at the genus level. A total of 1893,797 reads were obtained after screening and taxonomic assignment identified 169 bacterial ASV at the genus level (Supplementary Table 2-A).

Principal Coordinate Analysis (PCoA) was performed with the aim of discriminating between watery mouth and healthy lambs. The Bray-Curtis distances of the abundance profiles were used as the metric, with ellipses drawn at the 95 % confidence interval. The ADONIS analysis yielded a total variance of 58.5 % and shows that 19 % of the observed distance variation was explained (R2 = 0.192) for the groups tested (p = 0.001). Fig. 1A illustrates a distinct clustering of the lambs during the disease (During_illness), whereas the lambs that have overcome (Overcome_Illness) and those without the disease (Without_illness), do not show a clear clustering.

The *16S rRNA* sequencing study of each group of lambs reveals that *Escherichia-Shigella* and *Clostridium* are the most abundant groups, accounting for 36 % and 29 % of the total bacterial composition in lambs with symptoms (Fig. 1B; Supplementary Table 2-B). As shown in Fig. 1B and Fig. 1C, both groups are highly reduced in Overcome_illness and Without_illness lambs. As reported in Supplementary Tables 2-C and 2-D, the abundance of the genera *Bacteroides* and *Lactobacillus* increased in Overcome_Illness and Without_illness lambs compared to the During_illness lambs.

The evolution of bacterial composition over time was analyzed by *16S rRNA* sequencing, between 0 and 12 hours of life in seven lambs (WT1 to WT7), between 12 and 24 hours in seven lambs (WT8 to WT14) and 24–36 hours in three lambs (WT15 to WT17). After sequence screening, 265 ASVs and 466,243 reads were obtained at the genus level (Supplementary Table 3-A).

The *16S rRNA* sequencing analysis between 0 and 12 hours revealed

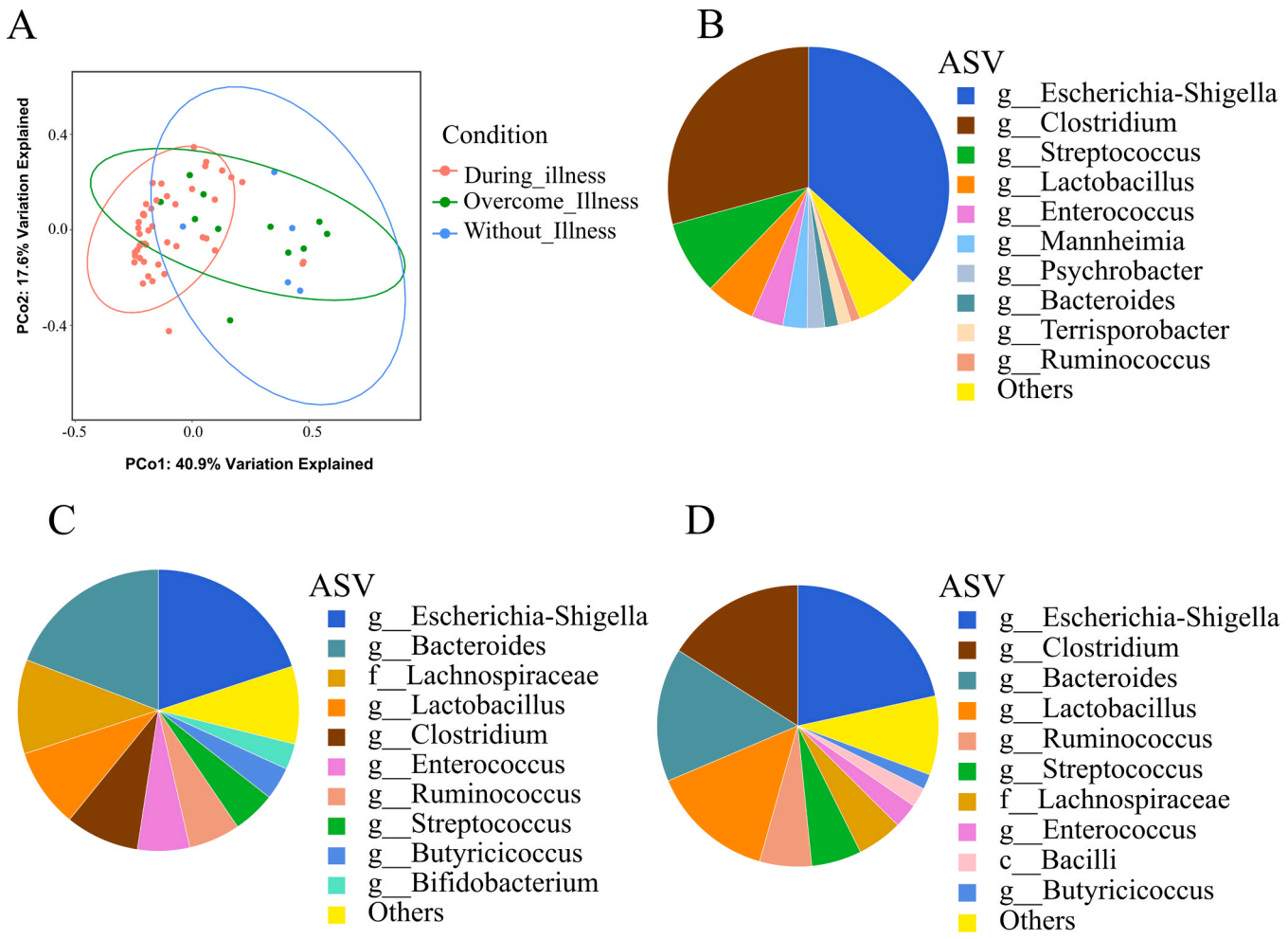


**Fig. 1.** Analysis of bacterial composition at genus level, of lambs during disease, when overcoming disease and without disease. (A) PCoA based on Bray Curtis distances for each group, the percentage of explained variation is plotted on the axes. Mean relative abundances of the 10 most abundant bacterial groups in watery mouth lambs (B); in healthy lambs (C); in lambs with past disease (D).

three distinct relative abundance profiles, (Supplementary Figure 1) from WT1 to WT5 with a high diversity represented by the "Others" group. On the other hand, in WT6, *Escherichia-Shigella* represents 92 % of the bacterial composition, while in WT7, *Clostridium* emerges as the dominant bacteria, accounting for 54 % of the total bacterial population. *Escherichia-Shigella*, however, retains a significant presence accounting for 36 % of the bacterial composition. The lambs between 12 and 24 hours present a very constant profile in which *Escherichia-Shigella* and *Clostridium* predominate occupy 42 % and 44 % on average in the group respectively. As shown in the Supplementary Figure 1, lambs between 24 and 36 hours exhibit more variable profiles between them. On average for this group, the dominant taxon is *Escherichia-Shigella* with 32 %, followed by *Lactobacillus* with 22 % and *Clostridium* with 13 % (Supplementary Table 3-B)

In order to study the spatial distribution of the bacterial composition, a *16S rRNA* amplicon sequencing approach was employed. Taking 56 samples of intestinal contents from nine lambs belonging to the same farm immediately after the death caused by Watery Mouth Disease. Samples were taken from the abomasum, duodenum, jejunum, ileum, cecum, colon and rectum whenever there were contents present in the intestinal segment of the sampled lamb. In lamb 5, two samples were taken from the jejunum, jejunum-1 (further away from the ileum) and jejunum-2 (closer to the ileum), due to the presence of bleeding in the second segment. After sequence screening, 95 ASVs and 2093,368 reads were obtained at the genus level (Supplementary Table 4-A).

The relative abundance of *16S rRNA* sequencing, has been indicated in Supplementary Table 4-B, which shows the relative abundance along the digestive tract. The genus *Escherichia-Shigella* being the predominant one with a homogeneous distribution along the intestine in the nine lambs studied (Fig. 2). *Escherichia-Shigella* also appears in the abomasum although, to a lesser extent, it is usually accompanied by the genus *Clostridium*. As shown in Supplementary Table 4-B, the second most prevalent group is the genus *Lactobacillus,* particularly in lambs 5 and 6 (Fig. 2). In these lambs, *Lactobacillus* has a high relative abundance in the abomasum which decreases along the intestine. It should be noted that in the analysis of jejunum-2 (with haemorrhage) the profile is slightly different from jejunum-1 (without haemorrhage) since *Clostridium* is the second most abundant genus, occupying 21 % of the total bacterial composition.

To confirm oral faecal contamination, buccal swabs were taken from 5 lambs with watery mouth disease. These were analysed by *rRNA 16S* sequencing. Two samples were collected at the onset of symptoms at 10 hours after birth (WB1 and WB2), two with the process at 24 hours after birth (WB3 and WB4) and one after recovery at one week of life (WB5). Following screening, 333,017 reads and 164 ASVs were obtained at the genus level (Supplementary Table 5-A). *Clostridium* and *Escherichia-Shiguella* groups were detected at all sampling times, but in low proportions (Supplementary Table 5-B). Also noteworthy is the large variation in the composition of the oral cavity between different sampling times (Supplementary Figure 3).

To corroborate the hypothesis that the abomasum is the portal of entry for bacterial pathogens in the early stages of life, abomasal contents were taken. Three of the lambs were sampled after the first colostrum feeding (WA1 to WA3) and the fourth one after several feedings at a very advanced stage of the disease close to death (WA4). These samples were analized using *16S rRNA* sequencing. After screening of the sequences 165,578 reads, and 58 ASVs were obtained at the genus level (Supplementary Table 6-A). The relative abundance shows how lambs, after the first colostrum intake (WA1-WA3), contain the genus *Escherichia-Shigella* but with varying proportions (7 % in WA1, 61 % in WA2, 9 % in WA3). The microbial profile of the lamb after several feedings (WA4) is different with a much lower percentage of *Escherichia-Shigella*, occupying 3 % of the total bacterial composition and in this case, the dominant group is the genus *Bacteroides* occupying 47 %. Interestingly, *Clostridium* appears with its highest proportion of 10 % in WA2, whereas *Escherichia-Shigella* is the most abundant genus (Supplementary Figure 2; Supplementary Table 6-B).

### 3.2. *Identification of the components of the Escherichia-Shigella and Clostridium groups in watery mouth lambs*

As *Escherichia-Shigella* and *Clostridium* were taxa identified with high relative abundance in lambs with Watery Mouth Disease, these groups were selected for isolation and further characterization. For the study of isolates of the genus *Escherichia-Shigella*, 72 rectal swabs and 11 samples of intestinal contents were taken from lambs with characteristic

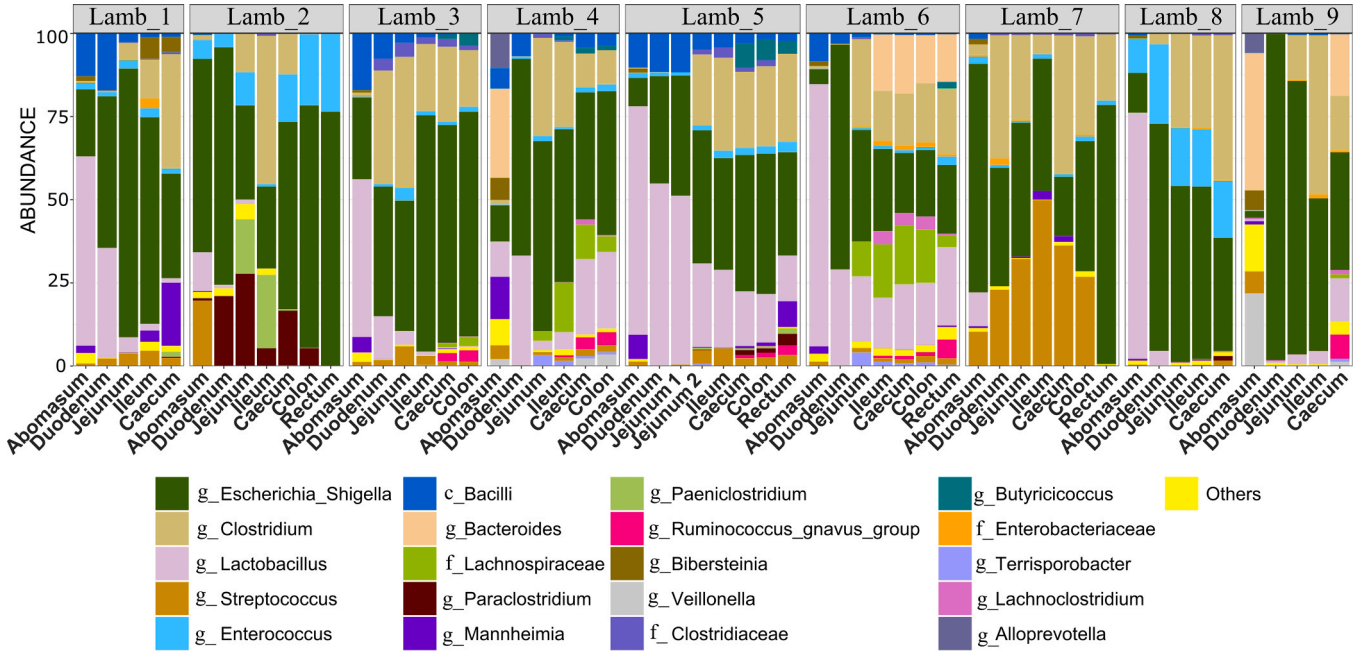


**Fig. 2.** Relative abundance of the 20 most abundant bacterial groups along the digestive tract. In 9 lambs (Lamb_1 to Lamb_9) which died as a result of Watery Mouth Disease.

symptoms of Watery Mouth Disease, belonging to 12 farms collaborating with the project. From which 111 isolates were obtained. All isolates were sequenced by shotgun sequencing, and their complete genome was analysed bioinformatically. A total of 101 isolates from the *Escherichia-Shigella* group were identified using Kraken2, as shown in the Supplementary Table 7-A. Of the total number of isolates, 100 were identified as *E. coli*, while only one was identified as *Escherichia fergusonii*. From each bacterial genome, its multilocus sequence type (MLST) and serotype were identified. The most commonly identified sequence types in the isolates were ST-88 (12 %). A total of 188 distinct virulence factors were identified in the Virulence Factor Database (VFDB) for *E. coli*. The most frequently identified toxin was the heat-stable enterotoxin EAST-1, observed in 20 % of the isolates (Supplementary Figure 4).

To analyse the Clostridia group a total of 54 isolates were obtained following processing of 29 intestinal samples, 11 rectal swabs and 18 intestinal contents (Supplementary Table 7-B). Of these isolates, the following genomes were sequenced by shotgun sequencing and were identified using Kraken2: 15 as *Clostridium paraputrificum*, 14 as *Paraclostridium bifermentans*, 10 as *Clostridium tertium*, 5 as isolates as *Clostridium perfringens,* 5 as *Clostridium cadaveris* and 5 as *Clostridioides difficile.* No dominant MLST characteristics were observed in *Clostridium and Paraclostridium* species. The most significant virulence factor gene identified was *plc*, which encodes alpha-toxin. This gene was present in four of the five *C. perfringens* strains examined. The presence of this toxin defines the pathotypes as pathotype A (Uzal et al., 2010). Moreover, a substantial number of accessory toxin genes were identified in conjunction with the *plc* gene, including those encoding Kappa-toxin, Mu-toxin, sialidase, and Theta-toxin (Supplementary Figure 5-A). In the case of *C. difficile*, all isolates were identified as ST-11 and exhibited the tox B gene. Two of the isolates also demonstrated the presence of the tox A gene (Supplementary Figure 5-B). The remaining members of the Clostridia species lack the presence of toxins or a distinctive genome.

### 3.3. Study of contamination hotspots

The potential sources of environmental contamination which the lambs could be exposed to in the first hours of life were investigated through microbiological counts on straw cover from the floor (B1-B5), mother's feaces (F1-F7), colostrum (C1-C9) and placentas (P1-P3) in farm B (Supplementary Table 1). The results are shown in Supplementary Figure 6 and are listed in Supplementary Table 8. The highest levels of contamination were found in the bedding, specifically in the lambing area where they reached a maximum value of 3.87 × $10^8$ CFU/g in B3

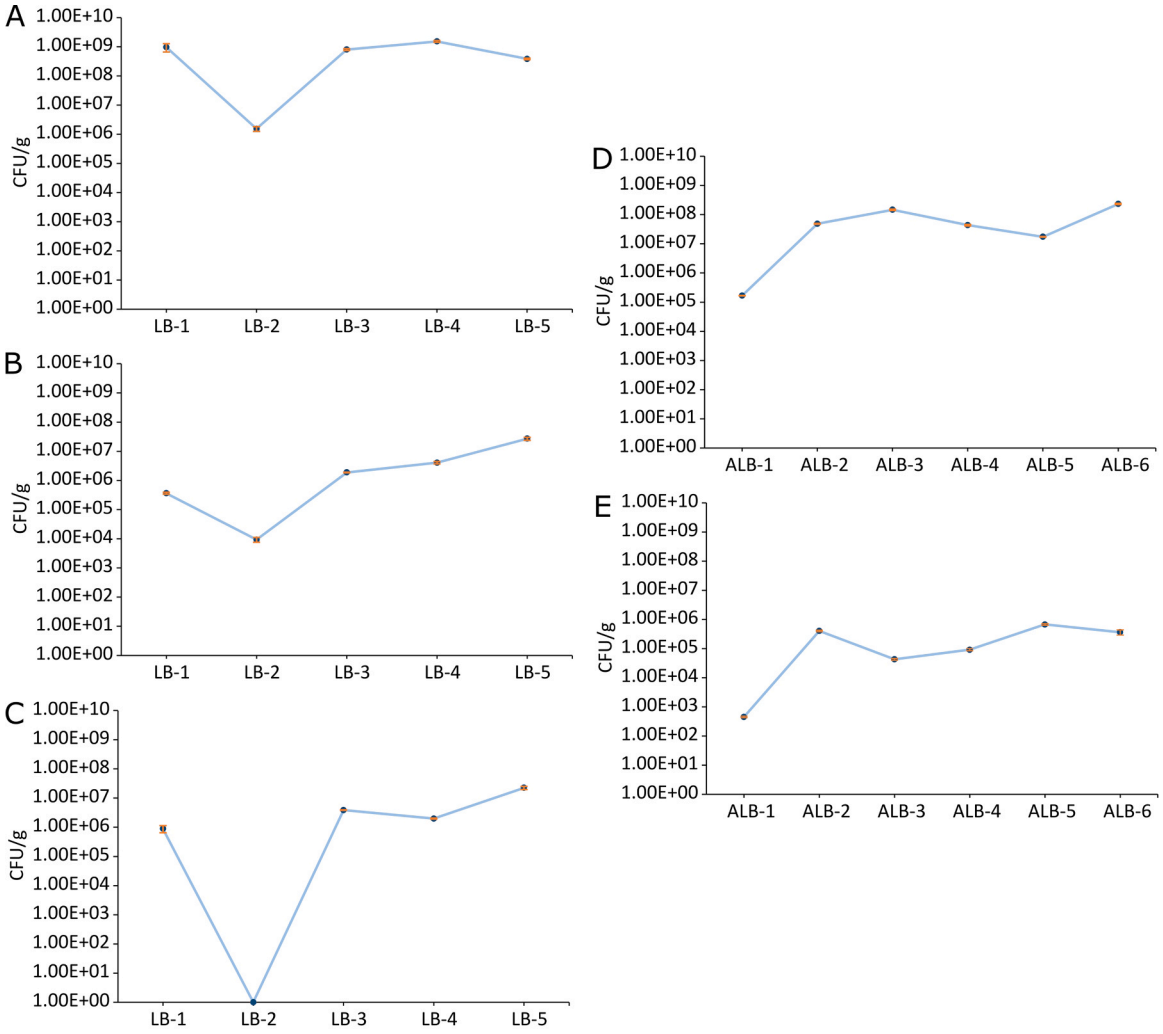


**Fig. 3.** Bacterial study along the lambing beeding (LB): A) Mesophilic aerobic count, B) total coliforms, C) Escherichia coli. In bedding samples before manure removal (LB-1), in clean bedding (LB-2), straw 48 h after cleanning (LB-3), one week after cleanning (LB-4) and straw 4 weeks after cleanning (LB-5). Counts for anaerobic bacteria during lambing bedding (ALB): D) mesophilic anaerobic count and E) sporulate count, in clean bedding sample (ALB-1), 48 h after cleanning (ALB-2), 1 week after cleanning (ALB-3), 2 weeks after cleanning (ALB-4), 3 weeks after cleanning (ALB-5) and 4 weeks after cleanning (ALB-6). The orange error bars represent the standard deviation of the means.

for coliforms and 7.67 × $10^8$ CFU/g in B2 for mesophilic anaerobes.

As the bedding in the lambing area was identified as the hotspot with the highest bacterial load, the evolution of the load along the lambing process was studied in two separate sampling events. The first sampling event focused on the study of aerobes in the lamb bedding (LB), while the second sampling event was dedicated to the investigation of anaerobic bacteria from the lamb bedding (ALB). After 48 hours of bedding the greatest bacterial increase was identified, for mesophilic aerobes (Fig. 3A), total coliforms (Fig. 3B), *E. coli* (Fig. 3C), anaerobes (Fig. 3D) and sporulates (Fig. 3C), being: 8.03 × $10^8$, 1.88 × $10^6$, 3.88 × $10^6$, 4.85 × $10^7$ and 4.00 × $10^5$ CFU/g, respectively (Supplementary Table 9). Thereafter, the bacterial counts remained relatively stable.

### 3.4. Study of the bacterial composition of the sources of contamination

To complete the study of the main sources of contamination, a *16S rRNA* sequencing analysis was carried out in two farms affected by the disease (Supplementary Table 1). For this purpose, 11 samples of faeces from pregnant mothers (MF1 to MF11) were collected from farm A and five samples of faeces (MF12 to MF16) were taken from farm B. Also from farm A, five placenta samples were taken, three of which were fresh (PL1 to PL3) and two dry (PL4 and PL5), five maternal vaginal swabs (VS1 to VS5) and five buccal samples from postpartum mothers (BS1 to BS5).

Sequencing of the samples yielded 855,234 reads and 409 ASVs for maternal faeces, 121,647 reads and 234 ASVs for maternal buccal swabs, 36,939 reads and 191 ASVs for placentas and 202,051 reads and 184 ASVs for vaginal swabs, at the genus level (Supplementary Table 10).

The genera *Clostridium* and *Escherichia-Shigella* were identified in all sampled environments (MF, BS, PL and VS), although at low levels as shown on Supplementary Table 11-A, 11-B, 11-C and 11-D respectively. Maternal faeces, in general, show a high bacterial diversity, without the dominance of some groups over others (Suplementary Figure 7-A). The oral bacterial composition in the mothers shows other pathogen groups such as *Streptococcus*, *Bibersteinia* and *Manhemia* show on average 16 %, 14 % and 8 % (Supplementary Figure 7-B). The major groups were *Jeotgalicoccus* and *Corynebacterium* with 12 % and 8 % in fresh placentas, whereas in dry placentas, they were *Psychrobacter* and *Jeotgalicoccus* with 30 % and 19 % (Supplementary Figure 7-C). In vaginal samples the most abundant groups were *Streptobacillus* with 30 %, *Fusobacterium* with 11 % and *Histophilus* with 10 % of the total bacterial composition (Supplementary Figure 7-D).

In parallel to the anaerobic bedding counts, the evolution of the bacterial composition was established in two separated pens on farm B: zone A (ZA) and zone B (ZB), using *16S rRNA* sequencing. Five samples were taken from zone A and four samples from zone B. Specifically, bedding two days after cleaning (ZA0), at one week (ZA1 and ZB1), at two weeks (ZA2 and ZB2), at three weeks (ZA3 and ZB3), and at four weeks (ZA4 and ZB4). In addition to a clean straw sample (Z), it is common to both zones. In Supplementary Figure 8 it can be seen that the biggest differences in composition occur between Z and ZA0 after two hours of cleaning; the proportions remain very stable throughout the lambing period, and it is very similar between the two zones. Supplementary Table 12 shows that only *Escherichia-Shigella* is not present in Z, but even reaches the maximum level after 48 hours of cleaning (4 %) with an average of 1 % for ZA and 0.63 % for ZB. *Clostridium* was not detected in: ZA1, ZA2, ZA3, Z, ZB1, ZB2, ZB3 and ZB4, reaching an average for ZA of 0.03 % and absence in ZB.

## 4. Discussion

The rectal bacterial composition of the lambs with the disease showed that 65 % were occupied by only two groups: *Escherichia-Shigella* (37 %) and *Clostridium* (28 %). The joint occurrence of *Escherichia-Shigella* and *Clostridium* is recurrent throughout the study as we found it in the temporal study of the syndrome mainly between 12 and 24 hours, in the study of the spatial distribution along the digestive tract and in the study of the abomasum of live lambs specifically in the lamb WA2. In calf studies, the high relative abundance of *Escherichia-Shiguella* and *Clostridium* taxa has been identified as characteristic of diarrhoeal calves (Li et al., 2023; Jessop et al., 2024). *Escherichia-Shigella* and *Clostridium* taxa, were reduced in healthy lambs. When observed recovered lambs both groups were reduced, other groups appearing such as *Bacteroidetes* characteristic of healthy lambs (Bi et al., 2019).

The identification of the components of the *Escherichia-Shigella* and *Clostridium* group through the study of isolate genomes, has allowed us to determine *E. coli* as the most abundant species in the first group and *C. paraputricum* in the second group, together with other species of clostridia such as *P. bifermentans*, *C. cadaver*is, *C. perfringens*, *C. tertium* and *C. difficile*. Previously, only *E. coli* has been given weight as an aetiological agent of Watery Mouth Disease (Collins and Carson, 2022) specifically endotoxaemia associated with undefined pathotypes of *Escherichia coli*. However, the findings of our study highlight the presence of species from the clostridia group, which have not previously been reported as an abundant group in animals with Watery Mouth Disease. Furthermore, their role is well known in sheep diarrhoeal processes mainly caused by *C. perfringens (*Kiu and Hall, 2018*)* and *C. difficile (*Keessen et al., 2011*)*. It is also known that a high relative abundance of pathogenic bacteria, such as *Clostridium* spp., and the detriment of commensal or probiotic bacteria, such as *Ruminococcus* spp. causes diarrhoeal episodes in livestock. This imbalance is commonly referred to as 'dysbiosis' (Zeineldin et al., 2018).

The temporal evolution of the disease has allowed us to determine the colonisation profile in Watery Mouth Disease. This starts with a diverse microbiota between the first 12 hours of life, which is then replaced at 12–24 hours of life by an increasing abundance of the genera *Escherichia-Shigella* and *Clostridium* coinciding with the onset of symptoms, and both groups are reduced to 24–36 hours of life giving way to other commensals or beneficial bacteria such as *Lactobacillus*. These rapid changes in bacterial composition seem to indicate a change from an aerobic to an anaerobic environment. Initially, we find bacterial genera (preferably strict aerobes) such as *Salinococcus*, *Dietzia*, *Acinetobacter* or *Psycrobacter* and facultative anaerobes such as *Staphylococcus* or *Jeotgaliococcus*. Oxygen depletion could be greatly favoured by a massive growth of *Escherichia*, consuming oxygen and allowing *Clostridium* to grow between 12 and 24 hours. The WT6 lamb could represent an intermediate stage marked by massive growth of *Escherichia*, consuming oxygen and making the environment suitable for the entry of *Clostridium*. Finally, between 24 and 36 hours, other strict anaerobic genera such as *Rumninococcus_gnavus_group* or Lachnospiraceae appear together with facultative anaerobes such as *Enterococcus* or aerotolerant genera such as *Lactococcus*. This succession from an aerobic to anaerobic environment has been reported before, although over periods of more than one week (Bäckhed F et al., 2015).

On the other hand, the *16S rRNA* amplicon sequencing study of the bacterial distribution along the digestive tract of nine lambs that died by Watery Mouth Disease shows that *Escherichia-Shigella* presents a high relative abundance along the whole digestive tract, including the abomasum. No specific location for the group was found. In contrast, *Clostridium* has a more limited distribution, with a higher relative abundance in the jejunum (20 %), ileum (25 %) and cecum (24 %), but is very scarce in the abomasum (0.85 %). A previous study by Zhuang et al (Zhuang et al., 2020). in the jejunum and colon of healthy lambs at 24 hours of life showed how *Escherichia-Shigella* is described as an abundant group, although the relative abundances for this study were much lower, occupying 22 % in the jejunum and 39 % in the colon. In addition, the genus *Clostridium* was not abundant in either the jejunum or the colon, and the genus *Lactobacillus* represented in this study as the majority group at 24 hr of life in the jejunum (70 %).

The *16S rRNA* amplicon sequencing analysis performed in the present study revealed that the three lambs sampled after the first

colostrum intake (WA1, WA2, WA3) all had *Escherichia-Shigella*. This points in favour of the oral pathway of *Escherichia coli* in the first hours of life, taking advantage of the relatively neutral abomasal pH of the newborn (Eales et al., 1986). After several intakes of colostrum (WA4), the genus *Escherichia-Shigella* in our analysis ceases to be an abundant group for the lamb and is replaced by other genera such as *Bacteroides* or *Porphyromonas*. These two genera have previously been identified as abundant in the abomasum of healthy three-day-old goats (Lei et al., 2018).

The study of the sources of contamination through coliform and mesophilic anaerobic counts established bedding as the main source of contamination. Exceeding the concentration of coliforms within the faeces of pregnant mothers by four orders. This agrees with previous studies in dairy farms, where bedding reaches levels of $10^6$ CFU/g in the soil for coliforms and sporulates (Ward WR et al., 2002; Bradley et al., 2018) suggesting that this medium is ideal for the growth of these bacterial groups.

Although previous studies have examined the bacterial count on dairy cow bedding and its influence on milk quality (Ward WR et al., 2002; Bradley et al., 2018), this study was the first to determine the evolution of aerobic, anaerobic and mesophilic anaerobic counts during a lambing period. We can say that after 48 hours of removal and deposition of new straw, the highest bacterial growth is observed, after which the levels remain stable with variations in an order of magnitude from this initial value. Therefore, the removal of straw is considered ineffective in reducing the bacterial load.

*Escherichia* and *Clostridium* do not appear within the 20 most abundant groups within the environments determined as high risk for lambs: bedding, maternal faeces, mothers' mouths, placentas and vaginal tract. However, both can be detected in all the environments sampled with the highest relative abundance in bedding for *Escherichia-Shigella* and maternal faeces for *Clostridium*, with the highest bacterial increase after 48 hours of cleaning for *Escherichia-Shigella*. There are other pathogenic groups, associated with respiratory diseases such as *Birsbersteinia* and *Manhemmia (*Sahay et al., 2020*)* on the mother's mouth, or bacteriemia complications such as *Psychrobacter* on placentas (Ortiz-Alcántara et al., 2016). Other genus such as *Histophilus* and *Fusobacterium* genus on vaginal swabs stand out as being involved in ovine infertility (Serrano et al., 2020; Boye et al., 2006).

Despite having established the bacterial profiles in lambs with Watery Mouth, it is necessary in future studies to increase the number of lambs without the presence of the disease in order to determine the profile in healthy lambs with greater accuracy. It is also necessary to rule out the presence of other pathogens, especially viruses, by means of metagenomic studies not associated with *16S rRNA*. It would also be interesting to include quantitative approaches to determine the load of the species identified, using qPCR or other quantitative methods. In studies of bedding as a source of contamination, it would be necessary to reduce sampling times between 0 and 48 hours after cleaning to establish the exact time of greatest bacterial increase, as well as to sample other areas of the livestock farms and the personnel themselves as a vector agent.

## 5. Conclusions

In this article we have studied Watery Mouth Disease from a comprehensive approach, analysing the composition of the bacterial community, identification of isolates, as well as contamination hotspots, resulting in a comprehensive understanding of the microorganisms involved and dynamics of the disease. This approach enabled us to include the *Clostridium* group with *E. coli*, as well as their temporal and intestinal distribution. Furthermore, this study demonstrates the need for implementing new control measures on farms, especially treatment of bedding 48 hours after cleaning. Finally, this study complements the previously scarce information on the disease and highlights the use of massive sequencing technology in epidemiological control in livestock farming.

## Ethics Statement

Not applicable

## Data Availability

All data are included in the research article. The sequences employed in this article have been deposited in GenBank, raw read in the Sequence Read Archive (SRA) and the complete genomes in Banklt. They are under the following Bioproject accession number, PRJNA1147586 (https://dataview.ncbi.nlm.nih.gov/object/484PRJNA1147586?reviewer=s691vmk9mr4vd10lpir09i5c26).

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## CRediT authorship contribution statement

**Álvaro Cañete-Reyes**: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Jorge Gutiérrez González**: Supervision, Methodology, Conceptualization. **Lauren V. Alteio**: Writing – review & editing, Validation. **David Rodríguez-Lázaro**: Writing – review & editing, Methodology, Investigation. **Marta Hernández**: Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marta Hernandez reports financial support, article publishing charges, and equipment, drugs, or supplies were provided by Spain Ministry of Science and Innovation. Alvaro Canete-Reyes reports financial support, equipment, drugs, or supplies, and travel were provided by Spain Ministry of Science and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2025.110542.

## Data availability

All data are included in the research article. The sequences employed in this article have been deposited in GenBank, raw read in the Sequence Read Archive (SRA) and the complete genomes in Banklt. They are under the following Bioproject accession number, PRJNA1147586 (https://dataview.ncbi.nlm.nih.gov/object/484PRJNA1147586?reviewer=s691vmk9mr4vd10lpir09i5c26).

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