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Importance of on-farm management practices on lactate-fermenting *Clostridium* spp. spore contamination of total mixed ration of Manchega ewe feeding. Determination of risk factors and characterization of *Clostridium* population

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ABSTRACT

This work studies the on-farm management practices that increase the risk of developing high lactate-fermenting *Clostridium* spp. spore counts in Total Mixed Ration (TMR) of Manchega sheep. Moreover the ecology of the *Clostridium* population was studied in relation to those management practices. A total of 136 TMR samples belonging of 23 Manchega sheep flocks were analyzed for lactate-fermenting *Clostridium* spores by the Most Probable Number technique (MPN). Information about the feeding characteristics was also collected from the 23 flocks. A logistic regression analysis indicated silages and wet brewerís grains used for feeding as the on-farm management risk factors that lead to an increase of *Clostridium* spp. spore counts. A total of 155 *Clostridium* isolates from TMR samples were typed using Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR). The 56 different genotypes obtained were subsequently identified by restriction analysis of 16S-rRNA gene (16S-ARDRA), so three populations were observed: 92.90%, 5.81% and 1.29% corresponding to *Clostridium* (*C.*) *sporogenes, Clostridium beijerinkii* and *Clostridium butyricum* respectively. The risks factors of high *Clostridium* spp. spore counts also increased *Clostridium* population diversity and favoured the presence of *C. butyricum*. The results confirmed that these risk factors should be taken into account in developing strategies in the control of *Clostridium* spp. spores contamination in TMR.

1. Introduction

Spain is one of the largest producers of dairy sheep in European Union. Sheep milk production in Spain has increased to 457.6 thousand tonnes in 2014 (MAGRAMA, 2014). Manchega sheep is one of the most important native dairy sheep in Spain (529.505 sheep) and its principal production area is Castilla-La Mancha region, with an estimated milk production of 73 million liters of milk in 2015 (ESROM, 2015). The sheep milk production is influenced by many factors: genetic, season, management, health, feeding, etc. The quality of feeding is very important in current's dairy sheep production systems. Feeding is one of the highest costs in these herds and directly affects the quality of milk. In commercial farms, Total Mixed Ration (TMR) in milking sheep is used. In its preparation it is common to use silage and other wet products. Previous studies in cattle (Colombari et al., 2005; Vissers et al., 2006; Julien et al., 2008) and in dairy sheep (Arias et al., 2013) have shown that these raw materials are sources of lactate-fermenting *Clostridium* spp. spores contamination in milk and these microorganisms are responsible of the late-blowing defect in ripened cheeses (Garde et al., 2011).

Studies in cattle demonstrate that it is necessary to use good management practices in feeding to prevent the butyric spores contamination increase (Gaggiotti et al., 2007; Vissers et al., 2007a; Borreani and Tabacco, 2008). In ewe milk, there are few studies (Salmerón et al., 2002; Scintu et al., 2004), and they have not established the management practices affecting *Clostridium* spp. spore contamination in TMR. For this reason, the aims of this study are to determine the management practices in dairy sheep production systems considered as risk factors to obtain high *Clostridium* spp. spore counts (>5 10^3 spores g^{-1}) in TMR and to determine how these practices affect *Clostridium* populations.

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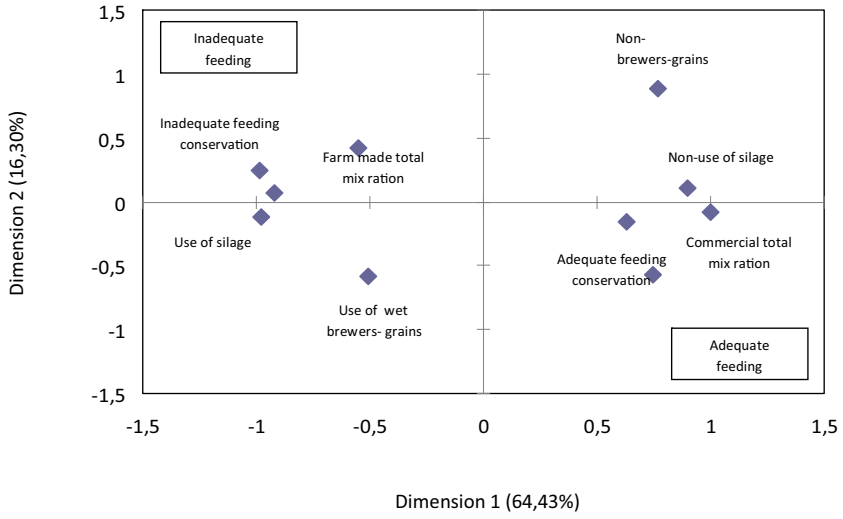

Fig. 1. Multiple correspondence analysis of risk factors for *Clostridium* spp. spore contamination of TMR.

2. Materials and methods

2.1. TMR samples

Every 2 months over 1-year period (March 2008–March 2009) TMR samples (n = 136) were taken from 23 farms belonging to the National Manchega Breeders Association (AGRAMA). TMR samples were transported to the laboratory and analyzed on the day of collection.

Samples were collected from the already prepared and in-barn dispensed TMR, using 500 g bags as recommended Vissers et al. (2007a).

2.2. Enumeration of lactate-fermenting Clostridium spp. spore

Lactate-fermenting *Clostridium* spores were enumerated by the most probable number (MPN) technique. The TMR samples were prepared following the procedure recommended by Te Giffel et al. (2002) and Vissers et al. (2007a, 2007b). Briefly, 10 g of the homogenized sample was mixed with 90 mL of sterile 0.1% (w/v) peptone solution (Cultimed, Panreac, Barcelona, Spain). This mix was homogenized by stomacher (IUL S.A., Barcelona, Spain) during 3 min and then filtered. Then, decimal dilutions of TMR samples were prepared in sterile 0.1% (w/v) peptone solution (Cultimed). Aliquots (1 mL) from serial dilutions of samples were inoculated into three tubes series containing 9 mL of Bryant and Burkey broth (BBB; Merck, Darmstadt, Germany). The tubes were overlaid with 2 cm of sterile melted paraffin and heated at 75 °C for 10 min to kill vegetative cells. Incubation was carried out at 37 °C for up to 7 days and daily tested for gas production. MPN counts were expressed as spores g^{-1}.

2.3. Obtaining pure cultures

A culture from each BBB gas positive tube from each dilution was transferred onto a plate of Reinforced Clostridial Agar (RCA; Difco, Detroit, MI, USA). Plates were incubated at 37 °C for 72 h in anaerobic jars with an H_2 plus CO_2 generating kit (AnaeroGen; Oxoid, Basingstoke, UK). Two isolates of each colony morphology type were randomly picked up from each TMR sample and were grown in tubes with RCM at 37 °C for 72 h.

In order to obtain pure cultures, isolates were alternatively grown in tube and in plate using RCM and anaerobic conditions as described above. Then isolates were Gram stained, tested for inability to grow aerobically on RCA and morphologically examined for the presence of endospores by using a phase contrast microscope.

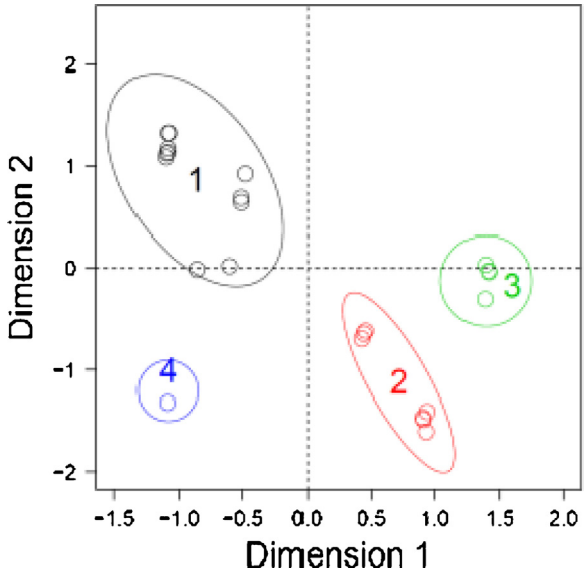

Fig. 2. Cluster analysis of farms in relation to risk factors for *Clostridium* spp. spore contamination of TMR. Group 1: farms with inadequate feeding practices; Group 2: farms with adequate feeding practices; Group 3: farms with adequate feeding practices but wet brewers' grains are used; Group 4: farm with inadequate feeding practices but wet brewers' grains are not used.

The purified isolates were stored in Reinforced Clostridial Medium (RCM, Difco, Detroit, MI, USA) with 5% glycerol at −80 °C until use.

2.4. DNA extraction from pure cultures

DNA extraction was performed as indicated by Garde et al. (2011). Colonies from the pure culture plate were collected with a swab and suspended in 1 mL of water MiliQ. The suspension was subjected to centrifugation at 12,000 rpm at 5 °C for 5 min, and the pellet was washed and suspended in 50 μL water MiliQ500. Then, 30-min incubation at 95 °C and 500 rpm in a Grant Bio PHMT Thermoshaker (Nirco S.L., Barcelona, Spain) was performed to break up the cells. Later the suspension was subjected to centrifugation at