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Bovine fecal biomarkers of intestinal inflammatory process: Calprotectin and lactoferrin, a comparative study

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ABSTRACT

Fecal lactoferrin and fecal calprotectin have been proposed as biomarkers of intestinal inflammation in several animal species. The main objectives of this work were to validate an analytical procedure for the measurement of lactoferrin in calf feces, to study the correlation between lactoferrin and calprotectin concentrations, and to evaluate the influence of fecal water content in the determination of these proteins. This knowledge is essential for effectively using these biomarkers in young calves exposed to inflammatory gastrointestinal diseases.Seventy-eight male Holstein dairy calves between two and three weeks of age were included in the study. Lactoferrin was determined with a bovine milk lactoferrin ELISA kit and calprotectin was measured using a human immunoturbidimetric method previously validated in bovine feces. Analytical validation of the lactoferrin assay achieved good results, with intra and inter assay CV < 10 %, recovery rates between 80 and 120 %, and optimal linearity under dilution. A robust correlation was observed between fecal calprotectin concentrations in dry and wet feces ($r = 0.903$), while a moderate correlation was observed for fecal lactoferrin concentrations ($r = 0.648$). Correlation between both biomarkers was moderate in fresh feces ($r = 0.514$) as well as in dry feces ($r = 0.561$). In conclusion, the lactoferrin ELISA kit is valid for its use with calf fecal samples, both biomarkers showed a moderate correlation between them, and fecal lactoferrin concentration is more influenced by feces moisture than fecal calprotectin concentration.

Inflammation of the gastrointestinal tract impairs organ functionality causing diarrhea that compromises the calves' health, welfare, and performance. Dairy calves and dairy-beef cattle are exposed to a wide number of conditions that might affect gastrointestinal function, such as transport, commingling, and changes in nutrition (Soares et al., 2022). More than 70 % of the immune system cells are found in the intestinal tract, therefore an intestinal inflammatory process can cause the release of their cellular components into the blood and/or feces (Celi et al., 2019; Soares et al., 2022). The quantitative determination of these components specifically released under gut inflammatory conditions, could be used for diagnostic or prognostic purposes. This, in turn, could help defining better practices for the transport, management, and nutrition of young livestock.

Fecal calprotectin (fCAL) and fecal lactoferrin (fLF) are biomarkers of gastrointestinal inflammation widely used in human medicine (Lamb and Mansfield, 2011). They are resistant to proteolysis, unaffected by multiple freeze-thaws, and stable in stool for as long as five to seven days (Yamamoto, 2015). Calprotectin is a calcium-bound protein complex expressed and released by neutrophils, monocytes, activated macrophages, and dendritic cells that accumulate at sites of inflammation. On the other hand, lactoferrin is an iron-binding glycoprotein secreted by neutrophils and epithelial cells at inflammation sites. Both proteins have antimicrobial effects and are part of the innate immune response (Soares et al., 2022; Wang et al., 2017).

Fecal samples are non-invasive and easy to collect, making them a very useful tool to identify intestinal inflammation. We have previously validated an immunoturbidimetric assay for fCAL in bovine and porcine feces (Pato et al., 2023). In the present work, the main objective has been to validate an ELISA (enzyme-linked immunosorbent assay) initially developed for the measurement of lactoferrin in bovine milk, for its application to calf bovine feces. Furthermore, we aimed to elucidate the influence of the water content of feces in fCAL and fLF concentrations, and the correlation between fLF and fCAL.

For this experiment, seventy-eight male Holstein dairy calves

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between two and three weeks of age were used. Calves belonged to another study which evaluated calf recovery after long-distance transportation (approved by the Animal Care Committee of the Government of Catalonia, project number 12075). Fecal samples were collected at arrival (day 0), and on days 2 and 7 after arrival via rectal stimulation and were previously stored at −80 °C until analysis.

The lactoferrin ELISA kit used in the present work (E11–126, Bethyl Laboratories) was designed for bovine milk, and validation for its use in calf fecal extracts was necessary, as stated in the recommendations of the European Medicines Agency (European Medicines Agency, 2011). The extraction procedure for fLF was based on the methodology described for adult cows (Cooke et al., 2020) with some modifications due to the higher concentration of lactoferrin in calves than in adults. On the day of extraction, samples were thawed at room temperature, and a 1:100 dilution was prepared by weighing 50 mg of fresh feces in 5 mL of buffer A (0.37 M NaCl, 10 mM NaH_2PO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) with a protease inhibitor cocktail (cOmplete, EDTA-free Protease Inhibitor Cocktail, Roche, Germany). Tubes were vigorously vortexed for 1 min and centrifuged at 4 °C for 5 min at 3000 rpm. The supernatant was centrifuged again at 12000 rpm for 5 min at 4 °C. To ensure the complete recovery of lactoferrin, a second extraction was performed from the first fecal pellet by adding 5 mL of buffer A and protease inhibitor, followed by two centrifugation steps, at 3000 and 12,000 rpm. The two extracts were combined and stored at −80 °C. A third extraction did not improve the recovery of fLF. Lactoferrin was measured in the extracts following the instructions of the ELISA kit.

Validation of the lactoferrin ELISA kit was performed following the ASVCP Guidelines, comprising the assessment of linearity, recovery, and precision (Arnold et al., 2019). Accuracy of the assay was assessed by a) linearity under dilution and b) recovery experiment (spiking). Linearity was determined by using two different fecal extracts serially diluted (1/2, 1/4, 1/8, 1/16) and results are shown in Fig. 1, with a coefficient of determination of $r^2 = 0.9976$. For the recovery, within-run accuracy was performed by spiking pure lactoferrin (standard of the ELISA kit) in three samples of known concentration (high, medium, and low) by duplicate, and between-run accuracy by spiking a random sample with a known amount of lactoferrin in two different days. Recovery results in the within-run accuracy test ranged from 87 % to 96 %, and results in the between-run accuracy test were 107 % and 113 %, respectively.

The intra-assay precision was calculated by measuring eight times two samples of known concentration (low and high) in the same ELISA plate, and the CV (coefficient of variation) was 5.2 % for the low concentration sample (56.3 ng/mL) and 6.4 % for the high concentration sample (392.2 ng/mL). The inter-assay precision was calculated by measuring two samples (low and high concentration) once on five different days (5 different ELISA plates), and CVs were 9.5 % and 5.7 %, respectively.

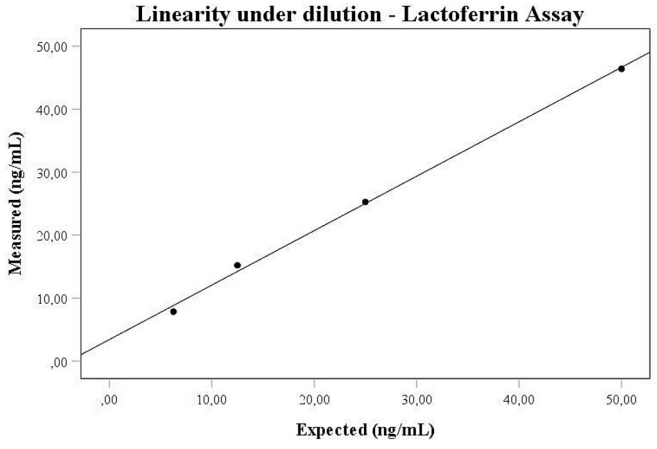

Fig. 1. Linearity test of Bovine Lactoferrin ELISA kit used with calf fecal samples. Concentrations correspond to 1/2, 1/4, 1/8 and 1/16 dilutions.

These results demonstrate that both accuracy (linearity under dilution and recovery) and precision showed optimal results. Recovery results showed that the detection of fLF was not affected by the biological matrix of the sample, since they fell within the acceptability criteria: 80–120 % (Andreasson et al., 2015). Linearity under dilution, with a r^2 close to 1, indicated the absence of scattering. On the other side, repeated individual measures of lactoferrin showed good precision since the CV of both within-run and between-run precision did not exceed 10 % (FDA, 2018). In conclusion, the lactoferrin ELISA kit from Bethyl Laboratories is valid for its use with fecal samples in newborn calves, providing that samples are adequately diluted during extraction.

Next, we wanted to correlate fCAl and fLF and check whether the water content of the samples could affect the results. For this, sample preparation and determination of fCAL were performed following the methodology described in (Pato et al., 2023), and the water content of stools was calculated by weighing feces before and after placing the samples in an oven at 105 °C for 24 h.

The influence of fecal water content in fCAL and fLF measurements was studied using Spearman's correlation between results obtained in dry and fresh samples (Fig. 2A, B). A very strong correlation was observed between fCAL concentration in both fresh and dry feces ($r = 0.903$, $p < 0.01$). The fCAL concentrations ranged from 2.7 to 936.0 μg/g of dry feces and from 0.5 to 135.8 μg/g of fresh feces. A moderate correlation was observed between fLF concentration in both fresh and dry feces ($r = 0.648$, $p < 0.01$). The fLF concentrations ranged from 22.2 to 668.6 μg/g of dry feces and from 3.8 to 93.1 μg/g of fresh feces.

To analyze the correlation between both biomarkers, fCAL and fLF concentrations were plotted, and a moderate correlation with an $r = 0.514/0.561$ ($p < 0.01$) was found in fresh and dry feces, respectively (Fig. 2C, D), indicating that this correlation was not influenced by fecal moisture. It must be taken into account that the extraction carried out for each protein was different. It is observed that fLF concentrations (Fig. 2B) were more influenced by feces moisture than fCAL concentrations (Fig. 2A) and one reason could be that the distribution of fLF in calf feces is not as homogeneous as fCAL. This fact must be considered when preparing fecal samples for fLF assay.

Colostrum and milk are rich in lactoferrin content, and sometimes supplemental lactoferrin is included in the diet to increase preweaning weight gain in calves (Robblee et al., 2003), and this could interfere with the determination of lactoferrin in feces. In the present case, calves were fed with milk replacer or acidified milk in similar percentages, and our results showed that fLF content is not affected by the type of nutrition. Thus, feeding treatment in the present case does not influence the fLF content of the samples.

In conclusion, both proteins can be determined in feces with good analytical performance in fresh or dry stools and, consequently, they can be proposed as good candidates to be evaluated as biomarkers for intestinal inflammatory disorders in calves. Since correlation was not strong, caution should be taken when comparing results obtained in fresh and dry feces.

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CRediT authorship contribution statement

Yolanda Saco: Conceptualization, Supervision, Writing – original draft. **Núria Crusellas-Villorbina:** Formal analysis, Methodology. **Raquel Peña:** Data curation, Methodology. **Raquel Pato:** Methodology. **Sonia Marti:** Investigation, Writing – review & editing. **Lucía Pisoni:** Investigation, Writing – review & editing. **Maria Devant:** Investigation, Writing – review & editing. **Anna Pelegrí-Pineda:** Investigation, Writing – review & editing. **Anna Bassols:** Resources, Supervision,

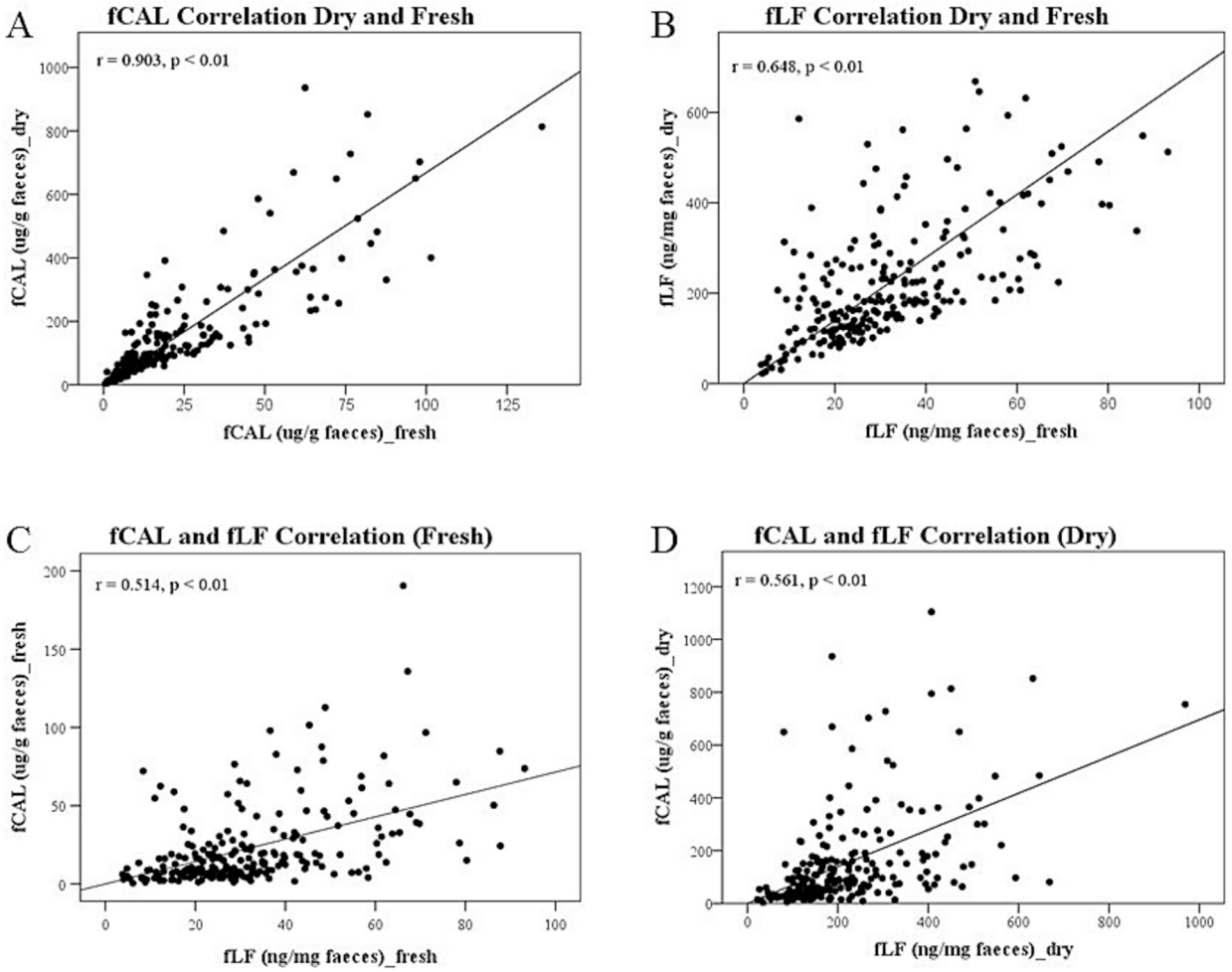

Fig. 2. (A) Influence of fecal water content on fCAL and (B) fLF concentrations. Correlation of fCAL and fLF in (C) fresh feces and in (D) dry feces.

Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest. The authors have no affiliations or final involvement with any organization or entity with a financial interest in, or in financial competition with the subject matter discussed in this article. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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