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Lactoferrin quantification in cattle faeces by ELISA

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Background: Promoting and maintaining health is critical to ruminant welfare and productivity. Within human medicine, faecal lactoferrin is quantified for routine assessment of various gastrointestinal illnesses avoiding the need for blood sampling. This approach might also be adapted and applied for non-invasive health assessments in animals.

Methods: In this proof-of-concept study a bovine lactoferrin enzyme-linked immunosorbent assays (ELISA), designed for serum and milk, was applied to a faecal supernatant to assess its potential for quantifying lactoferrin in the faeces of cattle. Faecal lactoferrin concentrations were compared to background levels to assess the viability of the technique. A comparison was then made against serum lactoferrin levels to determine if they were or were not reflective of one another.

Results: The optical densities of faecal samples were significantly greater than background readings, supporting the hypothesis that the assay was effective in quantifying faecal lactoferrin ($T_{13, 115} = 11.99$, $p < 0.0005$, n = 115). Lactoferrin concentrations of faecal and serum samples, taken from the same animals on the same day, were significantly different ($T_{21} = 2.49$, $p = 0.022$) and did not correlate (r = 0.069, $p = 0.767$).

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Lactoferrin quantification in cattle faeces by ELISA

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i. Abstract

Background: Promoting and maintaining health is critical to ruminant welfare and productivity. Within human medicine, faecal lactoferrin is quantified for routine assessment of various gastrointestinal illnesses avoiding the need for blood sampling. This approach might also be adapted and applied for non-invasive health assessments in animals.

Methods: In this proof-of-concept study a bovine lactoferrin enzyme-linked immunosorbent assays (ELISA), designed for serum and milk, was applied to a faecal supernatant to assess its potential for quantifying lactoferrin in the faeces of cattle. Faecal lactoferrin concentrations were compared to background levels to assess the viability of the technique. A comparison was then made against serum lactoferrin levels to determine if they were or were not reflective of one another.

Results: The optical densities of faecal samples were significantly greater than background readings, supporting the hypothesis that the assay was effective in quantifying faecal lactoferrin ($T_{13,\ 115} = 11.99$, $p < 0.0005$, n = 115). Lactoferrin concentrations of faecal and serum samples, taken from the same animals on the same day, were significantly different ($T_{21} = 2.49$, $p = 0.022$) and did not correlate (r = 0.069, $p = 0.767$).

1 Introduction

Ruminant gastrointestinal health is central to ensuring animal welfare and to facilitating productivity and sustainability in commercial ruminant systems. Gut inflammation can be symptomatic of poor health and cause economic losses related to reduced feed conversion and productivity [1–5]. As worldwide demand for meat increases [6], along with pressures on the natural resources that support its production [7], it is essential that gut health is optimised to improve the efficiency and sustainability of livestock production systems. This calls for the urgent development of economically viable diagnostic tools for the rapid diagnosis of gut disease, to support prevention and rapid correction of poor gut function.

Recently, Watt *et al.* [8] and Cooke *et al.* [9] both demonstrated that enzyme-linked immunosorbent assays (ELISA), designed for use on serum and milk, can be utilised for quantifying anti-parasite antibodies in the faeces of sheep and cattle [8, 9]. Such techniques can

provide valuable insights into the health of livestock, particularly in relation to parasitic diseases and potentially gastrointestinal health in general. Another advantage of faeces-based methods is that samples can be collected non-invasively and without negative impacts on welfare. Wider potential benefits include the immunological assessment of animals that cannot be directly sampled, e.g. if they are evasive or dangerous.

Lactoferrin is an inflammatory marker and key indicator of gut damage. Lactoferrin binds to iron, preventing its utilisation by bacteria and producing a bacteriostatic effect [10]. Furthermore, it can regulate immune responses against infection, preventing inflammation by modulating immune cell function, migration and maturation [11, 12]. Although predominantly found at mucosal surfaces, lactoferrin can be detected in milk and serum [13]. In human medicine, faecal lactoferrin is used as an inflammatory marker in the diagnosis of gastrointestinal conditions such as inflammatory bowel diseases and Crohn's disease [14–17]. In contrast, quantification of lactoferrin is not routinely conducted within veterinary medicine, other than for the analysis of bulk-tank milk [18–21].

The purpose of this study is to apply the principles of this common practice in human medicine to veterinary medicine, using techniques analogous to those presented by Watt *et al.* [8] and Cooke *et al.* [9]. That is, to assess if lactoferrin can be quantified in the faeces of ruminants by using ELISA. If successful, this proof-of-concept would demonstrate potential for the future development of ruminant faecal lactoferrin ELISA as an indicator of animal health and gut function.

2 Methods

2.1 Sample populations

Faecal samples were collected from three herds of beef cattle located in Cornwall, Angus, and Hertfordshire in the UK (C1, C2, C3 respectively) (**Error! Reference source not found.**). Groups C1 and C3 were fed on grass silage for at least one month prior to sampling, and group C2 was permanently grazed on pasture. A total of 117 faecal samples were collected from the three farms (65, 30 and 22 from C1, C2 and C3 respectively), and 22 blood samples were collected from C3.

Sheep and deer samples (faeces and blood) were available from other experiments. No commercial lactoferrin ELISA was available for analysis of those samples, so they were subject to the bovine lactoferrin ELISA protocol. Further details of the analysis of sheep and deer samples is available in the supplementary material.

2.2 Sample collection & preparation

2.2.1 Faecal samples

Fresh faeces were collected from the ground immediately after defecation was observed. Faecal samples were stored in screw-top 100 mL plastic containers and stored at -18°C until processing. Samples were defrosted at room temperature and mixed with a protease inhibitor (cOmplete™, EDTA-free Protease Inhibitor Cocktail) at a ratio of 1:1 - 1:2 (w:v) with phosphate buffered saline, depending upon consistency and moisture. This mixture was homogenised and centrifuged at 3-6°C and 12000 x g (Sorvall SLA-3000 rotor in a Sorvall RC-5B centrifuge, ThermoFisher Scientific, USA) for 5 min. The faecal supernatant was then removed by pipette and stored at -18°C.

2.2.2 Blood samples

Blood samples were collected by tail venepuncture into glass Vacutainers® (Becton Dickinson, USA). Samples were left >30 min to clot and were centrifuged at 1056 x g (Sorvall SLA-3000 rotor in a Sorvall RC-5B centrifuge) for 15 min to separate serum, which was removed by pipette and stored at -18°C.

2.2.3 Serial dilutions

Test plates were conducted to determine the optimum concentration of faecal supernatants and serum (diluted with Tris-buffer saline with 0.05% Tween™ 20 (TBST)) to achieve optical densities within the detection limits of the plate reader and to best show the variation in the datasets. The optimum dilution was qualitatively determined as the dilution at which no notable plateauing or data clumping had begun, which can both be features of more dilute samples. If two dilutions presented similar qualities, the least concentrated was chosen to ensure the capture of lactoferrin samples higher than those on the trial plates and to preserve sample quantity. Test plates

were conducted on 63 cattle faecal samples at concentrations of 1/1, 1/2, 1/8 and 1/32, and on 25 cattle serum samples at concentrations of 1/5, 1/10, 1/20, and 1/40.

On each plate, a seven-point halving series dilution of bovine lactoferrin standard was included (Bethyl Laboratories; RC10-126-8) for reference and as a positive control. Stock solution was 1000 μg mL^{-1}, and for the first standard in the series was diluted to 0.5 μg mL^{-1} with TBST. Subsequent dilutions added 500 μL of the previous solution in the series to 500 μL of TBST. Each plate also included three negative control blanks of TBST.

2.3 ELISA protocol

The ELISA were conducted using a commercially available bovine lactoferrin ELISA set (Bethyl Laboratories Inc., E10-126) which is produced primarily for use on bovine milk samples.

The plate coat was made by mixing affinity purified antibody (Bethly Laboratories Inc. A10-126A) with carbonate buffer at a ratio of 1:100 (v:v). Then, 100 μL of the formed coat was added to each well and the plates (Nunc-Immuno Maxsorp 96-well) were covered in cling film and incubated at 20°C for 1 h.

After the first incubation, the plates were washed 5 times with TBST using an automated plate washer. Two hundred μL of TBST was added to each well as a blocking solution and plates were covered in cling film and incubated at 20°C for 30 min.

After the second incubation, the plates were washed 5 times in TBST before 100 μL of sample was added to each well (except blanks) and plates were covered in cling film and incubated at 20°C for 1h.

After the third incubation, the plates were washed 5 times in TBST before 100 μL of horseradish peroxidase (HRP) detection (0.5% with carbonate buffer) antibody was added to each well and the plates were covered in cling film and incubated at 20°C for 1h.

After the fourth incubation, the plates were washed 5 times in TBST before 100 μL of enzyme substrate (SureBlue™ TMB Microwell Peroxidase Substrate Kit) was added to each well before the plates were placed in opaque boxes and incubated at 20°C for 15 min. Then, 100 μL of stop solution, 0.18m H_2SO_4, was added to each well and plates were immediately read for optical density at 450nm by a plate reader.

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2.4 Dry matter and optical density assessment

The moisture content of fresh faeces depends on animal diet and state of hydration. Temporal variation in faecal moisture content may significantly influence the concentrations of components, including lactoferrin, within the faeces. Thus, a simple test was conducted to investigate whether faecal moisture content or sample background optical density were related to lactoferrin concentrations.

For 56 of the cattle faecal samples (from groups C1, C2, and C3), faecal supernatants at 1:2 (w:v) ratio of faeces to protease inhibitor, were measured to determine optical density. This was conducted twice using 100 µL and 50 µL of supernatant. Blank 96-well plates were initially read to determine background optical density. Aliquots of each supernatant were pipetted into individual wells, avoiding the outer two rows and columns, to avoid potential edge effects. Plates were then read using a plate reader, to determine optical density, from which the background value was subtracted. Supernatant optical densities were then correlated to lactoferrin concentration and subsequent regression analysis conducted.

Twenty-nine of the faecal cattle samples (from groups C1, C2, and C3) were analysed for dry matter content by gravimetric loss at 65°C to a constant weight.

2.5 Statistical analysis

All statistical analyses were performed to a confidence level of 95% in Minitab 18 (Minitab Ltd., UK). Prior to statistical testing outliers were identified using a Grubb's outlier test and subsequently removed from relevant analysis. Two-sample *T*-tests were used to determine if faecal supernatant ODs were significantly above background levels (TBST blanks). Pearson's correlations were performed to determine if the test results for serum and faecal samples, matched per individual and taken on the same day, were correlated. A Pearson's correlation was also performed to assess if faecal sample moisture correlated to lactoferrin concentration.

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3 Results

3.1 Controls, references, and calibration

Negative controls of TBST were consistent across all plates and had a mean background optical density of 0.0486, ranging from 0.0467 to 0.0506, with a relative standard error of 0.77%. Reference material gave consistent curves with a mean relative standard error of 1.51% across all dilutions.

Based on the results of three initial test plates, it was determined that a sample concentration of 50% was optimum for cattle faecal samples. At this dilution, all samples yielded optical densities significantly above background levels, as determined using 2-sample *T*-tests that compared background levels to faecal supernatants ($T_{13, 115} = 11.99$, $p < 0.0005$) (Figure 1).

3.2 Lactoferrin concentrations

A Grubbs' test found one outlier value within the cattle faecal data set (1.937 µg mL-1). This sample was removed from consideration during statistical analyses.

The mean lactoferrin concentration across all cattle faecal samples was 0.258 µg mL^{-1} (S.E. 0.027) (Figure 2). For serum samples, the mean concentration was 0.075 µg mL^{-1} (S.E. 0.004) (Figure 3). A paired *T*-test comparing matched faecal and serum samples from the same individuals, taken on the same day, found a statistically significant difference between serum and faecal lactoferrin ($T_{21} = 2.49$, $p = 0.022$). Furthermore, no statistically significant correlation was found between faecal and serum lactoferrin concentrations taken from the same individuals on the same day ($r = 0.069$, $p = 0.767$).

3.3 Faecal dry matter and optical density assessment

Optical density of faecal supernatants correlated significantly with lactoferrin concentration at 100 µL ($\rho = 0.377$, $p = 0.004$) but not 50 µL (Pearson's correlation = 0.135, $p = 0.135$) (Figure 4). Subsequent regression analysis of lactoferrin concentration as a response to optical density at 100 µL yielded an r^2 of 14.2%.

Dry matter content of faeces was not significantly correlated with lactoferrin concentration ($\rho = -0.148$, $p = 0.161$) (Figure 5).

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4 Discussion

The optical densities of faecal and blood samples, isolated according to the extraction methods, significantly exceeded those from blank controls (TBST). It is, therefore, concluded that the ELISA protocol was successful for the detection of lactoferrin in faeces of cattle. Furthermore, we observed similar results for samples from sheep and deer sampled in Scotland (see supplementary material). We, therefore, accept the hypothesis that lactoferrin can be quantified in the faeces of ruminants using commercially available ELISA products.

The lack of correlation between faecal lactoferrin and serum lactoferrin taken from the same individual cattle on the same day suggests that faecal lactoferrin quantification cannot necessarily be used as a proxy for serum or milk lactoferrin. This finding strongly indicates the potential further development of the assay to specifically measure gut health, as opposed to systemic health. This lack of correlation between lactoferrin concentrations from blood serum and faecal supernatant is likely due to multiple sites of lactoferrin production within the body, including at the mucosal surface of the gut, and the contrasting metabolic sources of milk, serum, and faeces. This has been previously described for lactoferrin production in the human body [22, 23]. Confirmation of the gut as the physiological source of the lactoferrin in the faeces of ruminants could be achieved by taking swab samples for analysis along gastrointestinal transects of recently slaughtered individuals and comparison with matched faecal samples taken immediately prior to death. However, the contrasting chemistries of ruminant faeces and blood, and the manner in which lactoferrin reacts to different organic and inorganic molecules therein, may affect the successful extraction of the immune-marker from the different substrates. For example, inconsistent avidity of the ELISA to different physical forms of lactoferrin has been identified [24–27]. Based on the complexities related to the presentation of lactoferrin in faeces, future methodological development should include comparison with complementary established techniques in molecular biology that detect specific proteins, e.g. western blot, to ratify the results obtained by ELISA to establish its reliability. However, lactoferrin ELISAs are established and recognised [28], and widely used to quantify human lactoferrin in faeces [14, 15, 29] and bovine lactoferrin in milk and serum [30], supporting the use of this technique for quantifying bovine faecal lactoferrin.

The comparison of faecal sample dry matter and background optical density to lactoferrin concentration highlighted an important consideration when using faecal material for any molecular

quantification techniques. The physical composition of faeces can vary greatly [32], not just between individuals, but for the same individual at different times, diluting or concentrating immunomarkers within sampled faeces. Furthermore, diarrhoea (high faecal moisture content) can be symptomatic of gastrointestinal disease or pathology or infection [33–35], creating a confounding factor. The mechanism driving the significant relationship between supernatant optical density and lactoferrin is unknown but may relate to the effects of gut damage on faecal composition. Further investigation is required to determine any systematic adjustment or interpretation of ELISA results related to potential confounding factors.

5 Conclusion

The objective of this research was to assess the feasibility of quantifying lactoferrin in the faeces of cattle. The development of a rapid and non-invasive test for the gut health of ruminants using faecal lactoferrin quantification has potentially wide-reaching benefits. Immunological assessments of mammals are typically invasive and can be logistically difficult due to animal aggression, evasiveness and animal welfare legislation. Faecal sampling, therefore, offers an opportunity for the wide-ranging assessment of gut health in mammals.

This study trialled an existing ELISA developed to quantify lactoferrin concentrations in milk through the application to the supernatant of faecal samples of ruminants (beef cattle) from three geographically distinct regions of the UK. There was no relationship between the concentrations of lactoferrin in faecal supernatant and serum, suggesting different metabolic sources of lactoferrin, or differences in the success of lactoferrin extraction from two different substrates. Therefore, faecal lactoferrin may provide novel information that can provide new insight into animal health. Robust interpretation of faecal lactoferrin ELISA results will require substantial future work. Nevertheless, this successful proof-of-concept highlights the how lactoferrin, and potentially other immune-markers, can be quantified non-invasively for the assessment of animal health.

6 Abbreviations

ANOVA – analysis of variance

EDTA - ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

GIN – gastrointestinal nematode(s)

H_2SO_4 – sulfuric acid

HRP – horseradish peroxidase

OD – optical density

TBST – tris-buffered saline with Tween 20

7 Declarations

7.1 Ethical approval and consent to participate

All cattle blood samples were taken by a trained and qualified veterinary surgeon who was conducting routine analysis in support of animal health under the UK Veterinary Surgeons Act 1966. Samples were analysed on request of the veterinary surgeon using excess samples and did not require excess blood being drawn in addition to what was required under routine practice.

Sheep blood samples were taken under the Animal Scientific Procedures Act (1986). Samples used in this study were remnant samples taken under project license no: PPL 60/4211, personal license no: PIL 60/623.

The single deer blood sample was taken with the landowner's permission from an animal shot for food.

7.2 Consent for publication

Not required

7.3 Availability of data and material

Data generated and or analysed during this study are included in this published article [and its supplementary information file].

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7.4 Competing interests

The authors declare that they have no competing interests.

7.5 Funding

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7.6 Author's contributions

A.S. Cooke instigated the research project and the collaboration between Rothamsted Research/University of Bristol and the University of Edinburgh. He was involved in all the cattle sample collection, protocol development, and all laboratory and statistical analyses, and led the writing of the manuscript.

K. Watt was central to developing the ELISA protocols within the study, laboratory analysis of samples, and assistance with manuscript preparation.

G. Albery collected deer faecal samples and extracted them and provided input and feedback on the manuscript.

E.R. Morgan contributed by gaining the funding for the project with J. A. J. Dungait and advising on and contributing to manuscript content, especially to contextualise the work from a veterinary perspective.

J. A. J. Dungait contributed by winning the funding which was used for this project. J. A. J. Dungait also facilitated the development of the project by gaining support from the farms within the study. Professor Dungait provided valuable support and scientific input throughout the length of the experiment and manuscript preparation.

7.7 Acknowledgements

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Figure 1

Bar chart showing average optical densities, after ELISA process, of faecal, serum, and TBST blank samples. Error bars represent standard error.

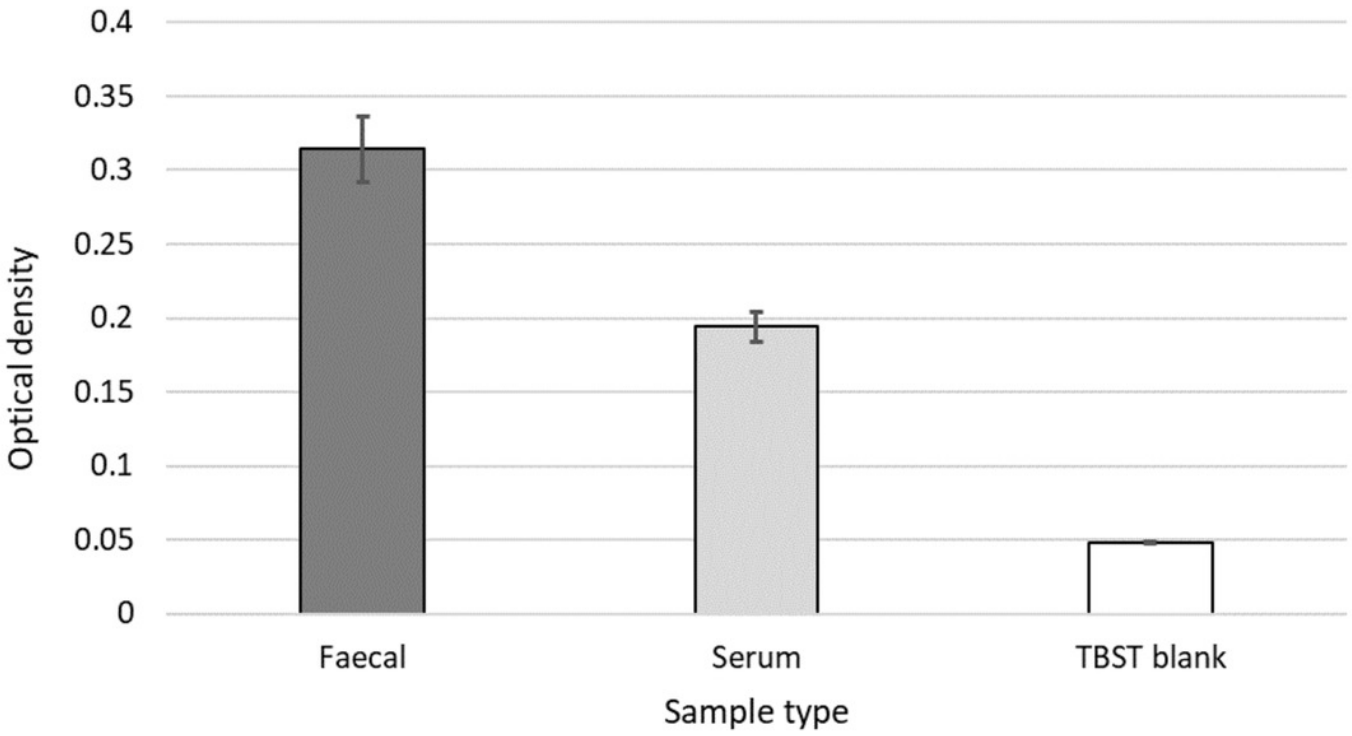

Figure 2

Kite graph showing the distribution of cattle faecal lactoferrin concentrations (µg mL^{-1}) (n = 115).

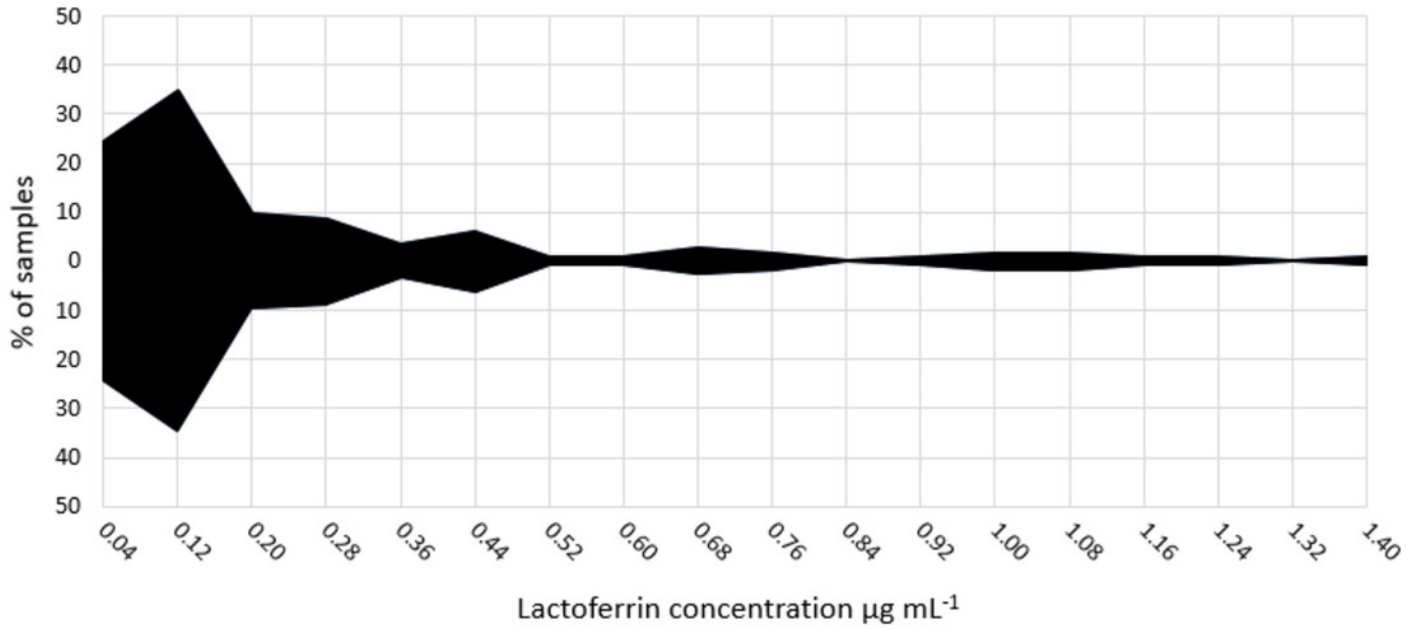

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Figure 3

Kite graph showing the distribution of cattle faecal lactoferrin concentrations (µg mL^{-1}) (n = 22).

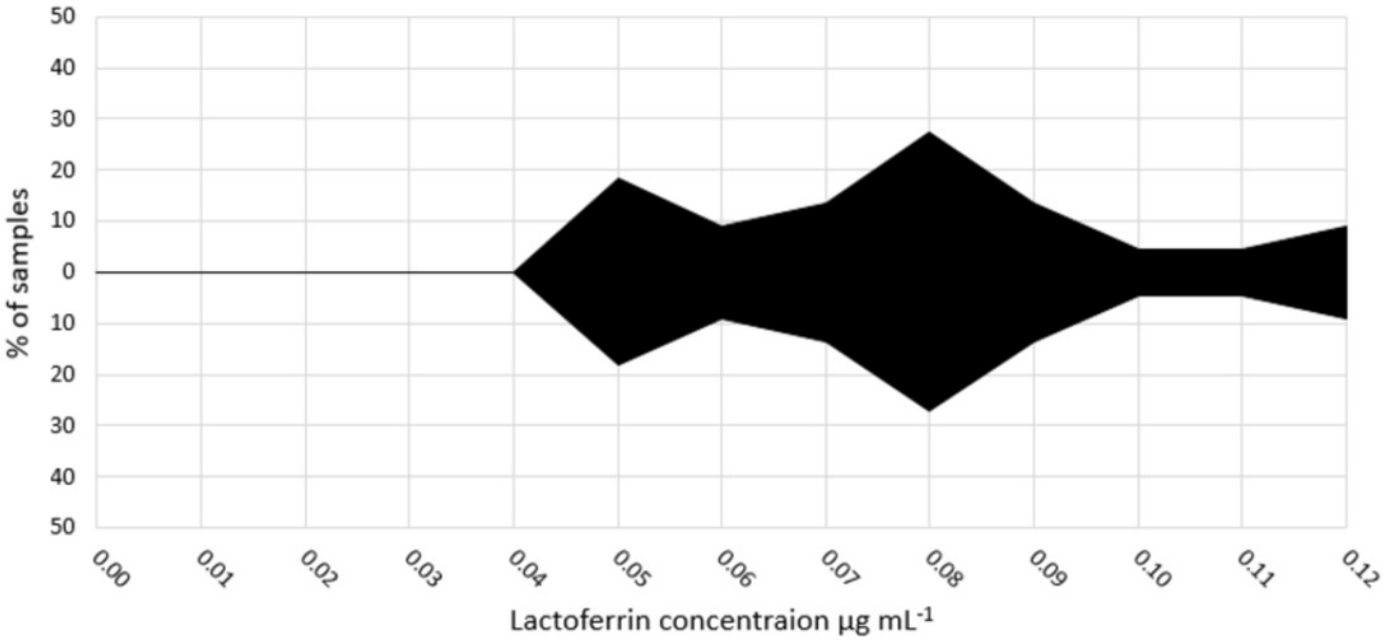

Figure 4

Scatterplot with trendlines showing the relationship between faecal supernatant optical densities (at 50 µL and 100 µL) and lactoferrin concentration (µg mL^{-1}).

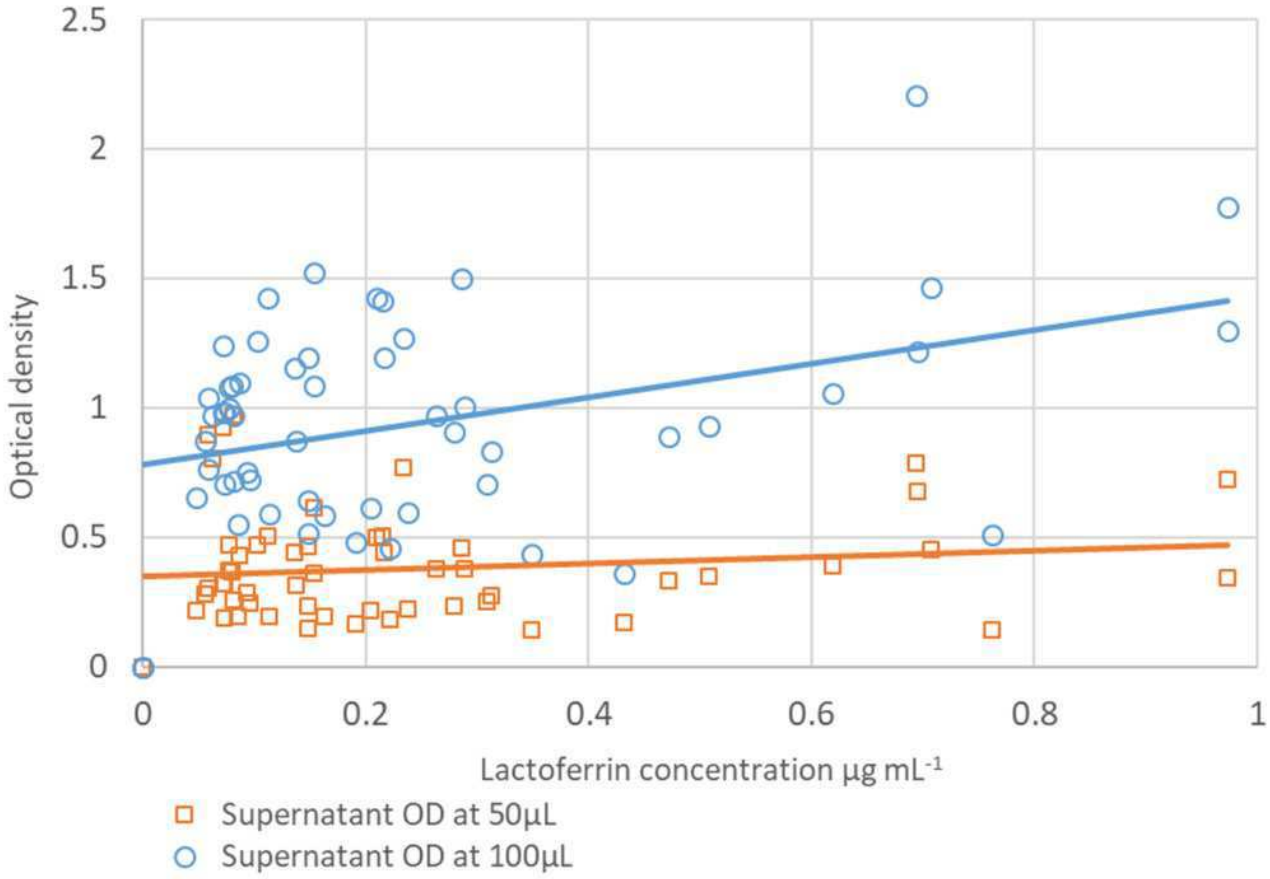

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Figure 5

Scatterplot with trendlines showing the relationship between faecal dry matter content (%) and faecal lactoferrin concentration (µg mL^{-1}).

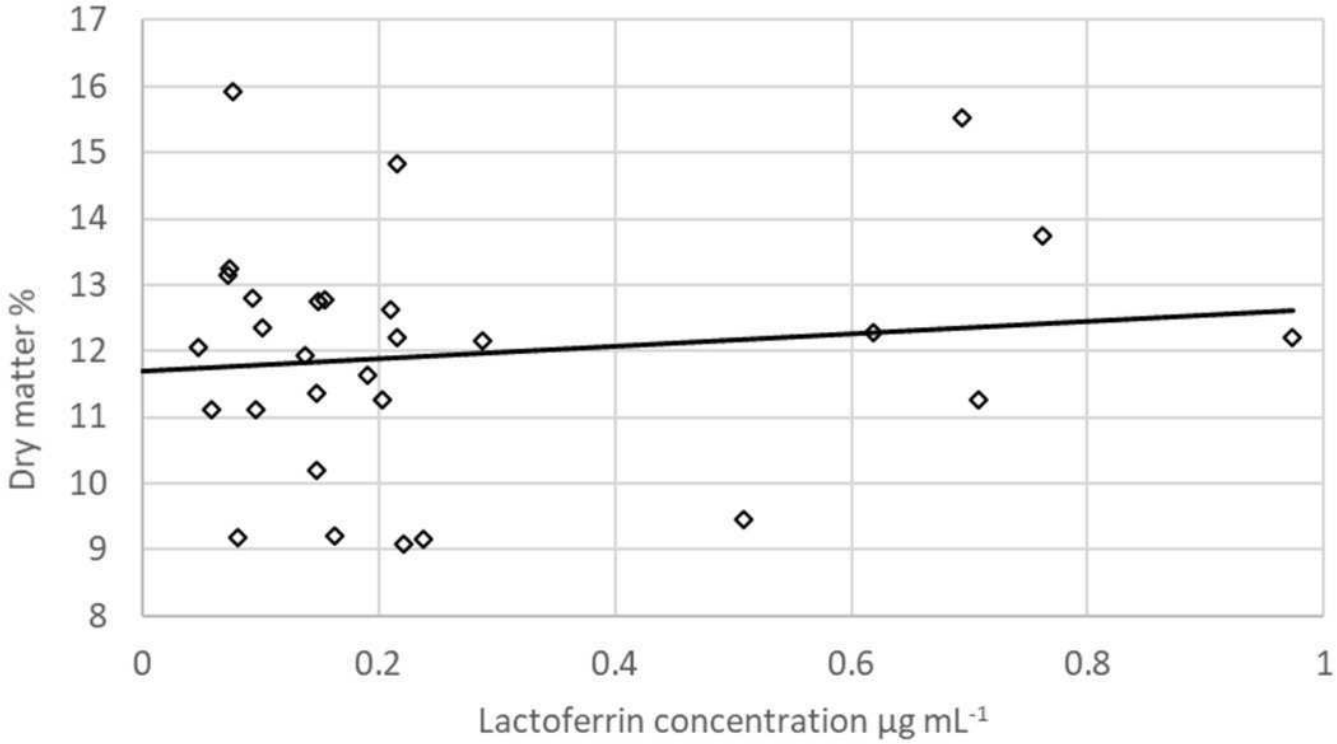

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Table 1(on next page)

Sample numbers and associated information herds within the study. Cattle were kept in traditional farm settings, with regular human interaction, controlled grazing patterns, and fenced fields.

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Group	Species	*n* faecals	*n* bloods
C1	Cattle	65	0
C2	Cattle	30	0
C3	Cattle	22	22

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