Evaluation of Human Fecal Calprotectin Detection Kits and Their Potential use as Non-Invasive Intestinal Biomarker of Infectious Enterocolitis in Pigs

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Abstract

Background: Fecal calprotectin is largely applied as a non-invasive intestinal inflammation biomarker in human medicine. Previous studies in pigs investigated the levels of fecal calprotectin in healthy animals only. Thus, there is a knowledge gap regarding its application during infectious diarrhea. This study investigated the usefulness of fecal calprotectin as a biomarker of intestinal inflammation in *Brachyspira hyodysenteriae* and *Salmonella* Typhimurium infected pigs.

Results: Fecal samples from pigs with colitis (n=18) were collected from animals experimentally inoculated with *B. hyodysenteriae* G44 or from sham-inoculated controls. Fecal samples from pigs with enteritis (n=14) were collected from animals inoculated with *Salmonella enterica* serovar Typhimurium or from sham-inoculated controls. For both groups, fecal samples were scored as: 0 = normal; 1 = soft, wet cement; 2 = watery feces; 3 = mucoid diarrhea; and 4 = bloody diarrhea. Fecal calprotectin levels were assayed using a sandwich ELISA, a turbidimetric immunoassay and a point-of-care dipstick test. Fecal calprotectin levels were greater in colitis samples scoring 4 versus ≤ 4 using ELISA, and in feces scoring 3 and 4 versus ≤ 1 using immunoturbidimetry ($P < 0.05$). No differences were found in calprotectin concentration among fecal scores for enteritis samples, regardless of the assay used. All samples were found below detection limits using the dipstick method.

Conclusions: Fecal calprotectin is a potential non-invasive biomarker of infectious colitis, but it is not suitable for detection of enteritis. While practical, the use of commercially available human presents sensitivity limitations. Further studies are needed to validate the field application of calprotectin as a marker.

Background

The indiscriminate use of antimicrobials in pork production have been globally discouraged due to the emergence of multi-drug resistant bacterial strains which can impose risks to human and animal health [1, 2]. Collectively, the current restrictions on antimicrobial agents available to treat pigs demanded improved biosecurity, nutrition, farm management, animal welfare, and the development of tools for the judicious use of antimicrobials [3]. A non-invasive biomarker associated with intestinal inflammation would help direct more judicious therapeutic and nutritional interventions during episodes of enteric diseases in commercial operations.

Swine dysentery (SD) and porcine salmonellosis are intestinal disorders of global relevance in grower-finisher pigs. Both diseases are associated with significant economic losses due to increased production costs and poor animal performance [4, 5]. Mucohemorrhagic diarrhea and colitis are the main clinical signs of SD caused by *Brachyspira hyodysenteriae*. *B. hampsonii* and *B. suanatina* [6]. Currently, antimicrobials are the only strategy to prevent and treat this disease [7]. *Salmonella enterica* serovar Typhimurium causes enteritis and watery diarrhea in pigs [5, 8]. Even though studies have evaluated

vaccination to control salmonellosis in pigs, protection is variable due to poor cross-protection across serovars [9, 10], and antimicrobials are still used metaphylactically.

Calprotectin is a 24 kDa calcium binding protein of the S100 family. It accounts for approximately 60% of the cytosolic protein in neutrophils and is also found in monocytes [11, 12]. It is released upon neutrophil activation and displays antimicrobial, antiproliferative and apoptotic properties [12, 13]. Interestingly, calprotectin is resistant to intestinal bacteria proteases [14]. In human medicine, calprotectin has been used to assess the extent of intestinal inflammation [15]. Its concentration in feces is correlated with inflammatory bowel disease (IBD) [16, 17], and necrotic enterocolitis in infants [18]. Fecal calprotectin is used to identify and aids in distinguishing IBD from irritable bowel syndrome (IBS) [19, 20], and is specifically useful to predict disease activity and relapse during treatment [21, 22]. Increased fecal calprotectin levels were associated with endoscopic and histological lesions during episodes of IBD [23, 24] and can be used to distinguish between inflammatory and non-inflammatory colitis in humans [25]. Thus, there is a plethora of commercially available kits aimed at detecting human calprotectin in feces, ranging from laboratory-intensive ELISAs to point-of-care dipsticks.

Studies focused on swine have investigated calprotectin levels in the feces of healthy animals only, suggesting it may be involved in intestinal homeostasis [26, 27]. However, there are no reports on the use of calprotectin as a biomarker of intestinal inflammation in disease-challenged pigs. We hypothesized that, similar to what is observed in humans, pigs with intestinal inflammation have increased levels of fecal calprotectin. The swine calprotectin S100-A8 subunit amino-acid sequence is 72% similar to the human protein, and the S100-A9 subunit is 66% similar. Thus, we also hypothesized that commercial kits aimed at human calprotectin should also detect the swine protein. Therefore, the objective of this study was to evaluate the usefulness of fecal calprotectin as a biomarker of colitis and enteritis in swine using commercially available human kits.

Results

A summary of the fecal samples (n = 32) and their associated consistency scores is shown in Table 1.

Table 1
Summary of fecal consistency scores from pigs challenged with *B. hyodysenteriae* (CS) or S. Typhimurium (ES)

Score	Classification	Colitis samples[a] (CS; n = 18)	Enteritis samples[b] (ES; n = 14)
0	Normal feces	3	4
1	Soft, wet cement	5	5
2	Watery diarrhea	1	5
3	Mucoid diarrhea	5	N/A
4	Bloody diarrhea	4	N/A
N/A– not applicable.			
[a]Colitis samples collected on days 13, 19, 22, 29, and 33 post-inoculation			
[a]Enteritis samples collected on day 4 post-inoculation			

Colitis samples assessment

Using ELISA, fecal samples that scored 4 (bloody diarrhea) had significantly higher calprotectin levels than those that scored 0, 1 or 3 ($P = 0.037$, Fig. 1A). Using immunoturbidimetry, fecal samples that scored 3 and 4 had significantly higher calprotectin levels than those that scored 1 (score 3 $P = 0.039$, score 4 $P = 0.044$ respectively, Fig. 1B). Fecal calprotectin level was significantly and positively correlated with fecal consistency scores using ELISA ($\rho = 0.728$; $P = 0.001$, Fig. 2A) and immunoturbidimetry ($\rho = 0.80$; $P = 0.001$, Fig. 2B). Immunochromatographic dipstick tested negative for all samples.

Enteritis samples assessment

No differences were found in calprotectin concentration among fecal score groups when measured using ELISA ($P = 0.098$; Fig, 3A) or immunoturbidimetry ($P = 0.579$; Fig, 3B). However, fecal scores 1 and 2 did have numerically higher fecal calprotectin concentrations than score 0 using either method. Fecal calprotectin concentration was not correlated with fecal consistency scores when analyzed by either ELISA ($\rho = 0.536$; $P = 0.59$; Fig. 4A) or immunoturbidimetry ($\rho = 0.268$; $P = 0.376$; Fig. 4B). All samples tested negative when the immunochromatographic dipstick test was used.

Discussion

Grower-finisher infectious diarrhea in commercial swine operations leads to decreased performance and increased production costs associated with treatment and mortality, directly impacting profits [4, 5]. To help direct immediate therapeutic and nutritional interventions following observation of diarrhea, a non-

invasive intestinal inflammation biomarker test that can differentiate inflammatory from non-inflammatory causes of diarrhea would be beneficial. In this study, we showed that fecal calprotectin levels detected by ELISA or immunoturbidimetry can be used as a biomarker of colitis in pigs challenged with *B. hyodysenteriae.* However, both methodologies were ineffective in discerning between mild, watery diarrhea and normal feces during colitis or enteritis.

Calprotectin is a calcium binding protein secreted by neutrophilic granulocytes and has a role controlling bacterial growth during inflammation [12, 28]. Recruitment of neutrophils to the intestinal mucosa leads to cell shedding and active secretion of calprotectin to the intestinal lumen [12]. Once secreted, calprotectin sequesters essential micronutrients such as iron, zinc, and manganese, inhibiting bacterial growth [29, 30]. Fecal calprotectin concentration has been shown to be correlated with the number of neutrophils released in the intestinal lumen during inflammation, which in turn can be associated with the severity of inflammation [19]. Previous studies focused on swine have investigated calprotectin levels in the feces of healthy pigs, suggesting it may play a role in intestinal homeostasis [26]. Lallès et al. [27] observed that the average fecal calprotectin concentration in sows (13 ± 38 mg/kg of feces) was close to the ranges of healthy adult humans (range 2–47 mg/kg), but the concentrations found for piglets at birth were very low (24 ± 60 mg/kg) compared with adult newborns (145 ± 78.5 mg/kg). They also found values of 38 ± 78 mg/kg for conventional grower pigs weighting 40 kg, and 2 ± 5 mg/kg feces for specific pathogen-free grower pigs weighting 80 kg, suggesting that calprotectin is very low in healthy pigs with high sanitary conditions. Elevated levels of fecal calprotectin were reported in humans with IBD [16, 22], and in dogs its concentration has a good ability to discern between animals with different causes of chronic inflammatory enteropathies such as steroid-responsive/-refractory enteropathy and immunosuppressant-responsive/-refractory enteropathy, and dogs with food-responsive enteropathy or antibiotic-responsive enteropathy before treatment [31, 32].Calprotectin is already extensively used in human medicine as a biomarker of IBD, as it can help distinguish IBS from IBD, and detect recurrent IBD during treatment[20, 25].

Here elevated fecal calprotectin levels in pigs are associated with diarrhea and mucohaemorrhagic colitis. While further studies using larger populations are needed to validate these results, our data suggests that fecal calprotectin could be a potential tool used to diagnose severe inflammatory colitis, particularly by untrained observers who may, for example, miss blood staining in feces when pigs are house in large groups. It may also help distinguish bacterial colitis from other causes of diarrhea in pigs, thus, contributing to a more judicious use of antimicrobials for pork production. We found that mucoid or mucohemorragic feces from pigs with colitis contained the highest calprotectin concentration. Multiple previous reports have characterized the accumulation of neutrophils, a source of antimicrobial peptides such as calprotectin, on the surface of the colonic mucosa during *B. hyodysenteriae* and *B. hampsonii* infection in pigs [33–36]. Here we showed evidence that severe SD clinical signs are associated with increased fecal calprotectin levels, providing further evidence of the importance of neutrophils in the pathogenesis of swine dysentery.

S. Typhimurium invades epithelial cells of the distal small intestine and colon, leading to inflammatory diarrhea with a marked increase in mucosal neutrophil infiltration [5, 8, 37]. Despite this, we did not observe a significant increase in fecal calprotectin levels following inoculation with *S.* Typhimurium, regardless of the assay used. Our findings differ from previous studies that found increased fecal calprotectin concentration during *S.* Typhymurium infection in rats [38, 39], and *Salmonella* spp. infection in humans [40]. Human patients with severe or moderate bacterial gastroenteritis and fecal mucus have increased fecal calprotectin, but those with mild diarrhea do not [41]. Mucoid feces is not a feature of swine salmonellosis, but it is associated with *Brachyspira* spp. [5]. Moreover, it has been shown that *S.* Typhimurium overcomes the antimicrobial effect of calprotectin by expressing a high affinity zinc transporter (ZnuABC) [42, 43]. We recognize that the lack of histopathology data from either sample cohort is a limitation here, and suggest the collecting of such samples in future studies.

The literature is contradictory with respect to the association between high fecal calprotectin levels and lesion site. There are reports showing that either ileal or colonic lesions can both be monitored using fecal calprotectin as an indicator of endoscopically active Crohn's disease (CD) [16, 44–46]. In contrast, other studies have found that the discriminatory power of fecal calprotectin is greater in ileocolonic and colonic CD, than in jejunal or ileal CD [47–49]. Zittan et al. [48] postulated that the slow intestinal transit in the colon could increase calprotectin degradation through intestinal proteases, thereby reducing its concentration in feces. We believe that the lack of difference in calprotectin levels in enteritis samples was due to the proximal location of the lesions, which were most likely associated with the small intestine [50]. Differently from humans, pigs have a functional cecum that may contribute to this disappearance effect by luminal proteases.

Interestingly, higher concentrations of fecal calprotectin were found when measured using the immunoturbidimetry assay compared to ELISA in both sample cohorts. For human samples, ELISA based on monoclonal antibodies is the gold standard used to quantify fecal calprotectin levels. It is specific to calprotectin heterodimeric and polymeric complexes. However, ELISA is is laborious and time-consuming [13, 51] when compared to the a particle enhanced turbidimetric immunoassays (PETIA), based on polystyrene nanoparticles coated with calprotectin-specific antibodies binding to their specific target within the extracted samples. Subsequent quantification of the agglutinated calprotectin-nanoparticle complex detected by light absorbance (turbidity) can be adapted to several commercially available clinical chemistry analyzers and has been proposed as a rapid response test [52]. Labaere et al. [53] compared different calprotectin detection methods (three rapid quantitative immunochromatografic tests, two enzyme-linked immunosorbent assays, and one automated fluoroimmunoassay), and reported significant variations in the calprotectin levels detected. Juricic et al. [54] reported fecal calprotectin concentrations using a commercial ELISA kit to be significantly lower than a turbidimetric immunoassay. Oyaert et al. [55] observed satisfactory diagnostic performance between six different fecal calprotectin immunoassays (two ELISA, two chemiluminescent immunoassays (CLIA), one fluoroenzyme immunoassay (FEIA), and one PETIA), even though there were discrepancies in calprotectin values detected between these kits. These reports are consistent with our findings that different assays resulted in different values for fecal calprotectin. It is worth mentioning that the kits evaluated in this study used

monoclonal antibodies specific for human calprotectin, therefore, the low calprotectin levels found by ELISA may be due to the lack of cross reactivity with swine calprotectin, as previously reported [26]. While we understand the limitation of this approach, commercial kits for fecal calprotectin detection are only available for humans. In addition, there are multiple home test kits commercially available that could be translated into farm-friendly tools. Nevertheless, we still found evidence that human tests can be used in veterinary medicine, taking advantage of this previously developed infrastructure.

While we understand that there are multiple other causes of enteritis and colitis in pigs, we believe that *B. hyodysenteriae* and *S.* Typhimurium are also representative of these syndromes. We also recognize that a limited number of samples were utilized in both COL and ENT groups. This likely limited some of our findings related to the less severe fecal scores.

Conclusions

This initial data suggests that fecal calprotectin may be a useful non-invasive biomarker to diagnose infectious colitis from swine fecal samples. However, it was unable to discern between mild-diarrhea and healthy feces, or when pigs only developed enteritis. Nevertheless, further development is suggested as this approach has the potential to support the judicious use of antimicrobials for pork production through the differentiation of infectious from non-infectious causes of colitis.

Methods

Animal trials and fecal samples

In both trials, pigs were obtained from a PRRSV negative, high health herd with no gastrointestinal clinical signs. Animals were housed and allowed to acclimate in a BSL-2 animal care facility for 7 days prior to inoculation. Colitis samples (COL, n = 18) were obtained from 9-to-10-week-old barrow pigs experimentally inoculated thrice over 72 hours with *Brachyspira hyodysenteriae*, the etiologic agent of swine dysentery, or from sham-inoculated controls. In this study, strain G44 was used to induce disease in a cohort of 24 pigs intragastrically inoculated with 50 mL liquid media averaging 1.69 x10^9 genome equivalents/mL as previously described [35]. Fecal samples were collected from pigs on all post inoculation days and a random selection of samples, representing all fecal scores were used for calprotectin analysis (Table 1). Enteritis samples (ENT, n = 14) were collected from a cohort of 64 pigs (eight weeks old, barrows n = 32, gilts n = 32) experimentally inoculated with *Salmonella enterica* serovar Typhimurium var Copenhagen, or from non-infected controls. After the acclimation period, challenged pigs were orally inoculated twice within 4 hours with 1 mL containing 3.3 x 10^9 CFU/mL/pig of *S.* Typhimurium, as previously described [56], and control pigs were inoculated with 1 mL of sterile saline solution. Random fecal samples scoring 0–2 were obtained on day four post-inoculation (Table 1). Samples from both trials were collected free fall or by digital stimulation, and were scored according to a previously developed fecal consistency rubric [35]: 0 = normal; 1 = soft, wet cement; 2 = watery feces; 3 =

mucoid diarrhea; and 4 = bloody diarrhea. All fecal samples were obtained from individual pigs and stored at -80ºC until extraction.

Fecal sample extraction

Fecal samples were processed according to the kit manufacturers' instructions, with minor changes as described below (Bühlmann Calprotectin ELISA EK-CAL, Bühlmann Laboratories AG, Switzerland). For each sample, between 50 mg and 100 mg of feces were weighed into a sterile polypropylene tube (15 mL, VWR Scientific Products, Suwanee, GA, USA). Extraction buffer was added, adjusting the reaction volume to each sample weight to obtain a final 1:10 ratio. Extraction tubes were individually vortexed for 30 seconds (Fisher Vortex Genie 2, Fisher Scientific, Pittsburgh, PA, USA) at maximum speed and incubated for 30 minutes at room temperature on shaker at 400 rpm (G-25 Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA). Samples were vortexed again for 30 seconds, a 1.5 mL aliquot was transferred to a 2 mL sterile microfuge tube and centrifuged at 3000 *g* for 5 minutes. Finally, the supernatant was transferred to a 1.5 mL microfuge tube and stored at -20°C until analysed.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out following the manufacturer's instructions (Bühlmann Calprotectin ELISA EK-CAL, Bühlmann Laboratories AG, Switzerland). Fecal extracts were thawed and homogenized prior to analysis. Initially, 100 µL of incubation buffer (blank, negative control), five calibrator samples (100 µL/well, ranging from 30 to 1800 µg/g; Supplementary Table 1), and low and high control samples (100 µL/well) were included on each microtiter plate precoated with anti-calprotectin monoclonal antibodies (mAb). Finally, 100 µL of fecal extract per sample was analyzed. All samples were analyzed in duplicates, including extraction controls. Following dispensing of samples and controls, reaction plates were incubated for 35 min using an orbital plate shaker at 450 rpm, at room temperature. After incubation, plates were washed three times for 30 seconds with 300 µL of wash buffer per well. Next, each sample was incubated and mixed for 35 minutes with 100 µL of enzyme label anti- mAb conjugated with horseradish peroxidase (HRP). The wash step was repeated 5 times as described above and immediately after; the color reaction was induced using 100 µL of tetramethylbenzidine (TBM). The plate was covered with a plate sealer (Bühlmann Laboratories AG, Switzerland) to prevent TBM degradation due to exposure to light, and incubated for 15 minutes on a plate shaker at 400 rpm at room temperature. The reaction was stopped by adding 100 µL of 0.25 M sulfuric acid to each well and absorbance assessed at 450 nm using a microplate reader (Biotek Epoch, Biotek Instruments, Winooski, Vermont, USA). Calprotectin level was expressed as micrograms per gram (µg/g) of feces and values are reported as the mean value for both duplicates.

Immunoturbidimetry assay

Fecal extracts were thawed and analyzed using the fCal Turbo assay (BÜHLMANN, Laboratories AG, Switzerland). This assay was adapted to be performed on a plate reader. Reaction buffer (150 µL) and immunoparticles (30 µL) were pipetted into all wells of a test plate. Six calibrator samples (10 µL/well,

ranging from 0 to 2207.6 µg/g; Supplementary Table 2) were included in each plate. Ten µL of fecal extract per sample was tested in duplicate. Absorbance was measured at 546–580 nm using a microplate reader (Biotek Epoch, Biotek Instruments, Winooski, Vermont, USA) using the Gen5 Data Analysis software interface (Biotek Instruments, Winooski, Vermont, EUA).

Immunochromatographic assay

Samples were also analyzed using a point-of-care dipstick test for detection of calprotectin in feces (Actim calprotectin rapid test, Medix biochemica, Espoo, Finland) following the manufacture's instructions. This is a semi-quantitative test with a detection range of 12.5 to 10,000 µg of calprotectin/g of human feces. Briefly, 1g from each fecal sample was brought to room temperature and added to the dilution buffer container. The container was manually shaken, and the detection stick was inserted in the container once the sample was diluted. Results were read after 10 minutes contact between the test strip and the sample.

Statistical analysis

One fecal sample from the COL group (the only score 2) was removed from the analyses but is still shown in the plots for visual comparison only. Analyses were performed using SPSS (IBM-SPSS, Chicago, IL, USA). Differences in calprotectin levels among fecal score groups were analyzed using the Kruskal-Wallis test. When there was a significant overall group difference, the Dunn's *post-hoc* test was used to assess pairwise differences. The association between calprotectin concentration and fecal consistency score was assessed by determination of the Spearman's correlation coefficient (r). Results were considered significant if $P \leq 0.05$.

Abbreviations

CD: Crohn's disease; CLIA: Chemiluminescent immunoassay; COL: Colitis samples; ELISA: Enzyme-linked immunosorbent assay; ENT: Enteritis samples; FEIA: fluoroenzyme immunoassay; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; HRP: Horseradish peroxidase; mAb: Anti-calprotectin monoclonal antibodies; PETIA: particle enhanced turbidimetric immunoassay; PRRSV: Porcine reproductive and respiratory syndrome virus; SD: Swine dysentery; TBM: tetramethylbenzidine; ZnuABC: high affinity zinc transporter.

Declarations

Bühlmann Laboratories AG had no role in study design, data analyses and interpretation.

Ethics approval and consent to participate

Colitis and enteritis samples used in this study were collected during two independent experiments designed and conducted in accordance with the Canadian Council for Animal Care and approved by the

University of Saskatchewan Animal Research Ethics Board (AUP #20180046 and AUP #20190003).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed to reviewing the topics and writing the present article, and agree with the contents of the review. All authors read and approved the final manuscript.

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Figures

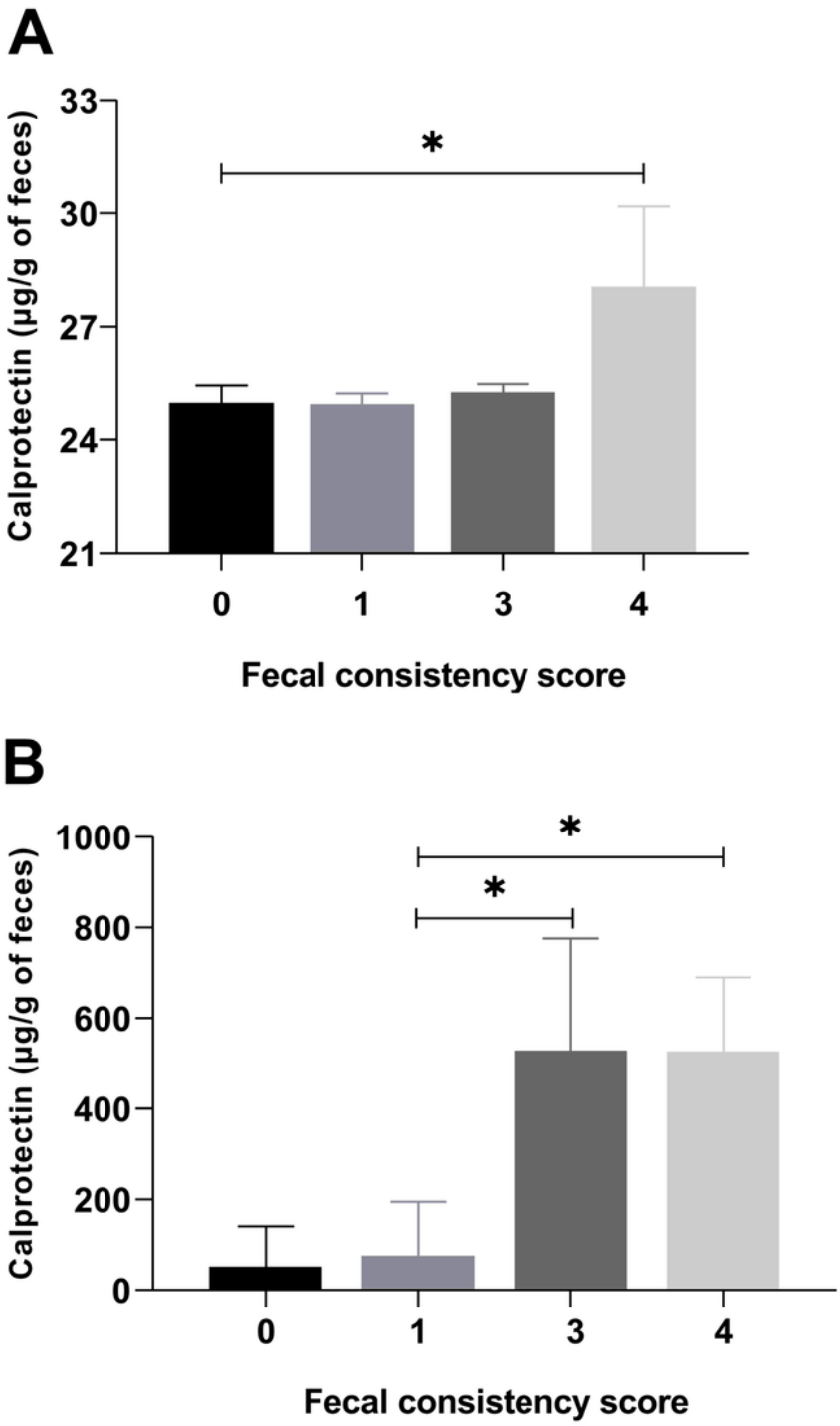

Figure 1

Calprotectin concentration in colitis fecal samples (CON, µg/g) from pigs challenged with B. hyodysenteriae. (A) ELISA assay; (B) Immunoturbidimetry assay. Stars denote a significant difference ($P<0.05$) between fecal scores. Bars denote means, with standard deviation represented by error bars.

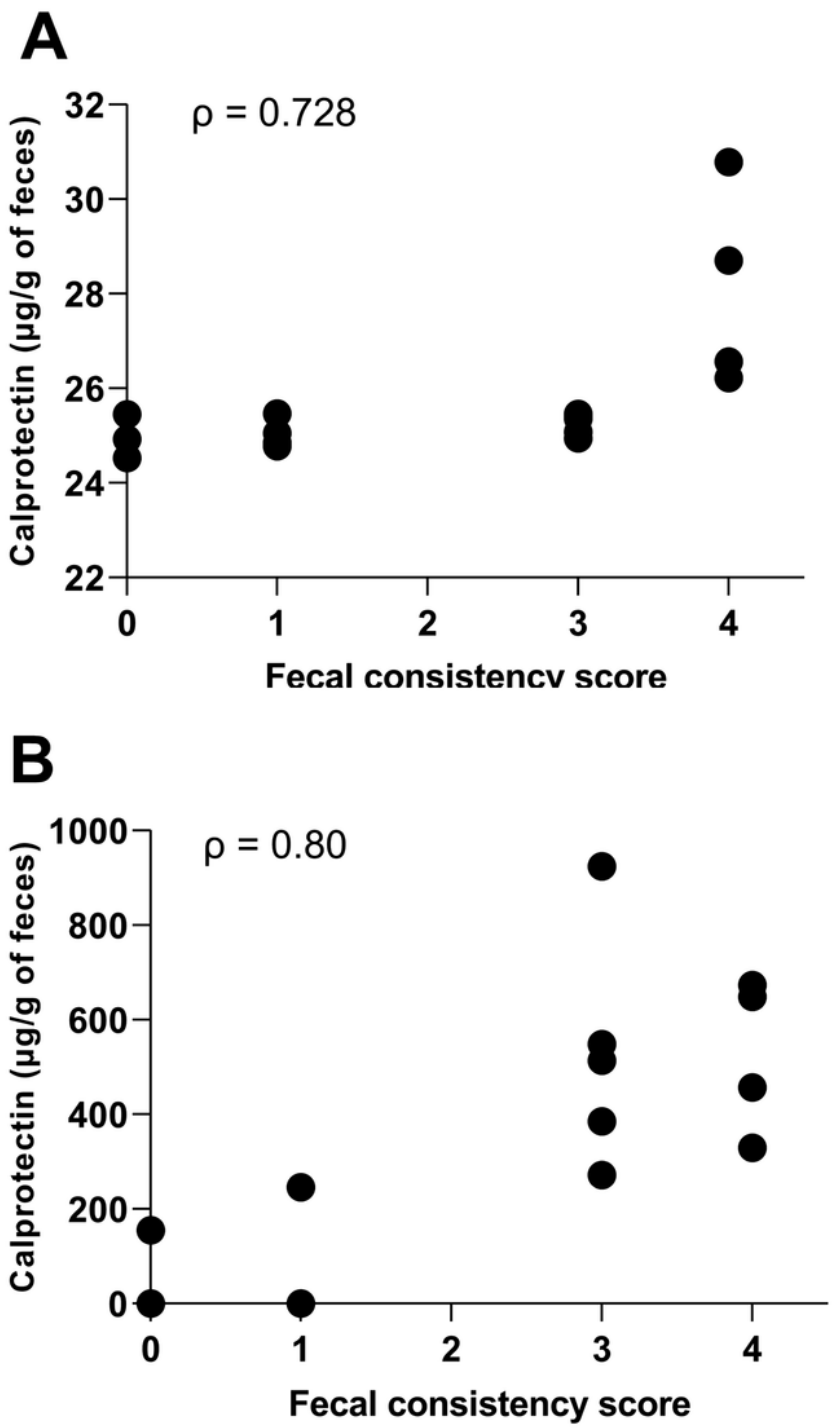

Figure 2

Correlation between calprotectin concentration and fecal consistency scores (CON,µg/g) from B. hyodysenteriae-challenged pigs. (A) ELISA assay; (B) Immunoturbidimetry assay (ρ = spearman's correlation coefficient).

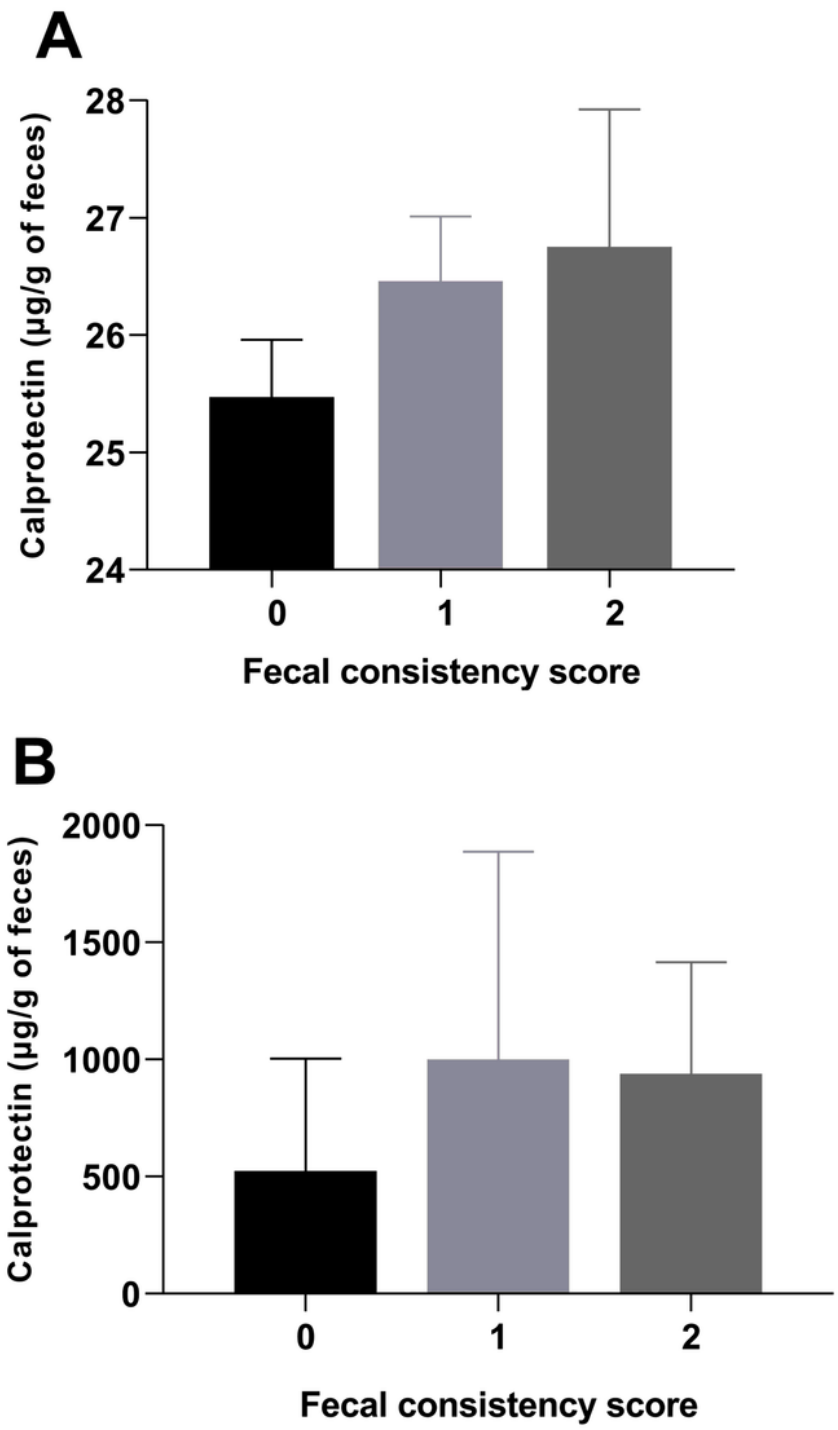

Figure 3

Calprotectin concentration in enteritis fecal samples (ENT, µg/g) from pigs challenged with S. Typhimurium. (A) ELISA assay; (B) Immunoturbidimetry assay. Bars denote means, with standard deviation represented by error bars.

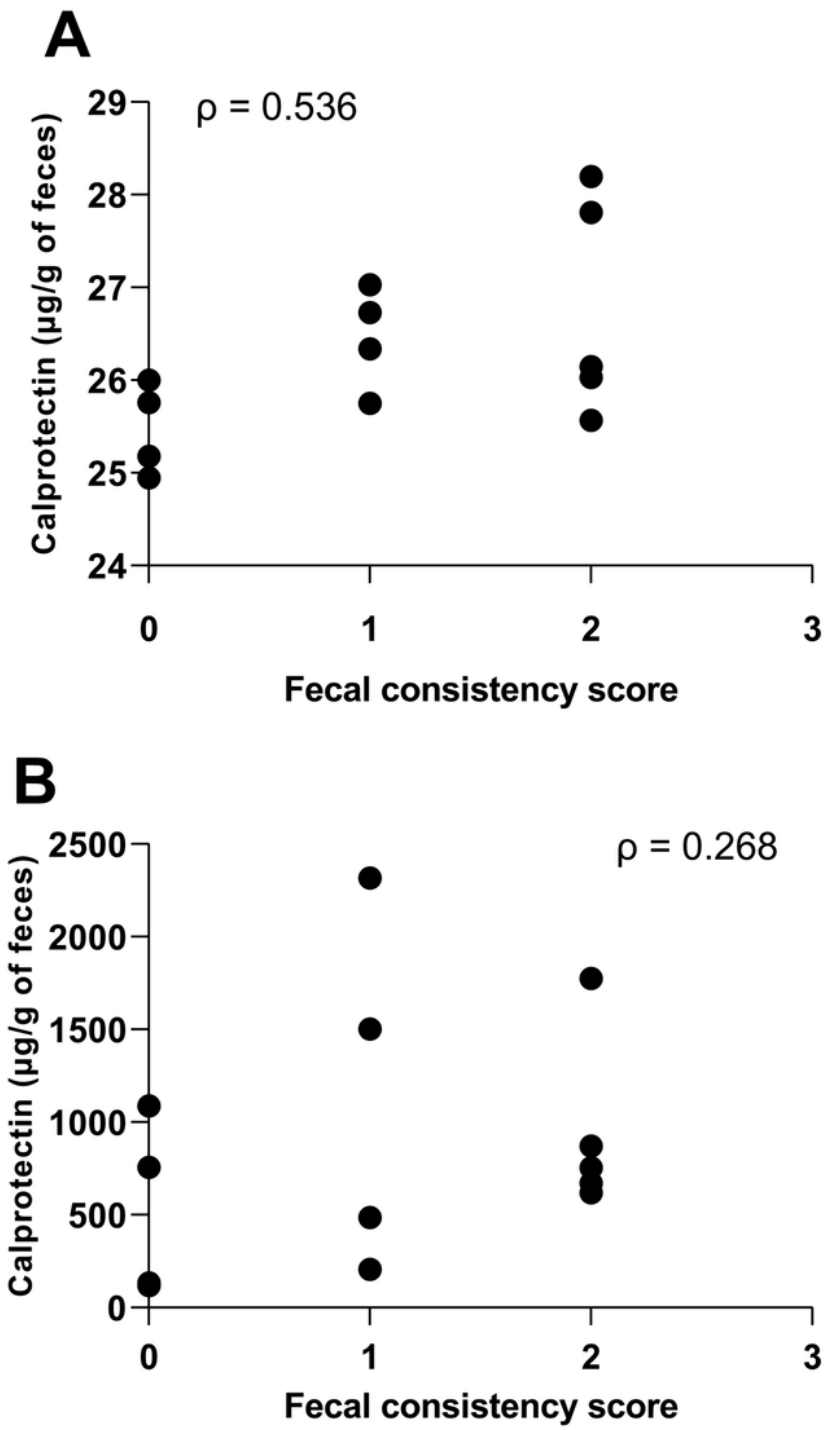

Figure 4

Correlation between calprotectin concentration and fecal consistency scores (ENT,µg/g) from S. Typhimurium-challenged pigs. (A) ELISA assay; (B) Immunoturbidimetry assay (ρ = spearman's correlation coefficient).

Supplementary Files

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