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Faecal inflammatory biomarkers as non-invasive indicators of feed intake status in weaned piglets

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ABSTRACT

Early weaning in piglets commonly leads to a postweaning (**PW**) fasting period, compromising intestinal integrity and triggering inflammation, overall affecting welfare and growth. Despite the well-documented consequences of fasting, no feasible and non-invasive method exists to monitor feeding patterns in postweaned piglets. Therefore, this study aimed to evaluate the use of faecal inflammatory biomarkers—calprotectin (**fCal**), lipocalin-2 (**LCN-2**), myeloperoxidase (**MPO**), and adenosine deaminase (**ADA**)—to detect whether an animal is eating or not by correlating fasting with its presence in faeces. The trial involved 623 weaned piglets (21 days) which were weighed at days 0, 3 and 10 PW. A blue-coloured creep-feed was provided before and after weaning to qualitatively trace feed consumption via rectal swabs. Piglets were assessed for feeding status at weaning and on day 3 PW. Additionally, 120 animals were selected to monitor feed status daily over the first 4 days PW. Animals were classified into three categories: creep-feed eaters (**CFE**), began feed intake preweaning and maintained it PW; PW eaters (**PWE**), started consuming feed PW; and non-eaters (**NE**), which did not consume any feed. Out of the 120 piglets, faecal samples were obtained from 77 piglets: CFE (n = 19 sampled from days 0–3), PWE (n = 7 on day 1, n = 14 on day 2, n = 33 on day 3), and NE (n = 30 on day 0, n = 28 on day 1, n = 27 on day 2, n = 25 on day 3). Among all piglets, 4.5% were classified as CFE, 69.5% as PWE, and 26% as NE. On average, CFE gained 44 g/day more than PWE, and 100 g/day more than NE in the first 10 days of PW ($P < 0.001$). The biomarkers fCal, LCN-2, and MPO differed by eater category ($P \leq 0.014$), while ADA increased over time ($P = 0.001$) without differences between categories. Calprotectin and LCN-2 were higher in NE than CFE, correlating with reduced growth ($r \leq -0.29$, $P \leq 0.044$). Conversely, MPO was higher in CFE than NE ($P < 0.001$), with no growth association. Calprotectin showed strong discriminatory power between CFE and NE, with an area under the curve of 0.86, sensitivity of 86%, and specificity of 69%. Calprotectin and LCN-2 increased with fasting and correlated negatively with growth, highlighting their utility as biomarkers of low feed intake–related inflammation, with fCal being the most sensitive. Myeloperoxidase and ADA showed no feeding-related associations.

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Implications

Early weaning reduces feed intake, potentially causing inflammation and affecting intestinal integrity and postweaning adaptation. This study evaluates non-invasive faecal inflammatory biomarkers to early detect fasting. Among the four biomarkers tested, calprotectin and lipocalin-2 were the most strongly linked to feeding patterns, with higher levels in fasted animals. Furthermore, calprotectin also showed greater predictive capacity, offering a potential cut-off point. In contrast, myeloperoxidase and adenosine deaminase levels appeared to reflect an immune response, regardless of feeding status. Measuring calprotectin can identify fasting animals, allowing early preventive supportive care to mitigate health, welfare and growth issues.

Introduction

The stress induced by weaning in piglets, including factors such as abrupt separation from the sow, transition from a liquid to a

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solid diet, and changes in environmental and social conditions, leads to a brief period of poor feed intake (Madec et al., 1998; Le Dividich and Sè, 2000). The small intestine is most affected by this reduced feed intake, as its cells primarily obtain their nutrients from the intestinal lumen (Alpers, 1972; Stoll et al., 2000). Moreover, numerous studies have correlated the lack of luminal stimulation with compromised intestinal barrier function (Montagne et al., 2007; Smith et al., 2010; Pluske et al., 2018). As a result of an increased intestinal permeability, bacteria and antigens from the intestinal lumen can translocate into subepithelial tissues, triggering an immune response that may lead to diarrhoea (Mccracken et al., 1999; Spreeuwenberg et al., 2001). Because weaning–induced fasting adversely affects gut health, growth and welfare, monitoring feeding behaviour during this critical window is essential; however, no practical, non–invasive method currently exists to assess piglet feeding status accurately.

In human medicine, faecal inflammatory biomarkers have gained attention as valuable tools for assessing intestinal health (Dragoni et al., 2021). These biomarkers provide localised information about gut inflammation while being non-invasive, making them a practical alternative to blood-based markers (Sands, 2015). Their detection in faeces allows for continuous monitoring with minimal manipulation, an aspect that is particularly relevant when considering animal welfare in research and swine production. The most common biomarkers used to assess intestinal inflammatory disorders are calprotectin (**fCal**) and myeloperoxidase (**MPO**), both as products of neutrophil activation. These biomarkers have been widely used to detect digestive tract inflammatory conditions, such as inflammatory bowel disease and Crohn's disease. They have shown strong correlations with other inflammatory parameters, including histopathological signs of intestinal impairment and an increased number of intraepithelial lymphocytes within the intestinal wall (Davies, 2021; Kapel et al., 2023). Furthermore, fCal is proven as a marker for tracking disease progression, evaluating treatment effectiveness, and assessing enteropathies caused by malnutrition in children (Guerrant et al., 2016).

Other neutrophil-derived products have also been employed in diagnosing inflammatory disorders, such as neutrophil gelatinase-associated lipocalin (**LCN-2**). Unlike fCal and MPO, LCN-2 is produced not only by immune cells but also by epithelial cells across various tissues, making it particularly relevant for detecting subclinical and chronic inflammation on the intestinal epithelium (Chassaing et al., 2012). Lastly, adenosine deaminase (**ADA**), an enzyme primarily produced by T lymphocytes, has been identified as a marker of immune activation and even lymphocyte maturation (Grześkowiak et al., 2023).

In this context and considering (a) the potential of these biomarkers to assess intestinal disorders and (b) the plausible association of postweaning (**PW**) fasting with an intestinal inflammatory condition (McCracken et al., 1999; Pié et al., 2004), it was hypothesised that higher faecal concentrations of these faecal biomarkers could reflect the impact of the fasting period undergone, thus serving as an indicator to assess PW feed intake in weanling pigs. Therefore, using population-level growth data to establish distinct feeding patterns and their potential impact on piglet development, the objective of this study is to evaluate whether faecal fCal, LCN-2, MPO, and ADA concentrations correlate with the different PW feeding patterns and piglet growth. Preliminary results have been published in an abstract form (Suppi et al., 2025).

Material and methods

Animals, housing and performance measurements

The current study involved 623 piglets [(Landrace × Large White) × Pietrain], containing 319 males and 304 females from litters of 50 multiparous sows, in commercial conditions. The individual farrowing pens measured 2.7 × 1.7 m^2 and included a heating pad sized 0.9 × 0.6 m^2. Each pen featured a slatted metal floor, complemented by a concrete lateral section. Sow feed was supplied via a farrowing electronic feeder activated wirelessly (Nedap, NV, the Netherlands) and water through a nipple waterer in the same hopper. Piglets had *ad libitum* access to creep–feed from day 10 of life via a pan feeder, and to fresh tap water from a separate nipple drinker. The temperature in the farrowing room was maintained at 24 °C (electronically controlled forced ventilation), and natural light was supplemented by artificial lighting from 0700 to 1800 h. Tail docking and iron injections were administered 48 h after birth.

At weaning, piglets (21 ± 1 days of age; average BW of 5.93 ± 0.05 kg) were transferred to the nursery facilities. They were then assigned to 24 pens (2.35 × 1.91 m^2), with approximately 30 piglets per pen. The allocation considered sex and BW to ensure initial homogeneity within each pen. The pens featured slatted plastic flooring and were equipped with a nipple waterer and a feeder with four individual entrances, each measuring 20 cm. The temperature was maintained at 26 °C (central heating and electronically controlled forced ventilation), and natural light was supplemented with artificial lighting from 0700 to 1700 h. Animals were ear-tagged and weighed individually at weaning, at day 3, and at day 10 PW (Fig. 1A). Average daily gain (**ADG**) was calculated for the periods from weaning to day 3, from day 3 to day 10, and from weaning to day 10. The ear tags and scales used for weighing piglets were provided by LeeO System (Deventer, The Netherlands).

Feed management and consumption evaluation

To qualitatively determine whether animals consumed the feed, 5 g/kg of the food dye Indigo Carmine (E132, PANREAC QUIMICA S. L.U., Spain) was added to the feed before pelleting. This dye remains largely undigested as it passes through the gastrointestinal tract (Tan et al., 2016), with a recovery rate of 60–80% depending on feed intake and intestinal motility (EFSA Panel on Food Additives and Nutrient Sources added to Food, ANS, 2014). Consequently, animals that consumed the dyed feed exhibited a distinctive blue or blue-green coloration in their faeces.

Dyed feed was provided from day 10 after birth until 4 days PW. During the suckling period, each farrowing unit received 300 g of fresh dyed feed daily. In the nursery, 12.5 kg of dyed feed was added to feeders to ensure sufficient feed per pen during the first 4 days PW. No monitoring of the feed disappearance was conducted either in the suckling or the PW period. Evaluation of feed consumption was performed via rectal swabs. The consumption evaluation was conducted every morning at approximately the same time to ensure a consistent time gap. Feed ingredient composition (as fed-basis) and analysed nutrient composition of the diet are presented in Table 1.

Faecal sample collection and classification of the different eater categories based on the onset and continuity of their feeding patterns

Classification of the whole piglet population

Feed consumption was evaluated in all 623 animals at weaning and at day 3 PW. At weaning, animals were classified as creep-feed eaters (**CFE**) if their swabs showed blue coloration, or non-eaters (**NE**) if not. Both CFE and NE animals coexisted within the same pens to resemble commercial conditions. On day 3 PW, CFEs that retained blue staining remained classified as CFE, indicating uninterrupted feed intake, while NEs without staining remained classified as NE, indicating 4 days of fasting. Non-Eaters that showed blue coloration on day 3 PW were reclassified as PW eaters

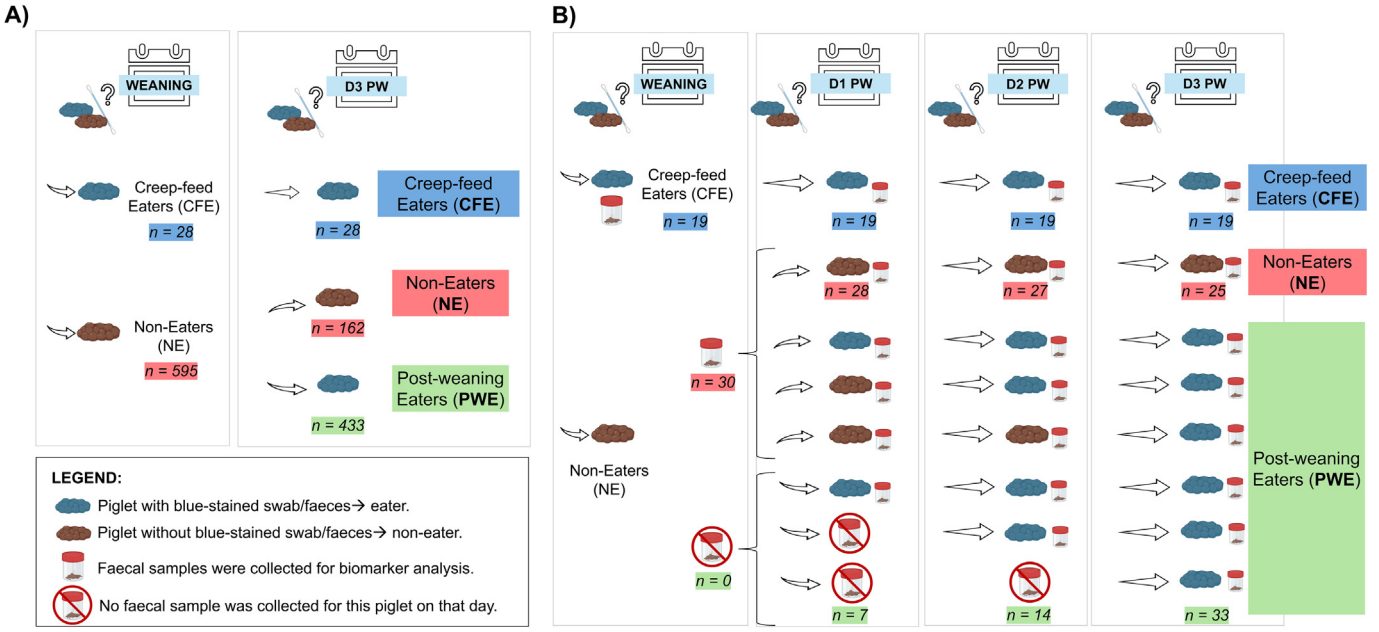


Fig. 1. Illustration of the experimental design for eater categorisation. (A) Schematic representation of piglet categorisation at weaning and day 3 postweaning (PW). At weaning, piglets were classified as creep-feed eaters (CFE) or non-eaters (NE). By day 3 PW, CFE piglets that continued eating remained classified as CFE, while NE piglets that began consuming feed were reclassified as postweaning eaters (PWE). Non-eater piglets that did not start eating remained classified as NE. (B) Eater categorisation for faecal sampling across weaning and the following 3 days (four pens with 30 piglets/pen). At weaning, faecal samples were collected from all CFE present (n = 19) and from a representative sample of NE piglets (n = 30). Piglets that maintained their initial categories throughout the next 3 days retained their original classification. Non-eater piglets that initiated feed consumption on days 1, 2, or 3 PW were classified as PWE. Because the entire pen was monitored daily but not all piglets were sampled at weaning, previously unsampled NE piglets that started eating within the first 3 days PW were also sampled and classified as PWE. A total of 77 piglets were sampled; this does not represent the entire pen population.

Table 1
Ingredient composition (as fed basis) and analysed nutrient profile (DM basis) of the experimental diet for piglets during the suckling and early postweaning days.

Ingredients (%)	Value	Estimated nutrient composition (%)	Value
Barley	19.0	DM	89.9
Maize	16.1	Net Energy (MJ/kg)	10.7
Wheat	15.2	CP	17.6
Sweet Whey	10.8	Ether Extract	4.9
Rice	7.0	Ash	5.0
Soybean Protein Concentrate	4.9	Crude Fiber	3.0
Extruded soybeans	4.8	NDF	9.1
Animal Plasma	4.0	ADF	3.4
Soybean Oil	2.2	Starch	34.6
Vitamin-Mineral Premix	2.0	SID Lysine	1.241
Wheat bran	2.0	Calcium (Ca)	0.526
Crude Glycerin	2.0	Phosphorus (P)	0.614
Hydrolysate Intestinal Mucosa	1.5	Sodium (Na)	0.374
Monocalcium Phosphate	1.2	Potassium (K)	0.689
Lignocellulose	1.0	Chlorine (Cl)	0.542
Hemoglobin	1.0		
Dextrose Monohydrate	1.0		
Acids	1.60		
L-Lysine HCl	0.48		
Valine Premix 25%	0.36		
Tryptophan Premix 25%	0.28		
Threonine	0.28		
DL-Methionine	0.24		
L-Isoleucine	0.13		
Calcium Carbonate	0.24		
Salt	0.15		
Food colouring[1]	0.50		

Abbreviations: SID = standardised ileal digestible.
[1] Indigo Carmine.

(**PWE**), indicating they initiated feed consumption within 3 days after weaning. The categorisation of the entire piglet population is shown in Fig. 1A.

Classification of the faecal sampled piglets

Four of the 24 pens were selected for daily faecal monitoring and faecal sampling on the weaning day and the subsequent 3 days. These pens contained only male piglets (n = 120) due to consistent evidence of greater challenges in the initiation of solid feed intake in males than in females (Delumeau and Meunier-Salaün, 1995). In the selected pens, 19 CFEs were intentionally added, taking part of the total 120 piglets, to increase the sample size for that category. The piglets belonging in these four pens were evaluated daily until day 3 PW, and they were assigned to the same three categories as the overall population: (a) Piglets which were CFEs at weaning which keep blue markings throughout the next 3 days were classified as CFE, indicating uninterrupted feed intake; (b) NE piglets at weaning which did not show blue faeces during the 3 days PW maintained their NE classification, indicating no feed consumption; (c) NE piglets of weaning which exhibited blue faeces in one of the evaluated 4 days PW were classified as PWE. Not all piglets housed in the four selected pens were included in the faecal sampling. Out of the 120 piglets, 77 were sampled: 19 classified as CFE, 25 as NE, and 33 as PWE. Creep-feed-eater piglets were consistently sampled across all 4 days. Non-eater piglets were initially 30 on weaning day (day 0), but five of them began eating during the following days (two on day 1, one on day 2, and two on day 3) and were subsequently reclassified as PWE. These five had faecal samples from weaning day as NE. The remaining PWE piglets were newly identified eaters who had not been sampled on previous days. This is represented in Fig. 1B. At the farm, faecal samples

were obtained by gentle anal stimulation, kept in plastic tubes and immediately stored in a refrigerated box at 4 °C. After collecting all samples, they are preserved at −80 °C until analysis.

Faecal biomarkers analysis

For the faecal extract preparation, a weight of 50 mg of faeces was measured into a polypropylene tube, and the extraction buffer was added at 1:10 (w/v) dilution. This consistent weight of faeces for all samples ensures comparability and effectively standardise the result. Each extraction tube was vortexed for 1 min at maximum speed and then mixed on a shaker for 1 h at room temperature to ensure thorough extraction. Subsequent centrifugation at 5 000 rpm for 10 min allowed for effective separation of the supernatant, which was collected, aliquoted, and frozen at −20 °C until analysis. Calprotectin was measured by Bühlmann fCAL® turbo test (Bühlmann Laboratories AG, Schönenbuch, Switzerland) on a Beckman Coulter AU400 Chemistry Analyzer (Beckman Coulter Inc., Brea, CA, USA), previously validated in the same work (Pato et al., 2023): CV intraassay 2.3–7.4%; CV interassay 4.4–8.3%; recovery between 80 and 120% and LoQ 1.34 μg/g faeces. Myeloperoxidase was determined spectrophotometrically using the o-dianisidine oxidation method (Bradley et al., 1982), slightly modified. Briefly, the two reagents were prepared fresh every day; reactive 1 (R1, 0.165 mg/mL O-dianisidine dihydrochloride, Sigma-Aldrich®, Merck Group diluted in 50 mM PBS, and reactive 2 (R2, 0.0005% hydrogen peroxide 30%, Sigma-Aldrich®, Merck Group diluted in distilled water). The autoanalyser configuration was 270 μL R1, 20 μL R2 and 10 μL sample in kinetic method analysis at 460 nm wavelength. For ADA analysis, an enzymatic colorimetric method described for human serum was used (from BioSystems, COD 23754, Barcelona, Spain). Calprotectin, MPO and ADA methods were adapted to Beckman Coulter® AU400 Chemistry Analyser. Lipocalin-2 was analysed using a commercial ELISA kit (Porcine Lipocalin-2/NGAL ELISA kit (Colorimetric) (NBP2-62163; Bio-Techne, Minneapolis, MN, USA). The results were read using a Multiskan Sky plate reader (Thermo Fisher Scientific, Waltham, MA, USA). For LCN-2 measurement, a previous validation in porcine faecal samples was needed, obtaining CV intraassay 3.5–14.8%; CV interassay 6.0–13.1%; and acceptable recovery results. To ensure the accuracy and reliability of the measurements, quality control measures included regular calibration of the analytical equipment, inclusion of blank samples to check for contamination, and running controls with known concentrations of the biomarkers to validate the assay's performance, alongside processing replicate samples to assess variability and reproducibility in the results.

Calculations and statistical analysis

For all analyses, the individual piglet was considered as experimental unit. Statistical analyses were performed by using R software (version 4.4.1, R Foundation, Vienna, Austria). Statistical significance was defined at $P \leq 0.05$, and a tendency was considered at $0.05 < P \leq 0.10$.

Growth performance and quantile analysis

Growth performance was analysed using an F-test within a linear model. A simple linear model was fitted for BW day 0 with "eater category" as the main factor. For the other growth performance data, the model included "eater category" as the main factor and BW day 0 as a covariate. Significant differences were corrected by using Tukey's test for multiple comparisons between each pair of means. Prevalence of eaters was calculated as a percentage of the total number of piglets.

Longitudinal analysis of biomarkers and differences between eater categories

Before conducting the biomarker analyses, a Shapiro-Wilk test to assess normality was performed. Since they did not exhibit normality, a logarithmic transformation was applied to achieve normalisation. To deal with correlated longitudinal data, linear mixed-effects models were considered, with day (x) for the time evolution, day squared (x^2) in second–order models, k^{-1} dummies to represent the k categories of the fixed effects factor and the individual as a random effect (Diggle et al., 2002). Additionally, the interaction between day and eater category was included in the model to assess differences in biomarker levels across days.

The model equation for the response (log-scaled fCal, LCN-2, MPO, and ADA) y_{ij} of individual i in day x_j can be written as: $y_{ij} = {}_{ij}\ \alpha + \alpha_1 D_1 + \alpha_2 D_2 + u_i + \beta_{xj} + \gamma x_j^2 + \delta_1(D_1 \cdot x_j) + \delta_2(D_2 \cdot x_j) + \varepsilon_{ii}$ (1), where α is the mean value of y at $x = 0$ in the reference category; α_1 and α_2 are the differential effect on the mean to be added if the individual belongs to the category represented by dummy variable D_1 or D_2, respectively; u_i the i-th individual random effect on the mean; $\beta x_j + \gamma x_j^2$ express the quadratic trend evolution on time; and ε_{ij} is the random noise. To account for potential differences in biomarker evolution over time between eater categories, interaction terms between day (x_j) and eater category (D_1, D_2) were included in the model. These interaction terms $\delta_1(D_1 \cdot x_j)$ and $\delta_2(D_2 \cdot x_j)$ allow assessing whether the effect of time differs across categories. All the random terms are assumed to be independent and centred Gaussian random variables, being σ_u^2 the common variance of the u_i and σ_ε^2 the common variance of the ε_{ij}. More complex models that assumed random effects on the day coefficient were considered and declared non–significant when tested against the random intercept model (1), which was the chosen model. Thus, the expected or mean response will be described in the three given categories by: in the reference category: $y = \alpha + \beta x_j + \gamma x_j^2$ (2); in the category defined by D1: $y = (\alpha + \alpha_1) + (\beta + \delta_1)x_j + \gamma x_j^2$ (3); and in the category defined by D2: $y = (\alpha + \alpha_2) + (\beta + \delta_2)x_j + \gamma x_j^2$ (4). The Linear Mixed Effects Model function from the nlme library in R (Pinheiro and Bates, 2023) was used to adjust the models and get the estimates and the P-values.

Analysis of the relationship between fasting duration and biomarker concentration, and its correlation with piglet growth

For the analysis of the faecal biomarkers on day 3 in relation to the days of fasting experienced by the animals, ANOVA F-tests were conducted separately for the two variables adjusting a GLM with a Gamma distribution and a logarithmic transformation, and a linear model for the ADG. The mean values are graphically represented, and significant differences are checked using Tukey's correction for multiple comparisons between each pair of means. A Spearman correlation test was conducted between the faecal biomarkers' concentration and the ADG, and to assess the correlations between biomarkers on each sampling day.

Evaluation of biomarker performance in discriminating feeding categories using machine learning techniques

To determine whether the selected biomarkers could predict whether an animal was in fasting or had consumed feed at least once during the first 4 days PW, a receiver operating characteristic and binary logistic regression analysis were performed. To increase the power of the model, all data from the 4 days for both CFE and NE animals were combined, considering the minimal variation in the day factor within these categories. A generalised additive mixed model was initially used to account for the day as a covariate and the piglet as a random effect, as repeated measures were present. However, the day factor was not significant for any biomarker. Following this, a receiver operating characteristic analysis

was conducted to calculate the area under the curve (**AUC**) and its significance using a bootstrapping test. Only biomarkers with an AUC above 0.7 and statistically significant were selected for further analysis, including fCal, LCN-2, and MPO, while ADA was discarded. Sensitivity, specificity, and accuracy were calculated from the receiver operating characteristic analysis, and the optimal cut-off point was determined using Youden's index (Perkins and Schisterman, 2006).

To assess the reliability of the model in predicting the two categories, the dataset was first split into a training set (70%) and an independent validation set (30%). Within the training set, a 10-fold cross-validation was performed to train and evaluate different models while minimising the risk of overfitting. Sensitivity (proportion of true NE), specificity (proportion of true CFE), and accuracy (overall proportion of correct predictions) were calculated during cross-validation to guide model selection. The final model, selected based on its cross-validation performance and kappa value, was then evaluated on the untouched validation set to estimate its generalisation performance.

Results

Eater and non-eater prevalence and piglets' growth

Prevalence of eaters and piglets' growth of all monitored piglets over the first 10 days PW are presented in Table 2. At weaning, 4.49% of piglets were classified as CFE, which tended to have lower BW than the NE group (5.49 vs 5.90 kg; $P = 0.092$). Of the 95.5% classified as NE at weaning, 69.5% began eating and were reclassified as PWE, while 26.0% remained NE. On day 3 PW, CFE piglets had the highest BW at 6.13 kg, followed by PWE at 5.96 kg, and NE at 5.66 kg ($P < 0.001$). Corresponding ADG values were 63.0 g/d for CFE, 19.9 g/d for PWE, and –63.4 g/d for NE, with CFE and PWE showing significantly greater growth than NE ($P < 0.001$). By day 10, CFE maintained the highest BW (7.05 kg), followed by PWE (6.61 kg) and NE (6.19 kg) ($P < 0.001$). Although no significant growth differences were found between CFE and PWE from day 3 to 10 ($P > 0.05$), CFE gained 44 g more per day than PWE and nearly 100 g more than NE ($P < 0.001$).

Faecal biomarker evolution throughout the first three days postweaning

Fig. 2 presents the evolution of faecal biomarkers over the first 3 days following weaning. All four biomarkers varied significantly over time ($P < 0.001$). However, while fCal (Fig. 2A), LCN-2 (Fig. 2B), and MPO (Fig. 2C) showed a non-linear association with time ($P < 0.014$), ADA (Fig. 2D) exhibited a proportional daily variation ($P = 0.179$).

Quadratic regression analysis revealed that fCal and LCN-2 had negative quadratic coefficients (–0.104 and –0.258, respectively), indicating a concave shape, where levels initially increased, reached a peak, and then declined. In contrast, MPO had a positive quadratic coefficient (0.178), indicating a convex shape, with levels decreasing initially, reaching a minimum, and subsequently increasing.

All biomarkers except ADA were significantly influenced by the eater category ($P < 0.001$). Non-eaters consistently showed eight times higher fCal concentrations than CFE piglets across all four sampling days, exceeding 30 μg/g of faeces, while CFE piglets remained consistently below 7 μg/g ($P < 0.001$). On day 1 PW, no significant differences in fCal concentrations were observed between NE and PWE piglets. Even so, both groups differed from CFE, maintaining levels 18 μg/g higher than CFE ($P < 0.05$). By day 2 PW, this pattern shifted, with PWE becoming statistically distinct from both NE and CFE, settling at an intermediate concentration of 15.1 μg/g of faeces ($P \leq 0.011$). By day 3 PW, PWE aligned with CFE, showing fCal levels 20 μg/g of faeces lower than NE ($P = 0.245$).

Lipocalin-2 levels consistently differed between NE and CFE piglets throughout the sampling period. At weaning, NE piglets exhibited concentrations 80 ng/g higher than CFE, rising to over 100 ng/g on days 1 and 2 PW before decreasing on day 3 PW, when the difference returned to 80 ng/g ($P \leq 0.040$). PWE piglets showed no differences from NE on days 1 and 2 PW, but their levels decreased notably over time, ultimately aligning with CFE by day 3 PW, with concentrations 70 ng/g lower than NE ($P < 0.05$).

Overall, MPO followed a distinct pattern compared to fCal and LCN-2. At weaning, no significant differences were observed between NE and CFE piglets ($P = 0.397$). From day 1 to day 3 PW, NE piglets exhibited lower faecal MPO concentrations compared to CFE. As the days progressed, this difference increased, with CFE reaching 1.4 U/g higher MPO levels than NE by day 3 ($P < 0.001$). Moreover, PWE piglets showed no significant differences compared to CFE throughout the period ($P > 0.05$).

Biomarker correlations

Fig. 3 presents the daily correlations among the four biomarkers. Lipocalin-2 and fCal exhibited a consistently significant positive correlation throughout the four-day period ($P \leq 0.035$), reaching its strongest point on day 3 PW ($P < 0.001$). Myeloperoxidase tended to positively correlate with ADA on day 1 PW ($P = 0.055$), while by day 3 PW, it displayed a negative correlation

Table 2
Prevalence and growth performance from weaning to day 10 postweaning of piglets according to the three eater categories (CFE, NE, and PWE)[1].

Item	Eater category			*P*-value[2]
	CFE	NE	PWE	
Prevalence at weaning (%)	4.49% (28/623)	95.5% (595/623)		
Prevalence at day 3 PW (%)	4.49% (28/623)	26.0% (162/623)	69.51% (433/623)	
BW day 0 (kg)	5.49 ± 0.24	5.90 ± 0.05		0.092
BW day 3 (kg)	6.13^{a} ± 0.10	5.66^{b} ± 0.04	5.96^{a} ± 0.03	<0.001
BW day 10 (kg)	7.05^{a} ± 0.13	6.19^{c} ± 0.05	6.61^{b} ± 0.03	<0.001
ADG days 0–3 (g/d)	63.0^{a} ± 23.6	–63.4^{b} ± 9.9	19.9^{a} ± 6.0	<0.001
ADG days 3–10 (g/d)	147^{a} ± 20	82.0^{b} ± 8.6	108^{a} ± 5.2	0.003
ADG days 0–10 (g/d)	117^{a} ± 13	27.0^{c} ± 5.6	72.8^{b} ± 3.4	<0.001

Abbreviations: ADG = average daily gain; CFE = Creep-feed Eaters; NE = Non-eaters, PW = postweaning; PWE = postweaning eaters.
[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$ (Tukey's test).
[1] Values are represented as Least-Squares Means ± SEM.
[2] *P*-values come from ANOVA F-test.

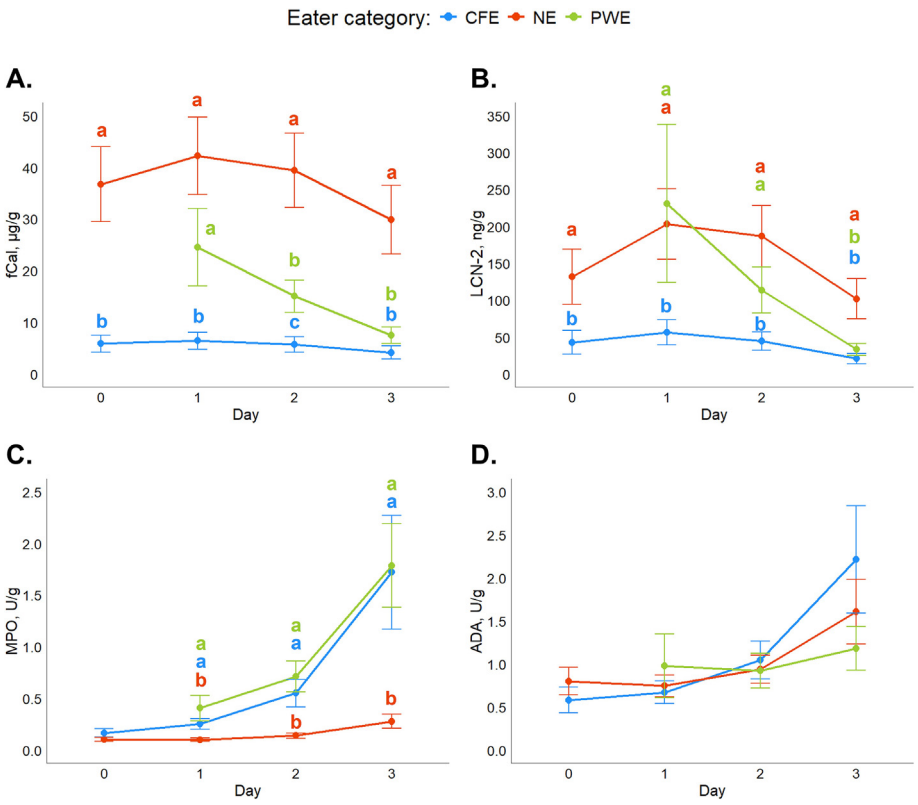


Fig. 2. Evolution of faecal biomarkers over the first 3 days postweaning according to the piglets' eater category: (A) calprotectin (fCal), (B) lipocalin-2 (LCN-2), (C) myeloperoxidase (MPO) and (D) adenosine deaminase (ADA). Eater categories: Creep–Feed Eaters (CFE; day 0–3 n = 19), Non–Eaters (NE; day 0 n = 30, day 1 n = 28, day 2 n = 27, day 3 n = 25) and PostWeaning Eaters (PWE; day 0 n = 0, day 1 n = 7, day 2 n = 14, day 3 n = 33). Values are presented as least–square means with error bars representing SEM. Different letters (a, b, c) indicate significant differences between groups ($P < 0.05$) according to Tukey's test.

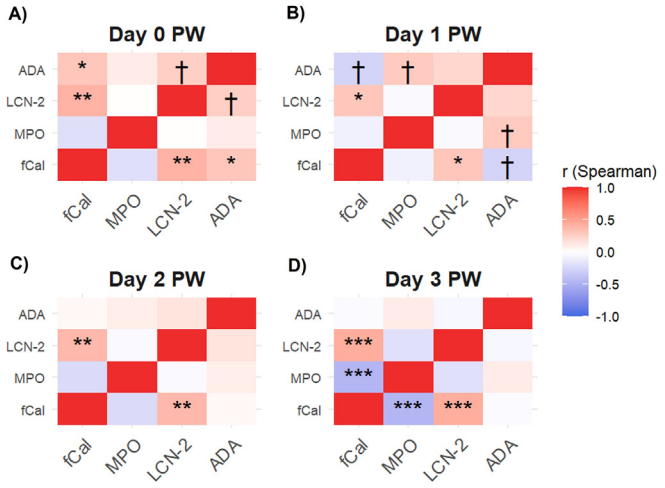


Fig. 3. Correlations between inflammatory biomarkers measured daily in piglets during the first 4 days postweaning (PW): calprotectin (fCal), lipocalin-2 (LCN-2), myeloperoxidase (MPO) and adenosine deaminase (ADA). Correlations were calculated with the Spearman correlation test. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, † $P < 0.1$.

with fCal ($P \leq 0.010$). At weaning, both fCal and LCN-2 showed a positive correlation with ADA ($P \leq 0.050$); however, by day 1 PW, this correlation was reversed between fCal and ADA ($P = 0.061$).

Association between biomarker concentrations on day 3 postweaning, postweaning growth, and fasting duration

Fig. 4 illustrates the correlation between different faecal biomarkers on day 3 PW and the ADG from weaning to day 3 PW, based on the number of days, the animals experienced fasting. Only fCal and LCN-2 had a significant negative correlation with the piglets' growth, where their concentrations increase as the ADG decreases ($r = -0.541$, $P < 0.001$ and $r = -0.292$, $P = 0.044$, respectively). On the contrary, MPO and ADA had no significant relationship with the ADG ($r \leq 0.197$; $P \geq 0.05$).

The biomarker that showed the highest sensitivity in differentiating animals based on their fasting duration was fCal ($P < 0.001$). No different fCal concentrations were observed between piglets fasted for 0 or 1 day, both showing fCal concentrations below 10 µg/g ($P > 0.10$). Similarly, no differences were observed between those fasting for 3 or 4 days, which showed average fCal between 30 and 60 µg/g. However, fCal concentrations were significantly increased in piglets that showed a fasting period of 4 days compared to those fasting for zero or one day ($P \leq 0.012$). Piglets experiencing 2 days of fasting showed intermediate values of 13.7 µg/g, overlapping with the 1, 3, and 4-day groups. Lipocalin-2 followed a similar pattern to fCal. A trend towards significance was observed between piglets that did not fast and those fasting for 4 days (65.5 vs. 495.2 ng/g, $P = 0.078$), and their concentrations only differed significantly between piglets fasting for one day and those fasting for 4 days (22.5 vs. 495.2 ng/g, $P = 0.009$). No differences were observed among piglets fasting for two, three, or 4 days ($P > 0.05$). In terms of MPO, values did not vary between piglets fasting for zero to 3 days. However, a significant reduction was

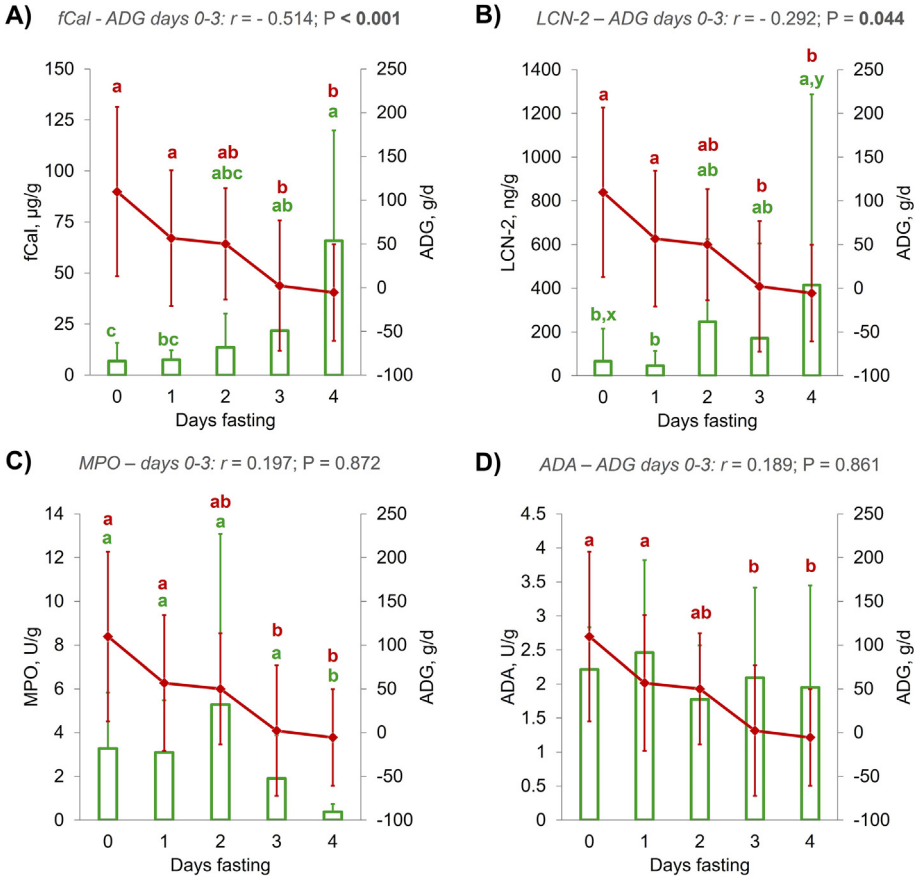


Fig. 4. Faecal biomarkers and average daily gain at day 3 postweaning (ADG days 0–3) in relation to days of fasting in postweaned piglets: (A) calprotectin (fCal), (B) lipocalin-2 (LCN-2), (C) myeloperoxidase (MPO) and (D) adenosine deaminase (ADA). Faecal concentrations are shown as bars (left axis) and ADG as lines (right axis). Sample sizes: 0 days fasting (n = 11), 1 day (n = 12), 2 days (n = 11), 3 days (n = 17) and 4 days (n = 15). Values are presented as least–square means ± SEM. The correlation between the biomarkers' concentrations and ADG was assessed using Spearman's correlation; significance was set at $P < 0.05$. Different letters (a, b, c) indicate significant differences between groups ($P < 0.05$), and letters (x, y) indicate trends towards significance ($P < 0.1$) according to Tukey's test.

observed in piglets fasting for 4 days, with MPO levels dropping below 1 U/g, whereas all other groups maintained average concentrations above 2 U/g ($P < 0.001$). The biomarker ADA did not have differences regarding the days of fasting ($P = 0.856$).

Diagnostic performance of biomarkers in distinguishing creep-feed eaters from non-eaters

The predictive models of the faecal biomarkers are presented in Table 3. Calprotectin demonstrated the highest capacity for predicting the two eater categories, showing a significant AUC of 0.86 (confidence interval: 0.81–0.92, $P < 0.001$). The model identified the cut-off point at 24.9 µg/g of faeces, where animals with higher concentrations were categorised as NE and animals below this threshold were categorised as CFE. The fCal model had a high capacity for identifying NE (Sensitivity: 0.86), but it faced some challenges in identifying CFE, as indicated by a high number of false positives in the confusion matrix (Specificity: 0.69), although the overall accuracy was acceptable (0.76). The training and validating models achieved AUC values above 0.8, with high sensitivities (0.95 and 0.97), but specificity was penalised, ranging between 0.55 and 0.58, reflecting the difficulty in classifying CFE. Overall, the fCal model had a 10-fold cross-validation accuracy of 0.7 and a Kappa value of 0.44, indicating moderate reliability of the model. Fig. 5A illustrates the graphical representation of the receiver operating characteristic analysis and the AUC calculation

Table 3
Binary logistic regression analyses predicting non-eaters (NE; piglets which did not consume feed during the first 4 days postweaning) from creep-feed eaters (CFE; piglets which had uninterrupted intake after weaning) during the first 3 days postweaning. Only analytes with a significant AUC were included ($P < 0.05$).

Biomarker	Model	AUC (SD)	95% CI	*P*-value	Cut-off	Se	Sp	Acc	Kappa
fCal	Cross-validation[1] (Training Model)	0.84 (0.03)	0.78–0.91	<0.001	>24.9 µg/g	0.95	0.55	0.77	0.44
	Validation Model	0.91 (0.04)	0.83–0.98	<0.001		0.97	0.58	0.83	
LCN-2	Cross-validation (Training Model)	0.74 (0.05)	0.65–0.83	<0.001	>298.7 ng/g	0.60	0.82	0.70	0.26
	Validation Model	0.63 (0.07)	0.47–0.79	0.410		0.62	0.55	0.58	
MPO	Cross-validation (Training Model)	0.68 (0.04)	0.57–0.79	0.007	<1.07 U/g	0.69	0.65	0.67	0.27
	Validation Model	0.78 (0.06)	0.66–0.91	0.010		0.77	0.63	0.72	

Abbreviations: AUC = Area under the curve; Acc = Accuracy; CI = Confidence interval; fCal = Faecal calprotectin; LCN-2 = Lipocalin-2; MPO = Myeloperoxidase; Se = Sensibility; Sp = Specificity.
[1] Sample size for the Training Model: CFE = 53, NE = 68; Sample size for the Validating Model: CFE = 22, NE = 29.

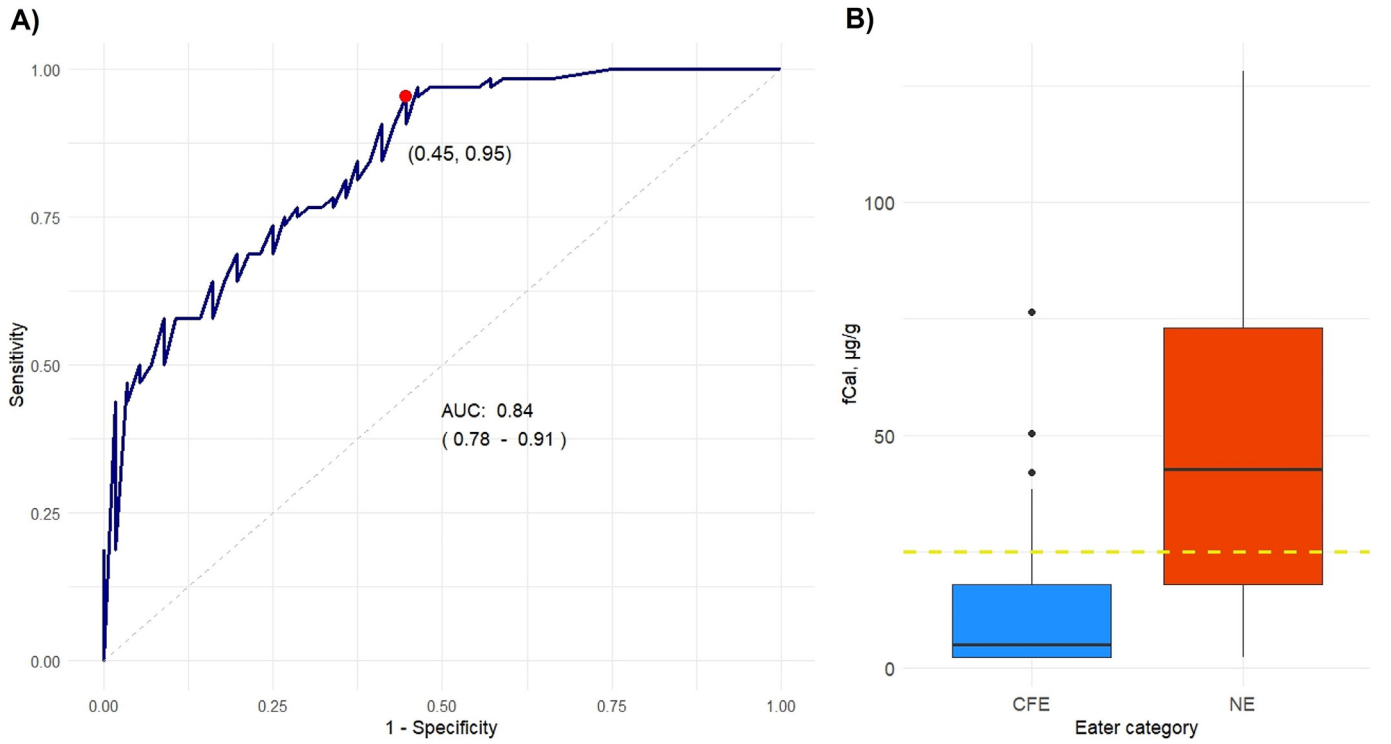


Fig. 5. Receiver operating characteristic (ROC) analysis for the most discriminant biomarker, faecal calprotectin (fCal), in distinguishing creep-feed eaters (CFE) from non-eaters (NE) piglets during the first 4 days postweaning. (A) ROC curve and optimal cutoff value were calculated using cross-validation on the training set. The area under the curve (AUC) is 0.84 (95% confidence interval, CI: 0.78–0.91). The red dot indicates the optimal cut-off point balancing sensitivity (0.95) and specificity (0.55). (B) Boxplot shows fCal concentrations in the independent validation set, with the optimal cutoff value of 24.9 µg/g previously calculated with the training set, marked by the yellow dashed line. Sample size: Training set: CFE = 53, NE = 68; Validating set: CFE = 22, NE = 29.

for fCal. In Fig. 5B, the actual data distribution is shown with the cut-off point determined by the binary logistic regression model, visualising how the data are classified based on this threshold.

On the other hand, the LCN-2 and MPO models did not achieve the same high capacity for identifying CFE as fCal, with AUCs around 0.7 and wider confidence intervals (between 0.6 and 0.8). Although both biomarkers showed higher sensitivity in identifying NE (0.85 and 0.81, respectively), they had higher false-positive rates, with specificity ranging from 0.54 to 0.49, respectively. The 10-fold cross-validation accuracy did not exceed 0.7, while the Kappa value was between 0.26 and 0.27, indicating that the reliability of these biomarkers in predicting NE versus CFE was not as high as that of the fCal model. Finally, ADA did not have a significant AUC, being discarded for further analysis ($P > 0.05$).

Discussion

Weaning is a critical phase involving dietary, environmental, and social stressors (Moeser et al., 2017). Piglets are abruptly separated from the sow and exposed to a cereal-based diet, often leading to temporary fasting. Studies report that some piglets eat less than 10 g/day in the first 48 h, and up to 10% may take more than 2 days to start eating (Bruininx et al., 2002; Smith et al., 2010). Consistent with this, in short lactation (21 days), only 4% of piglets ate continuously after weaning, while 26% did not eat at all in the first 3 days. This drop in intake causes negative energy balance and growth setback (Noblet and Etienne, 1986), as piglets require 700–800 kJ $\times$ kg$^{-0.75}$ $\times$ d^{-1} but often consume only ~250 kJ (Le Dividich and Sè, 2000; Close and Stanier, 1984). In addition, prolonged fasting has severe physiological consequences (Dong and Pluske, 2007). Without luminal nutrients, intestinal epithelial cells undergo apoptosis, leading to villous atrophy and increased intestinal permeability (Spreeuwenberg et al., 2001; Smith et al., 2010). This compromises gut integrity, allowing the translocation of antigenic compounds through the intestinal wall, possibly triggering an inflammatory response (Mccracken et al., 1999). Additionally, inflammation has high energy demands (Labussière et al., 2015), further diverting resources away from other physiological processes (Klasing and Johnstone, 1990). Overall, our findings illustrate this impact: piglets that fasted for more than 3 days gained 1 kg less than those with continuous feed intake after weaning during the first 10 days PW. Notably, even those who started eating within 3 days gained 40 g/day less than uninterrupted eaters. Fabà et al. (2024) also reported that higher early feed intake correlates with better growth throughout the nursery period.

Despite its impact on growth, health and animal welfare, feeding behaviour is challenging to monitor in commercial settings. Given the link between fasting and intestinal inflammation (Madec et al., 1998), we aimed to assess whether faecal inflammatory biomarkers could serve as indicators of feed intake and, consequently, animals facing greater challenges. These biomarkers could offer a practical non-invasive tool for identifying piglets at risk of PW growth retardation and diarrhoea. This study used faecal inflammatory biomarkers (fCal, MPO, LCN-2, and ADA), widely applied in human medicine and increasingly valued in animal research as indicators of intestinal inflammation (Celi et al., 2019).

Calprotectin and LCN-2 showed persistent differences between NE and CFE piglets throughout the four sampling days, showing increased values in the NE piglets. Calprotectin is the most used biomarker for intestinal inflammation (Kapel et al., 2023). Its abundance in neutrophils and its role in immune cell recruitment make it highly sensitive to early inflammatory responses (Bressler et al., 2015). Although LCN-2 is also produced in neutrophiles, its expression has been observed in intestinal epithelial cells during inflammation, correlating with its presence in faeces as a marker of

chronic or sub-clinical inflammation (Thorsvik et al., 2017). Our results support the idea that fasting after weaning may promote an inflammatory local effect, as suggested by others (McCracken et al. 1999; Pié et al., 2004). At weaning, LCN-2 and fCal values were clearly greater in NE than in CFE piglets. At this point (day 0), we were not assessing fasting but rather the difference between having consumed creep-feed or not. Some studies suggest that eating shortly after weaning may have a pro-inflammatory effect proved with increasing concentrations of plasma C reactive protein and haptoglobin (Engelsmann et al., 2023). Nevertheless, it is important to note that larger piglets (NE in our case) often experience more social conflicts PW. This leads to increased stress, which could explain the observed differences in plasma acute phase proteins (Meese and Ewbank, 1973). Despite variability, fCal was more sensitive in distinguishing PWE piglets by day two, with CFE averaging 12.5 μg/g, NE 63.7 μg/g, and PWE 21.4 μg/g, whereas LCN-2 did not differentiate this intermediate group.

Unlike fCal or LCN-2, the biomarker MPO exhibited distinct trends both in relation to the eater categories and its evolution over time. Myeloperoxidase is an enzyme released by neutrophils during oxidative stress, creating a hostile environment for pathogenic bacteria while affecting host cells (Davies, 2021). In our results, MPO levels did not differ between CFE and NE at weaning but remained consistently higher in CFE thereafter, following a non-linear increasing trend. No previous study has reported a negative correlation between MPO and fCal. Myeloperoxidase is known to respond to infectious challenges rather than non-septic inflammation (Botía et al., 2023), suggesting it may be more indicative of pathogenic stress than feed intake changes (Lemoine et al., 2019). While fasting increases the risk of secondary colibacillosis infections, these usually emerge in the following week PW (Carstensen et al., 2005). The short timeframe of this study, combined with the rapid physiological changes in piglets, may not have been enough to trigger MPO secretion. Its increase over time could be a response to a different stimulus unrelated to fasting.

Notably, ADA showed no differences between eater categories. This enzyme is mainly produced by T lymphocytes and is involved in the adenosinergic system (Ye and Rajendran, 2009). The function of ADA is breaking down the anti-inflammatory molecule adenosine, promoting the immune response (Antonioli et al., 2012). While ADA has not been extensively studied in piglets, Souza et al. (2018) reported increased levels in piglets exposed to mycotoxins, linking it to immune activation. In our study, ADA levels rose linearly over time regardless of feeding status, suggesting it reflects a broader immune response rather than fasting-induced inflammation. Moreover, ADA has been used as a marker of lymphocyte function (Contreras-Aguilar et al., 2019) and has been associated with stress-driven immune responses in pigs, alongside immunoglobulin A and alpha-amylase in saliva (Ott et al., 2014). Additionally, ADA levels have been shown to increase with age and high-fibre diets, indicating enhanced immune function (Grześkowiak et al., 2024). This suggests that the gradual rise in ADA observed in this study may reflect the maturation of the immune system, as this period coincides with lymphocyte migration into intestinal lymphoid tissue (Bailey et al., 2002). Given the evolution of the four faecal biomarkers, fCal and LCN-2 may be more specifically linked to local inflammation associated with fasting, while MPO and ADA appear to reflect a broader immune response.

To validate the role of these biomarkers in detecting fasting animals, a correlation with their growth during these first 4 days after weaning was performed. Both fCal and LCN-2 significantly negatively correlated with the piglets' ADG, with increased values in piglets with weight loss. As discussed earlier in this manuscript, fasted animals face challenges not only due to the lack of nutrients, which impairs growth, but also due to inflammation. This inflammatory response has been reported to contribute to growth loss, as it demands high energy expenditure (Le Floc'h et al., 2006). Neither MPO nor ADA shows a significant correlation with growth performance, again suggesting a different role of these biomarkers. Souza et al. (2018) also found no association between PW growth and faecal ADA concentrations. In contrast, Sun et al. (2020) reported increased MPO levels with higher BW, suggesting a positive correlation. This aligns with our findings, where MPO was numerically higher in faster-growing piglets. It might suggest that higher fCal and LCN-2 reflect more challenged animals during the first days after weaning, while MPO and ADA do not. Animals that fasted for more than 2 days showed reduced growth during the first 4 days PW, suggesting similar effects between three and 4 days of fasting. In contrast, those fasting zero to one day grew the most, indicating that exceeding 2 days without food notably impacts growth. Calprotectin levels closely mirrored the animals' growth patterns, effectively distinguishing those fasting for less than 2 days from those fasting for more than three. In contrast, MPO does not exhibit this same pattern, as it only differentiates animals that fasted for 4 days from the rest, decreasing its values.

After identifying the biomarkers that distinguish between eater categories, a binary logistic regression model was used to predict the likelihood of an animal being a CFE or NE. To achieve this, we focused on the two extremes (CFE and NE) and used data from the four sampling days, providing an approximation that could be applicable during the three critical days PW. The results from the binary logistic regression analysis revealed that the biomarker with the highest predictive capacity to distinguish between continuous eaters (CFE) and animals with prolonged fasting (NE) was fCal, with a cut-off value of 24.9 μg/g, which can be applied during any of the first 4 days PW. It is important to note that although the model was highly reliable in predicting animals that were not feeding, it was somewhat questionable in predicting those with continuous consumption, showing low specificity. Therefore, applying this fCal cut-off could result in misclassifying some animals that are CFE as NE. However, this is not a major concern, as it may mean that some animals that are eating would receive individualised treatments, being the most common strategy mixing the solid feed with warm water, which is not a significant issue economically (Missotten et al., 2015; Ferreira et al., 2024). The most crucial aspect is identifying the animals that are not eating, as this allows for individualised therapies at the pen level to encourage consumption and minimise prolonged fasting periods.

A potential limitation of using faecal biomarkers to compare eaters and NE is the influence of faecal mass and consistency, as NE produce less faeces. However, prior studies found no major differences between fresh and lyophilised samples, suggesting faecal composition may not strongly affect concentrations (Grellet et al., 2016; Lasson et al., 2015; Pato et al., 2023). In our study, faecal mass was not monitored, so this effect cannot be ruled out. Still, if lower faecal mass in NE explained higher biomarker levels, all markers should be similarly affected, which was not observed, where MPO and ADA showed similar or even lower levels in NE compared to eaters. Further research should clarify this confounding factor, validate findings with additional inflammatory markers, and expand sample size across diverse farms and conditions. To our knowledge, this is the first study to define a non-invasive faecal biomarker to distinguish fasting status in PW piglets.

Conclusions

Both fCal and LCN-2 were elevated in piglets undergoing prolonged fasting and lower in those with continuous feed intake. Their negative correlation with growth suggests that these biomarkers can distinguish between animals coping well with

weaning and those experiencing greater challenges. Among them, fCal proved to be the most sensitive biomarker, effectively differentiating between continuous consumers, animals that started eating within 3 days, and those fasting beyond this period. With a potential cut-off value, fCal demonstrated the highest predictive capacity for identifying fasting animals during the first 4 days PW. However, while the model was highly effective in detecting non-consumers, its ability to predict consumers was more limited. Otherwise, neither MPO nor ADA showed a direct relationship with feeding patterns, which may reflect other physiological processes unrelated to feeding behaviour.

Ethics approval

All procedures described in this study, conducted in commercial farm conditions, were approved by the Ethical Committee on Animal Experimentation of the Universitat Autònoma de Barcelona (CEAAH 6644).

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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