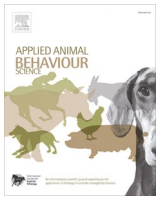
Applied Animal Behaviour Science 261 (2023) 105882

Contents lists available at ScienceDirect

Applied Animal Behaviour Science

journal homepage: www.elsevier.com/locate/applanim



Behavioural changes in weaned piglets orally challenged with *Escherichia coli* F4 and supplemented with in-feed protected acid salts

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ARTICLE INFO

Keywords:
Pig
Sickness behaviour
Escherichia coli F4
Butyrate, medium-chain fatty acid

ABSTRACT

Improvement of the intestinal health of piglets at weaning is a principal objective in pig farming in terms of performance and welfare benefits. Early indicators of disease are indispensable for evaluating animal health and the efficacy of interventions such as feed additive supplementation. This study evaluates behavioural changes in weaned piglets that are orally challenged with enterotoxigenic *Escherichia coli* F4, and which receive or not two different in-feed additives. Two independent trials were performed with early-weaned piglets in pens of three animals, which were fed a plain diet (N = 32) or one supplemented with sodium butyrate (N = 16; Trial 1) or sodium heptanoate (N = 16; Trial 2) and protected with coconut distillates. After one week of adaptation, piglets were challenged with a single oral dose of *E. coli* F4 (minimum 1.4 $\times 10^9$ cfu). Scan-sampling was used to evaluate individual behaviour (location in the pen, postures, contact with pen mates, and activities) on the day before (d-1) and two days post-inoculation (d+2 and d+3) at 2-min intervals. Behaviours were recorded in mornings (8 am to 10 am) and afternoons (4 pm to 6 pm). Faecal consistency was also recorded for each animal. Diarrhoea peaked at d+ 2. Regarding behaviour, on d+ 2 there was greater frequency of the animals lying inactively under the heat lamp, in contrast to d-1 when they were more frequently present in the feeder, standing and active ($P < 0.05$). Around the feeder, standing and active behaviour increased at d+ 3, especially in the afternoon ($P_{Day \times Time\ of\ day} < 0.05$). Piglets fed sodium heptanoate spent less time around the feeder ($P < 0.05$). The weight of the animal at weaning was also observed to have an impact on the effect of time or diet on behaviour. Medium size piglets spent more time lying with pen mates in the afternoon ($P_{Size \times Time\ of\ day} < 0.01$) and the smallest piglets increased their feeding behaviour when receiving the supplemented diets ($P_{Size \times Diet} < 0.05$). In conclusion, a lethargic response among piglets after the *E. coli* F4 challenge was evidenced, this response being slightly modified by the supplementation of in-feed additives and the size of the animals. These results are evidence of the potential of behavioural indicators as a useful tool to assess the health status of piglets at weaning and their responses to in-feed supplementation, but they should be regarded with caution before any transfer to farm conditions due to the limitations of experimental models.

1. Introduction

Enterotoxigenic *Escherichia coli* is the main pathogen responsible for post-weaning diarrhoea in pigs (Rhouma et al., 2017). Also called colibacillosis, it is an opportunistic disease that takes advantage of unfavourable conditions around weaning, enabling pathogenic serotypes to become dominant (Bessone et al., 2017; Li et al., 2020). This disorder represents major economic losses for the pig industry due to reduced growth and performance of weaned pigs, and also mortality (Luppi, 2017). However, it has been estimated that up to 66% of enterotoxigenic *E. coli* colonized nursery pigs can remain subclinical, but their welfare and productivity will nevertheless be compromised (Moredo et al., 2015).

Behavioural observations, such as decreased feeding and drinking

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https://doi.org/10.1016/j.applanim.2023.105882
Received 5 May 2022; Received in revised form 21 February 2023; Accepted 6 March 2023
Available online 11 March 2023

behaviours and generally reduced activity, have been shown to be particularly useful for detecting illness (Brown-Brandl et al., 2013; Escobar et al., 2007; Madsen and Kristensen, 2005; Rostagno et al., 2011). As a result of pathogen colonization, animals develop sickness behaviours that are characterized by anorexia and lethargy (Johnson and von Borell, 1994), associated to internal changes that occur sub-clinically and may go unnoticed by other clinical or analytical measurements (Dawkins, 2003; Escribano et al., 2019; Reiner et al., 2009). Animals that have been experimentally challenged with enterotoxigenic *E. coli* have presented mild changes in behaviour such as inactivity and unresponsiveness to stimuli (Spitzer et al., 2014), although an earlier study did not find any behavioural response (Jensen et al., 2006). These responses may be conditioned by the virulence profile (Krsnik et al., 1999) and probably many other factors.

In recent decades, and especially most recently due to real, urgent need to reduce the use of antimicrobials in livestock, different post-weaning in-feed strategies have been proposed to improve the intestinal health of pigs and reduce the risk of diarrhoea (Kim et al., 2022; Patience and Ramirez, 2022). These include the addition of bioactive ingredients to weaning diets, such as organic acids, which has been widely observed to improve weaning transition and to reduce the negative influence of stress factors from the environment, such as pathogen pressure and its negative consequences on animal performance, and digestive and immunological systems (Vanrolleghem et al., 2019). Short- and medium-chain fatty acids (SCFA, and MCFA, respectively) like butyric acid and lauric acid have been demonstrated to improve weaned pigs response by boosting stomach acidification and digestion of nutrients, and inhibiting the growth of pathogens (Lauridsen, 2020; Mroz, 2005; Zentek et al., 2012). Previous results published by our group have also demonstrated the impact of protected butyrate (López-Colom et al., 2019a) and medium-chain fatty acid (López-Colom et al., 2019b) salts on the intestinal barrier by modifying the immune response and modulating the intestinal microbial ecosystem.

The potential of behavioural observations as evidence of the benefits of in-feed additives around weaning has been explored previously by our group with promising results (Barba-Vidal et al., 2017). However, despite the potential of behavioural changes to provide information about pig health, this method has rarely been used to assess the effects of nutritional strategies to improve welfare around weaning (Liu et al., 2018; Upadhaya and Kim, 2021). Additives, such as protected acid salts, could have an impact on animal behaviour by fighting intestinal pathogens and reducing their deleterious effects, but also by modulating gut microbiota, which could have an impact on animals' behaviour by acting on the microbiota-gut-brain axis, whereby the microbiota, the enteric nervous system (ENS) and the gut endocrine and immune systems may communicate via neurotransmitters with a demonstrated impact on behaviour (Lyte, 2013).

In this study, we therefore aim to evaluate the usefulness of behavioural observations to assess the responses of weaned pigs that are orally challenged with *E. coli* F4 to different in-feed butyrate or heptanoate-based additives (acid salts of SCFA and MCFA, respectively) protected with coconut distillates. Additionally, since individual traits might interfere with social interactions and health outcomes (Brown-Brandl et al., 2013; Bruininx et al., 2001), we also evaluated possible differences in individual behavioural responses in consideration of live weight (LW) at weaning. Finally, we also discuss these responses in relation to previously reported results based on performance, clinical signs, intestinal health, and microbiota (López-Colom et al., 2020).

2. Material and methods

This study was part of a larger one that has been published previously (López-Colom et al., 2020). In this case, two different trials were performed to evaluate the effect of the two additives on the behaviour of piglets in response to an oral challenge with *Escherichia coli* F4. Both trials were performed at the Servei de Granges i Camps Experimentals at the Universitat Autònoma de Barcelona (UAB) and received prior approval (permit no. CEAAH2933/HR-10–13) from this institution's Animal and Human Experimental Ethical Committee. The treatment, management, housing, husbandry, and slaughtering conditions were also approved and conformed to European Union Guidelines (Directive 2010/63/EU).

2.1. Animals and housing

The two trials followed Level 2 – High Risk Biosecurity Procedures with appropriate training of the involved personnel. For both trials, 48 21-day-old male piglets ([Landrace × Large White] × Piétrain) were used, coming from *E. coli* non-vaccinated mothers and with previous creep feed provision. At the beginning of the experiments, animals weighed an average of 5.8 ± 0.57 kg of body weight (BW) in the first trial (Trial 1) and 5.6 ± 0.93 kg BW in the second trial (Trial 2).

In each trial, piglets were transported at weaning to the UAB facilities and placed in sixteen pens (three animals per pen). In each pen, animals were distributed according to weight: one low- (5.2 ± 0.30 kg BW in Trial 1 and 4.5 ± 0.42 kg BW in Trial 2), one intermediate- (5.7 ± 0.14 kg BW in Trial 1 and 5.7 ± 0.27 kg BW in Trial 2) and one high-weight (6.4 ± 0.24 kg BW in Trial 1 and 6.6 ± 0.44 kg BW in Trial 2), to obtain a homogenous final average weight among pens. The experimental treatments were distributed evenly to eight pens each.

Each pen (3 m^2) had a fully slatted floor, a round hog feeder and a nipple to provide food and water for ad libitum consumption. The weaning rooms were equipped with automatic heating and forced ventilation. Trial 1 and Trial 2 were conducted in the spring and winter, respectively (May and February), under a mean room temperature of 26.5 ± 1.29 °C. Both trials were maintained under a 13 h light/11 h dark lighting pattern.

2.2. Experimental diets

The pigs received pre-starter diets consisting of maize (28.1%), wheat (28%), and two-row barley (15%), sweet whey powder (10%) and whey powder 50% fat (3.3%), extruded soybean (12.2%), fishmeal LT (5%), and soybean meal 47 (5%), mono-calcium phosphate (2.1%) and calcium carbonate (0.8%), and a vitamin-mineral premix (0.4%; Vit-Min GPlus, Balsa, Les Masies de Voltregà, Spain). These diets were formulated to provide 2448 kcal/kg of net energy and 1.45% digestible lysine, to satisfy the nutrient requirement standards for pigs (NRC, 2012), and were administered ad libitum throughout the entire experimental period.

There were two experimental diets in each trial: a plain diet without additives (CTR), and the same diet supplemented with 3 kg/t of the feed additive. In each trial, the diets were manufactured in the same batch and feed additives were included in a second mixture, following the manufacturer's recommended dosages. Feed additives were supplied by Norel S.A. (Madrid, Spain), consisting of sodium salts of butyric acid (DIP in Trial 1) or heptanoic acid (HPP in Trial 2), both protected with a mixture of sodium salts of distilled coconut medium-chain fatty-acids (containing 50% of each salt).

2.3. Bacterial strain

The bacterial strain used was an enterotoxigenic *E. coli* F4 strain (positive to virulence factors F4ab, F4ac, LT, STb and negative to EAST1 and F6, F18, F41, STa, VT1, VT2 and EAE) that was isolated from 14-week-old pigs and provided by the Diseases Laboratory at the UAB (ref. COLI30/14–3). The oral inocula were prepared by overnight incubation at 37°C and 250 rpm in brain heart infusion (BHI) (Laboratorios Conda S.A., Torrejón de Ardoz, Spain). For the first trial, the final inoculum was 2.3 × 10^8 cfu/mL. For the second trial, the final inoculum was 2.5 × 10^8 cfu/mL. To confirm the cfu/mL, serial dilutions of the inocula were cultured in Luria agar by overnight incubation at

37°C.

2.4. Experimental procedure

Both experiments lasted for 16 days. After one week of adaptation, a single oral dose of 6 mL of *E. coli* F4 (1.4×10^9 and 1.5×10^9 cfu for Trial 1 and Trial 2, respectively) was administered on day 8 to the animals (d+0 post-inoculation). In order to ensure that stomachs were full at the time of inoculation, and to facilitate bacterial colonization, feed was withdrawn the previous day at 9:00 pm and returned 30 min before inoculation.

After the oral challenge, animals were checked daily for clinical signs to evaluate their status (i.e., dehydration, apathy and faecal score), always by the same person. Faecal score was also measured individually on d+ 0, d+ 1, d+ 2, and d+ 3 following the scale: 0 hard faeces, 1 solid and cloddy faeces, 2 soft to viscous faeces without firmness, 3 very viscous or liquid faeces without blood, and 4 watery or bloody faeces. Mortality was also recorded, and no antibiotic treatment was administered to any of the animals during any of the experiments.

2.5. Behavioural observations

Each piglet in a pen was identified individually with a dark permanent hair dye, and behaviours were recorded individually using live scan sampling at 2-min intervals (Martin and Bateson, 2007). Before beginning the scans, the trained observer ensured that all observed animals were standing up and after 5 min they were allowed to return to their previous behaviours. Behavioural observations were performed from the aisles twice a day (obtaining 10 scans per animal and time of day), mornings (from 8 am to 10 am) and afternoons (from 4 pm to 6 pm) on day 1 before inoculation (d-1) and on days 2 (d+2) and 3 (d+3) after inoculation. Behavioural observations are listed in Table 1, which consisted of noting piglet location in the pen, posture, and activities, based on the Welfare Quality, (2009) and complemented with parameters described by Escobar et al. (2007).

Table 1
Summary of recorded behaviours using a 2-min scan sample.

Behaviours	Description
Location in the pen	
Feeder area	Use of the third of the pen surrounding the feeder.
Heat lamp area	Use of the third of the pen surrounding the heat lamp.
Water nipple area	Use of the third of the pen surrounding the water nipple.
Posture	
Standing	Body supported by all four legs or walking.
Lying	Body in contact with the floor.
With pen mate	Lying with more than half of the body in contact side by side with other pen mates (one or two).
Without pen mate	Lying without contact or with less than half of the body in contact side by side with other pen mates.
Ventral	Lying with the four legs under the body.
Lateral	Lying with two visible legs on the floor on the opposite side.
Huddling	Lying with minimum more than half of the body over the top of another pig (i.e., virtually lying on top of another pig).
Sitting	Hindquarters on floor. "Dog sit".
Activities	
Active	
Negative social	Aggressive behaviour, including biting or aggressive social behaviour with a response from the disturbed animal.
Non-agonistic social	Sniffing, nosing, licking, and moving gently away from another pig without an aggressive or flight reaction.
Exploration	Searching of the pen by sniffing, nosing, licking or chewing items inside the pen.
Feeding	Head in the feeder and/or chewing next to it.
Others	If not resting, other active behaviours such as playing, drinking or sniffing the air.
Inactive	Lying at rest.

2.6. Statistical analysis

The results from both trials were pooled into a single dataset to evaluate consistency of faeces and behaviours and analysed with the free software R v3.4. To evaluate daily faecal score, a linear mixed model was applied using the lme4 package (Bates et al., 2015) considering candidate fixed factors to be diet, day, size and all possible pair interactions, as well as the trial. Size effect referred to the weight category assigned to each of the three piglets in each pen at the beginning of the experiments (low-, intermediate- and high-weight animal). The pen nested within trial was used as the random effect. The backward selection procedure was used to select the best fitted model. Model selection (fixed effect selection) criteria were based on the model with the lowest Akaike Information Criterion (AIC).

For the behavioural analysis, the proportion of each behaviour was summarized per individual, time of day, and day (10 scans as the denominator). The proportions of lying postures (with pen mate, without pen mate, ventrally, laterally, huddling, and sitting) and activities (negative and non-agonistic social, exploration, feeding, and others) were based on lying and active counts (as denominators), respectively. The same fixed factors and model selection procedures as for faecal score were used, with the addition of time of day effect and in this case under Poisson or negative binomial distributions.

For any further pairwise comparisons between days, treatments, and animal sizes, the lsmeans package (Lenth, 2016) was used for Tukey post hoc tests. The experimental unit was the individual animal (32 animals for CTR group and 16 animals for DIP and HPP groups). Faecal scores are presented as means and standard errors, and in the case of behavioural responses, only those factors included in the model and which are significant in most of the behaviours are displayed as percentage means and standard deviations for purposes of simplicity of results. For further details, the models with all the selected factors are provided in the Supplementary Material.

In the case of mortality, the proportion of dead animals for each diet was compared with a generalized linear model under binomial distribution using the stats package (R Core Team, 2013). The alpha level for the determination of significance for all the analyses was 0.05.

3. Results

This study relates to previously published results (López-Colom et al., 2020) from a larger study that is recommended for complementary information. That study included the detailed evaluation of growth performance, clinical signs, gut fermentation, intestinal morphology, inflammatory mediators, pathogen excretion, and colon microbiota.

Most of the animals used in both studies presented good health status at the beginning of both trials and were well-adapted to the facilities and feed. It should be mentioned that in Trial 1, four animals had diarrhoea on the day of arrival, and in Trial 2, three animals also had slight diarrhoea. After the challenge, there were a total of five animal casualties (three from CTR and two from the DIP group) in Trial 1 and four (one from CTR and three from the HPP group) in Trial 2. No statistical differences were observed between treatments in terms of mortality ($P > 0.10$).

The evolution of faecal consistency is presented in Fig. 1. Faecal score worsened at d+ 1 and improved afterwards, recovering initial values at d+ 3 ($P_{Day} < 0.001$). Feed additives were not related to diarrhoea, although faecal scores differed between animal sizes ($P_{Size} < 0.001$), low-weight piglets presenting inferior (better) scores than intermediate- and large-weight animals.

3.1. Behavioural analysis

On selection of the mixed models with the backward selection procedure, some behavioural responses were unaffected by the candidate fixed factors (mainly postures and activities). Therefore, only their

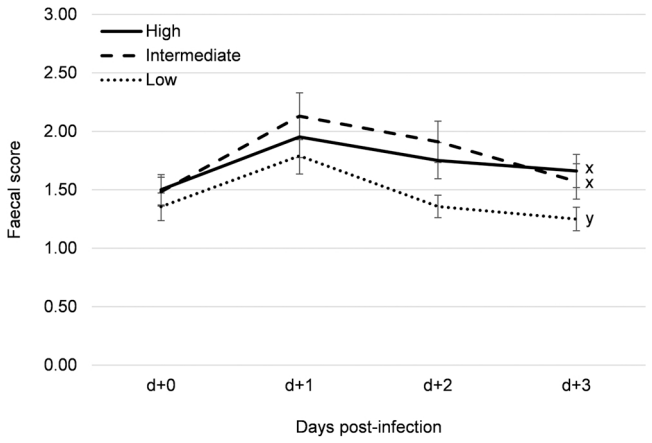

Fig. 1. Evolution of average faecal score (0–4 for hard to watery faeces, respectively) per day (relative to *E. coli* F4 challenge) and animal size (high, intermediate or low-weight piglets). [a,b] Different letters indicate significant differences between days (d-1, and d+2 and d+3) under Tukey adjustment ($P < 0.05$). [x,y] Different letters indicate significant differences between animal sizes (large, intermediate, or small) under Tukey adjustment ($P < 0.05$).

overall percentages are provided in the Supplementary Material and are omitted from the Results section.

The trial, considered a candidate fixed factor, showed significant effects in terms of different behavioural responses. Most specifically, the standing and lying postures, also ventrally and with pen mate, and the negative social, feeding, active, and resting activities were affected by the trial ($P < 0.01$). On average, compared to Trial 2, piglets from Trial 1 spent more time standing (62.6 ± 0.28% vs 34.9 ± 0.27%) and active (63.9% ± 0.28 vs 37.5 ± 0.28%), whereas in Trial 2 animals spent more time lying (65.1 ± 0.27% vs 37.4 ± 0.28%) and resting (62.5 ± 0.28% vs 36.1 ± 0.28%) than those in Trial 1. For further details, see the Supplementary Material.

3.1.1. Effect of challenge

Table 2 shows the differences in piglet behaviour recorded before (d-1) and after the oral challenge (d+2, d+3) and related to the time of day (morning and afternoon). The effect of day was evidenced in the place where piglets were located, in their posture, and in the activities they performed most of the time. At d+ 2, animals spent less time in the feeder (−8.7%), standing (−7.0%) and being active (−6.7%) and more time under the lamp (+6.5%), lying (+7%) and being inactive (+6.7%). The time spent next to the water nipple area increased over the days (+3.1% from d-1 to d+3). Differences in behaviour were also evidenced in relation to the circadian cycle, with animals spending more time around the feeder (+4.3%) and water nipple (+1.7%) and standing (+3.8%) and active (+3.7%) in the afternoon than in the morning. Finally, the interaction between day and time of day was observed for location in the feeder area, standing posture, and active animals. Whereas during the morning, responses to these parameters were observed shortly after the challenge, with decreases at d+ 1 but not at d+ 3, in the afternoon, this effect was not observed but there were significant increases in all these behaviours at d+ 3.

Table 2
Piglet behaviour depending on the day (relative to oral inoculation of *E. coli* F4 at days −1, and +2 and +3) and time of the day (morning from 8 am to 10 am and afternoon from 4 pm to 6 pm).

	Day			p value[1]		
	d-1	d+ 2	d+ 3	Day	Time of day	Day × Time of day
Location in the pen (%)						
Feeder area						
Morning	56.0[b]	43.2[c]	53.3[b]	< 0.001	0.012	0.005
Afternoon	53.5[b]	49 BCE	63.6[a]			
Daylong	54.8 [y]	46.1[z]	58.5[x]			
Heat lamp area						
Morning	40.9	51.1	39.5	0.002	0.041	
Afternoon	40.8	43.6	28.6			
Daylong	40.9 [y]	47.4[x]	34.1 [y]			
Water nipple area						
Morning	3.0	5.7	7.1	0.012	0.034	
Afternoon	5.6	7.4	7.7			
Daylong	4.3 [y]	6.5[xy]	7.4[x]			
Posture (%)						
Standing						
Morning	51.4[ab]	40.9[b]	47.5[b]	< 0.001	0.042	0.014
Afternoon	48.8[b]	45.3[b]	57.6[a]			
Daylong	50.1[x]	43.1 [y]	52.5[x]			
Lying						
Morning	48.6	59.1	52.5	0.031	0.359	
Afternoon	51.3	54.7	42.4			
Daylong	49.9[xy]	56.9[x]	47.5 [y]			
Activities (%)						
Active						
Morning	52.6[ab]	43.3[b]	49.9[b]	< 0.001	0.041	0.012
Afternoon	50.5[b]	46.6[b]	60.4[a]			
Daylong	51.6[x]	44.9 [y]	55.2[x]			
Inactive						
Morning	47.4	56.7	50.1	0.025	0.323	
Afternoon	49.5	53.4	39.6			
Daylong	48.4[xy]	55.1[x]	44.8 [y]			

[1] Empty space indicates the factor was removed from the model during the backward selection procedure.
[a,b,c] Different letters indicate significant differences between Day × Time of day interaction under Tukey adjustment ($P < 0.05$).
[x,y,z] Different letters indicate significant differences between days (d-1, and d+2 and d+3) under Tukey adjustment ($P < 0.05$).

3.2. Effect of diet

The effect of diet on the piglets' behaviour appeared to be dependent on the time of day, as shown in Table 3, including interaction between diet and time. The experimental diets had an impact on being in the feeder area (%) as CTR and DIP animals spent more time there than HPP (+14.0% and +18.8%, respectively). Regarding interactions, animals from the HPP group spent more time in the lamp area in the afternoons compared to CTR and DIP piglets in the mornings ($P_{Diet \times Time\ of\ day} = 0.02$). A similar pattern was observed for lying posture ($P_{Diet \times Time\ of\ day} = 0.02$) and inactivity ($P_{Diet \times Time\ of\ day} = 0.007$), although post hoc pairwise comparisons with Tukey adjustment did not reach statistically significant levels. Significant interactions were also observed in standing posture and active behaviours ($P_{Diet \times Time\ of\ day} < 0.02$), with clearer decreases observed for the HPP diet in the afternoon than in the morning, although post hoc pairwise comparisons did not show significant differences either. Finally, lying with pen mates (data not shown) was also affected by diet and time of day, the lowest value for HPP animals being in the afternoons (54.6% vs 66.1% as an average of the remaining combinations; $P_{Diet \times Time\ of\ day} < 0.001$).

3.3. Effect of weight at weaning

Table 4 presents the significant effects observed involving piglet size. On the one hand, piglet weight at weaning showed interaction with the time of the day ($P_{Size \times Time\ of\ day} = 0.008$), whereby intermediate-weight piglets spent more time lying with pen mates in the mornings than in the afternoons and the values were between those observed for large and small animals. On the other hand, the impact of the diet on feeding activity also depended on the size of the piglets ($P_{Size \times Diet} = 0.034$), small and intermediate piglets presenting greater feeding activity with

Table 3
Piglet behaviour depending on diet (control – CTR, butyrate supplementation – DIP, and heptanoate supplementation – HPP) and time of day (morning from 8 am to 10 am and afternoon from 4 pm to 6 pm).

	Diet			p value[1]	
Location in the pen (%)	CTR	DIP	HPP	Diet	Diet × Time of day[2]
Feeder area					
Morning	51.3	57.1	44.3	0.029	0.084
Afternoon	59.6	63.4	38.4		
Average	55.4^{x}	60.2^{x}	41.4 ^{y}		
Heat lamp area					
Morning	43.8^{ab}	37.6^{ab}	50.0^{ab}	0.062	0.020
Afternoon	33.8^{b}	30.1^{b}	53.8^{a}		
Average	38.8	33.9	51.9		
Water nipple area					
Morning	4.9	5.3	5.7		
Afternoon	6.6	6.5	7.8		
Average	5.8	5.9	6.7		
Posture (%)					
Standing					
Morning	45.1	62.2	34.2	0.482	0.018
Afternoon	54.0	66.2	27.4		
Average	49.6	64.2	30.8		
Lying					
Morning	54.9	37.8	65.8	0.494	0.009
Afternoon	46.0	33.8	72.6		
Average	50.4	35.8	69.2		
Activities (%)					
Active					
Morning	47.2	63.2	37.0	0.505	0.015
Afternoon	56.1	67.6	29.6		
Average	51.6	65.4	33.3		
Inactive					
Morning	52.8	36.8	63.0	0.485	0.007
Afternoon	43.9	32.4	70.4		
Average	48.4	34.6	66.7		

[1] Empty space indicates the factor was removed from the model during the backward selection procedure.
[2] Time of day effect is not shown as it is already provided in Table 3.
[a,b] Different letters indicate significant differences between Diet × Time of day interaction under Tukey adjustment ($P < 0.05$).
[x,y] Different letters indicate significant differences between diets (CTR, DIP, and HPP) under Tukey adjustment ($P < 0.05$).

Table 4
Piglet behaviour depending on size (low, intermediate, or high-weight), time of day (morning from 8–10 am and afternoon from 4–6 pm) or diet (control – CTR, butyrate supplementation – DIP, and heptanoate supplementation – HPP) and their interaction.

	Diet			p value		
Location in the pen (%)	Low	Intermediate	High	Size	Time of day	Size × Time of day
With pen mate						
Morning	77.9^{a}	71.1^{a}	67.6^{ab}	0.127	0.208	0.008
Afternoon	59.4^{ab}	53.7^{b}	53.8^{ab}			
Activity (%)	Low	Intermediate	High	Size	Diet	Size × Diet[2]
Feeding						
CTR	35	25.5	32.2	0.932	0.554	0.034
DIP	38.3	34.5	32.9			
HPP	50.4	36.6	33.2			

[a,b] Different letters indicate significant differences between Size × Time of day interaction under Tukey adjustment ($P < 0.05$).

the feed additives, although differences after post hoc pairwise comparisons were not significant.

4. Discussion

The present study aimed to evaluate the usefulness of behavioural observations to assess the health response of weaned pigs after being orally challenged with *Escherichia coli* F4, and moreover, to test the efficacy of feeding interventions, such as the inclusion of in-feed acid salts, to prevent or overcome post-weaning diarrhoea. In this section, we will first discuss the effects of the challenge on the behavioural responses of piglets and after that the effects on diet. In addition, due to the interest in assessing differences between animal sizes, we briefly discuss this issue in a separate section.

In the present study, data was pooled from two different trials in which animals received the same control diet but different supplemented ones. Before any further discussion, it is worth highlighting the possible impact that the trial could have on different behaviours. Although both trials were performed under the same conditions (facilities, environment, husbandry, and experimental protocol), experimental reproducibility with animal trials is difficult and has inherent drawbacks (Frommlet and Heinze, 2021). Whereas in Trial 1, the piglets showed generally greater levels of activity, in Trial 2 they were generally lying and inactive. This could be associated to the differential clinical and physiological parameters assessed previously (López-Colom et al., 2020). In general terms, it could be said that the severity of the challenge was higher in Trial 1 than in Trial 2, which is particularly supported by the milder peak of diarrhoea observed in Trial 2 compared to Trial 1 together with the lack of response observed in serum tumour necrosis factor alpha (TNF-α) (pro-inflammatory cytokine marker, measured on d+4), which significantly rose in Trial 1. Moreover, animals from Trial 2 also harboured lower counts of *E. coli* F4 in intestinal mucosa.

4.1. Challenge effect

By challenging piglets with *E. coli* F4 we aimed to reproduce the course of post-weaning colibacillosis that commonly occurs in pig practice. Our results showed that the pathogen challenge had no significant impact on social behaviours, as we did not encounter modifications to either non-agonistic or negative social contacts. However, other behavioural responses were observed after the *E. coli* challenge, whereby a generally more lethargic attitude was observed in the animals, involving less active behaviours accompanied by longer times in the lying posture as opposed to standing. Also, the feeder area was visited less than it was before the challenge, and the animals spent more time under the heat lamp. These changes are to be expected if animals have lost water due to diarrhoea and need to maintain body temperature (Nasirahmadi et al., 2015; Rostagno et al., 2011).

We also assessed the possible impact that the circadian cycle could have had on animal behaviour. Interestingly, before the challenge (d-1), the piglets generally presented similar activity in the morning and afternoon, but afterwards they were more active in the afternoon. In contrast to the present results, in a previous study by our group with an experimental infection with *Salmonella* Typhimurium and a similar ethogram (Barba-Vidal et al., 2017), pigs were more active in the morning. Bowden et al. (2008) also found that pigs were more active early in the morning (6–10 am). This could be due to differences in the observation schedule. Pigs are diurnal eaters (Quiniou et al., 2000) and in our study, we might have missed the earlier peak in feed consumption, which occurs at around 7 am (Boumans et al., 2017). Moreover, the few differences we observed in feeding activity in terms of time of day could also be due to the possibility of witnessing the larger peak at between 11 pm and 4 pm, as determined by Boumans et al. (2017). Despite these discrepancies, other authors found similar results to ours, such as Ahmed et al. (2014) who showed that weaned pigs experimentally infected with *Salmonella enteritidis* or *E. coli* are more active late in the

afternoon (as of 6 pm). Older studies also observed pigs to be more active between 2 pm and 4 pm (Young and Lawrence, 1994).

Regarding feeding activity, we were unable to observe important changes after the challenge (d+2), despite noting an upward tendency from d-1 to d+ 3. Although truncation of feeding in pigs is characteristic of sickness behaviour (Hart, 1991; Miller et al., 2019), Ison et al. (2016) suggested that some activity registries could get missed because of the scan-sampling method. Alternatively, piglets might express fewer sickness behaviours in the presence of observers. The use of an unchallenged control group in parallel would have helped to discriminate the impact of the challenge and the post-weaning moment itself. Activities such as exploration are also expected to decrease before others that are more critical for survival (i.e., feeding and drinking) (Brunberg et al., 2016; Murphy et al., 2014; Weary et al., 2009), hence, the absence of a decline in feeding or drinking may be associated with the degree of severity of the model. Another possible reason for the lack of changes recorded for specific activities at d+ 2 might have been because after the peak in diarrhoea and the decline in feed consumption on d+ 1, the piglets had already recovered by d+ 2 (López-Colom et al., 2020).

Behavioural assessment thus appears to be a sensitive tool that can be affected by the moment when observations are performed and the course and severity of disease. Different pathogens have been reported to cause varying changes in behaviour patterns. For instance, *Salmonella* produces more severe and retarded clinical signs (Ahmed et al., 2014; Barba-Vidal et al., 2017; Rostagno et al., 2011) compared to *E. coli* and lipopolysaccharide (LPS) (Jensen et al., 2006; Johnson and von Borell, 1994), and it is even possible to observe differences before clinical signs appear, i.e., sub-clinically. Another example is the study by Escobar et al. (2007), who detected less feeding and more resting behaviours in a PRRS infection in pigs whereas *Mycoplasma hyopneumoniae* did not alter behaviour.

In the same line, previous studies with LPS and *E. coli* challenges have related lethargic behaviours with the rapid response of cytokines and acute inflammation through hypothalamus-pituitary-adrenal (HPA) axis activation (Nordgreen et al., 2018; Zimomra et al., 2011). However, an older study by Krsnik et al. (1999) distinguished other factors for consideration with regard to the behavioural responses of pigs, such as *E. coli* serotypes (somatic, capsular, other antigenic variants). This factor might produce differences in later studies. Spitzer et al. (2014) reported similar outcomes to ours with an experimental *E. coli* F4 infection up to 3 days post-challenge but other authors did not even observe changes the day after (less than 24 hrs) (Jensen et al., 2006). In our case, we were able to link our observations with clinical outcomes of the disease, which as stated earlier is observed to be an acute process, with diarrhoea peaking on the day immediately after the challenge.

4.2. Diet effect

The supplementation of diets with sodium butyrate or heptanoate had some clear effects on behaviour, especially when considering the time of day. The most relevant effect was observed in the feeder area, where piglets supplemented with HPP spent less time and instead spent more in the heat-lamp area, particularly in the afternoons ($P_{Diet \times Time\ of\ day} = 0.02$). There was a recurrent effect of the interaction between diet and time of day with regard to standing and lying postures and to active and inactive behaviours, whereby HPP animals were more inactive and prone to lie down in the afternoons than CTR and DIP animals. Heptanoate, like other MCFA, presents an unpleasant odour (Lauridsen, 2020; Mroz, 2005; van der Aar et al., 2017). However, there is no information regarding its palatability, as there is for other fatty acids or additives. Also, the more lethargic state of HPP animals might be related to other confounding factors that we did not control. HPP was only supplemented in piglets from Trial 2, which were generally more inactive. Moreover, piglets supplemented with HPP presented declines in SCFA concentrations (López-Colom et al., 2020) that could be associated to antimicrobial activity and possible changes in the gastrointestinal ecosystem (Ríos-Covián et al., 2016). There is increasing evidence nowadays of the role that the gut microbiota may play in the mood and behaviour of animals and humans (Choudhury et al., 2022; Margolis et al., 2021). Microbe-host relationships are very complex (Lyte, 2013; Lyte and Lyte, 2019; Roura et al., 2019), and study of the gut-brain axis that communicates via neurotransmitters is only in its infancy (Parois et al., 2020).

In contrast, animals supplemented with sodium butyrate appeared to be more active, in standing postures, and surrounding the feeder. The additive DIP was designed to improve gut health by combining butyrate as an active molecule with a functional protection composed of MCFA. With DIP, we observed a global enhanced barrier through lengthening of intestinal villi and goblet cell proliferation (López-Colom et al., 2020), which could give positive feedback to animals' behaviour. Furthermore, butyrate has recently been shown to cause changes in metabolism and hippocampus plasticity without the need for changes to intestinal tract functionality or structure (Val-Laillet et al., 2018), which is claimed to have a direct impact on behaviour. However, these differences were merely numerical and interaction between diet and time of day did not provide sufficiently significant results to support these hypotheses. It should also be noted that the doses we applied and the numbers of replicates and factors that were included in the models might have limited the robustness of the results.

4.3. Animal size effect

A few effects were found on behaviour associated to piglet size. The interaction between size and time of day influenced lying with penmates. The greatest differences were found for intermediate pigs, which spent longer periods with pen mates in the mornings than in the afternoons. This could be an expected response, as these piglets would maintain their body temperatures through contact with pen mates or by generating heat with locomotion (Nasirahmadi et al., 2015). However, it is difficult to explain why these behaviours are particularly observed among intermediate pigs. Feeding activity also presented interaction between size and diet. Compared to control animals, low-weight animals increased their feeding behaviour with the supplementation of both additives, although pairwise comparison did not reach a significant level. In fact, lighter pigs are described as visiting the feeder more frequently (to possibly feed), and present better feed conversion ratios compared to heavier ones (Bruininx et al., 2001). Supplementing the diets with the tested in-feed additives could have caused this behaviour in the smallest pigs by increasing their interest in the diets (Blavi et al., 2016; Clouard and Val-Laillet, 2014; Sterk et al., 2008) and improving their adaptation to weaning. In our study, we also observed an improvement in the faecal scores of smaller piglets.

Finally, we should mention the limitations of experimental models to fully reproduce real farm conditions. In commercial conditions, animals are kept in larger groups, and their activity is influenced by social facilitation, what has been described as the engagement by third animals in certain identical behaviours (Hsia and Wood-Gush, 1984). Moreover, the way the animals are exposed to pathogens and the population dynamics of disease can be also different. Despite these limitations, experimental trials can also offer certain advantages, such as better control of external stimuli and environmental conditions (Boumans et al., 2016; Brunberg et al., 2016). In this study, we could take advantage of the low number of pigs per pen to analyse possible interactions driven by the weight of the animals at weaning, going one step further than the classical approach of the average pig (Berckmans, 2004). In this regard, although results did not reveal clear, consistent effects, they provide first insight into the different behavioural responses of pigs depending on their weight at weaning.

5. Conclusions

The experimental challenge with *Escherichia coli* F4 on weaned

piglets was able to induce some behavioural changes. Animals displayed a moderate sickness behaviour consisting of a general lethargy that was recovered at third day post-challenge. Differences could also be detected in behaviour related to in-feed supplementation with protected acid salts. These changes were found to be variable depending on the additive, the severity of the diarrhoea and also the weight of the animals at weaning. The use of these or other behavioural indicators in practice would therefore need to also integrate variables such as the relative weight of the animals, individual characteristics such as the hierarchy, or different husbandry practices.

Funding

This work was supported by the CDTI and Ministerio de Economía y Competitividad, Spain ["PORCDIGEST", IDI-20140262]; and the Agència de Gestió d'Ajuts Universitaris i de Recerca, Spain, awarded to Paola López-Colom [pre-doctoral grant, FI 2016].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the staff of the Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (UAB), for their support, and appreciate the assistance from Jaume Gou Sanchis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2023.105882.

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