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Consequences of environmental and social enrichments on the resilience of piglets experimentally challenged with enterotoxigenic E. coli

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Abstract

Post-weaning diarrhoea is a multifactorial disease associated with the weaning transition, but little is known about the effects of environmental and social enrichments on its occurrence. We investigated the effects of physical and social enrichments on health indicators of piglets challenged with enterotoxigenic *E. coli* (ETEC), as a model of post-weaning diarrhoea. We divided a total of 119 specific pathogen-free piglets between two contrasting housing conditions: minimal (MIN) versus enriched with increased space allowance, rooting material, a rotation of objects and early socialisation (ENR). Eleven days after weaning, piglets were orally inoculated with either ETEC bearing F4 adhesins (inoculated pens) or sterile broth (control pens). We monitored piglets' health for four weeks, with regular clinical observations, faecal and blood samplings. Regardless of inoculation, ENR pigs had overall more D-lactate and leucocytes compared to MIN pigs, suggesting different microbiota and immune systems functioning. ETEC shedding peaked at higher levels in ENR-inoculated pigs than in MIN-inoculated pigs, without detrimental effects on clinical signs, growth performance or metabolism. Therefore, environmental and social enrichments appeared to increase the susceptibility of ENR challenged pigs to the bacteria but not their disease resilience, i.e., coping with the consequences of the pathogen burden.

Introduction

Post-weaning diarrhoea (PWD) is a multifactorial disease [1] in which clinical signs of diarrhoea arise as a consequence of abruptly weaning the piglets [2]. PWD involves a microbial imbalance termed 'intestinal dysbiosis' [3] and is reported to occur most frequently within two weeks after weaning [1]. It is frequently associated with the proliferation of enterotoxigenic *E. coli* (ETEC), most predominantly bearing adhesins F4 or F18 which mediate intestinal colonisation [1]. However, other *E. coli* strains, non-*E. coli bacteria*, or even non-bacterial pathogens such as rotavirus may be involved [4]. PWD usually results in major economic losses for the producers due to reduced productivity, increased mortality and additional costs associated with medication and veterinary care [5]. Additionally, extensive use of antibiotics to control PWD has contributed to the emergence of multidrug-resistant *E. coli*, including strains resistant to last-resort antimicrobials such as colistin [1]. As a result, there is a growing demand for developing non-antibiotic approaches to control PWD [1, 6].

After weaning, piglets must adapt to numerous changes while they are still young and physiologically immature [7], which increases their vulnerability to PWD. At that time, piglets have to cope with the combined effects of physiological and psychological stress [1, 8]. Physiological stress includes nutritional changes with a switch from milk to solid, dry cereal-based feed. This switch is accompanied by a reduction in voluntary feed intake, and nutrient maldigestion and malabsorption due to low secretion of appropriate digestive enzymes [9]. Piglets also exhibit altered gut morphology with villi atrophy [10]. Undigested nutrients alter the gut microbial populations, resulting in a decrease in beneficial organisms such as *Lactobacilli* and a loss of microbial diversity, whereas opportunistic or pathogenic bacteria, including *E. coli*, increase [6]. When piglets are weaned, they are also abruptly separated from their mother, mixed with piglets from other litters and moved to a new environment [8]. During the first hours, they respond by showing clear signs of psychological stress such as restlessness, distress vocalisations, and escape attempts [11]. Social stress is

a known contributing factor to disease [12] and competition to establish the dominance hierarchy after mixing further contributes to reduce feeding behaviour, thus indirectly raising the risk of PWD [1]. Transportation is an additional stressor that should be minimised to control PWD [13]. Finally, the post-weaning environment is generally cooler and provides fewer sources of warmth than the farrowing room, where piglets benefit from both heat lamps and maternal contact. As a result, piglets are often exposed to suboptimal temperatures, which have been associated with an increased incidence of PWD [10, 13].

Growing societal and regulatory expectations regarding animal welfare in European pig production provide an opportunity to refine management to mitigate weaning-related stress and its wider implications for animal health. Enrichment of pigs' physical and social environment is commonly used to improve animal welfare on farms, by promoting natural behaviours and reducing abnormal ones [14]. Early socialisation of piglets is a social enrichment that has also proven effective in reducing damaging behaviours. When socialised at a young age, piglets tend to have shorter and less severe fights; moreover, the establishment of the new social hierarchy at weaning is facilitated [15, 16]. Successful enrichment should increase the ability of the animals to cope with behavioural and physiological challenges [14], which may extend to health outcomes. Wen et al. [17] showed that early socialisation and environmental enrichment accelerated the maturation of early-life gut microbiota, which may improve piglets’ health through the weaning transition. The provision of bedding material in a barren housing may also reduce post-weaning diarrhoea in piglets [18]. These previous observations lead to consider how enrichment may modulate piglets’ capacity to cope with stressors by enhancing their disease resilience. Clunies Ross [19] proposed a distinction between "resistance to infection", as reflected by parasite burden, and "resistance to the effects of infection", as reflected by disease severity in the host. The latter is what led to the concept of disease resilience [20, 21]. This concept has subsequently been refined to include non-infectious stressors [21]: resilience is "the capacity of the animal to be minimally affected by a disturbance or to rapidly return to the physiological, behavioural, cognitive, health, affective and production states that pertained before exposure to a disturbance."

In light of the aforementioned concepts, this study aimed at investigating the consequences of environmental and social enrichments on the resilience of piglets experimentally challenged with ETEC as a model of PWD. This provides a valuable framework to evaluate how welfare-oriented strategies may modulate stress responses, immune competence and susceptibility to PWD in piglets.

Results

Mortality and sample size

No mortality was observed prior to the challenge. After inoculation, we had to euthanise 6 piglets before the end of the experiment due to the severe deterioration of their health status (3 ENR-inoculated, 1 MIN-inoculated, 2 MIN-control). The potential causes identified were the ETEC colonisation for four of them, a persistent non-ETEC disease pre-inoculation for one pig, and a haematoma after blood sampling for the last pig. The final number of animals at the end of the experiment was therefore 27 ENR-inoculated, 30 ENR-control, 29 MIN-inoculated, and 27 MIN-control pigs. The descriptive statistics (mean ± SEM and range) for each variable are provided in Table 2. The numbers of observations and summary of p-values associated with fixed effects selected in the final models are presented in Table 3.

Table 2
Descriptive statistics of the measured variables (mean ± SEM and range min-max) over the total period of observation (D-3 until D28) by experimental group.

Category	Variable	ENR control	ENR inoculated	MIN control	MIN inoculated
Shedding	ETEC inoculum strain, log10(CFU/mL)		8 ± 8 (0–10)		8 ± 7 (0–10)
	Total *E. coli*, log10(CFU/mL)	7 ± 6 (5–8)	8 ± 8 (5–10)	7 ± 6 (5–8)	8 ± 7 (5–10)
Clinical signs	Rectal temperature, °C	39.31 ± 0.01 (38.2–40.1)	39.31 ± 0.01 (37-40.7)	39.351 ± 0.009 (38.7–40.1)	39.362 ± 0.009 (38.4–40.2)
	Days with diarrhoea, number of days	1.2 ± 0.2 (0–4)	2.7 ± 0.4 (0–7)	1 ± 0.3 (0–5)	2.1 ± 0.6 (0–9)
Histology	Villus height, µm	510 ± 14 (354–693)	463 ± 12 (351–596)	510 ± 14 (386–667)	502 ± 14 (369–720)
	Crypt depth, µm	242 ± 10 (95–331)	200 ± 13 (91–306)	227 ± 9 (110–295)	206 ± 12 (75–287)
	VH:CD ratio[1]	2.3 ± 0.2 (1.4–5.1)	2.6 ± 0.2 (1.6–4.5)	2.4 ± 0.1 (1.6–4.6)	2.7 ± 0.2 (1.5-6)
Performance	Average daily gain, g/day	687 ± 21 (-144-1175)	643 ± 24 (-160-1133)	693 ± 18 (125–1175)	703 ± 24 (-322-1742)
Biochemistry	D-lactate, mg/L	27.9 ± 0.8 (8.4–72.4)	28.9 ± 0.7 (10.3–77.5)	19.2 ± 0.6 (5.4–55.8)	21 ± 0.5 (4.2–51.4)
	Haptoglobin, mg/mL	0.3 ± 0.02 (0-3.23)	0.43 ± 0.05 (0-6.24)	0.25 ± 0.01 (0-2.07)	0.34 ± 0.03 (0-4.39)
	d-ROM, CARRU[2]	661 ± 8 (92-1246)	677 ± 10 (386–1600)	683 ± 9 (96-1132)	705 ± 10 (412–1437)
	FRAP, µg/mL	51 ± 0.7 (33.5-147.5)	53 ± 2 (32–433)	51.5 ± 0.8 (36.8-179.1)	53 ± 4 (33–981)
	D:F ratio[1], CARRU[2]/µg/mL	13.3 ± 0.2 (2.2–25.5)	13.6 ± 0.2 (1.4–30.6)	13.7 ± 0.2 (2.1–22.7)	14.6 ± 0.3 (0.6–29)

[1]VH:CD ratio: Villus height to Crypt depth ratio, D:F ratio: d-ROM to FRAP ratio, G:L ratio: Granulocytes to Lymphocytes ratio.

[2]CARRU: Carratelli Unit, 1 CARRU = 0.08 mg H_2O_2/100mL of sample.

[3]Gene expression is expressed as relative abundance of the target gene compared to the house-keeping genes. The normalisation factor was calculated with the GeNorm algorithm [54]

Category	Variable	ENR control	ENR inoculated	MIN control	MIN inoculated
	Cortisol, ng/mL	45 ± 1 (9-101)	43 ± 1 (7-124)	42 ± 1 (7-100)	40 ± 1 (9-108)
	Glucose, mg/L	1380 ± 10 (981–2154)	1316 ± 12 (776–2323)	1398 ± 11 (996–2901)	1317 ± 13 (418–1741)
	NEFA, µmol/L	83 ± 4 (12–436)	130 ± 13 (8-1717)	90 ± 5 (29–637)	151 ± 17 (18-2186)
	Creatinine, mg/L	8.2 ± 0.08 (5.29–13.2)	8.6 ± 0.1 (5.1–21.7)	8.07 ± 0.09 (4.85–17.15)	8.04 ± 0.08 (5.55–19.69)
Haematology	Red blood cells, 10E9/mL	5.9 ± 0.05 (3.56–7.31)	5.91 ± 0.06 (3.45–7.46)	5.88 ± 0.05 (3.87–7.59)	5.84 ± 0.04 (2.45–7.28)
	Haematocrit, %	35.5 ± 0.2 (23.3–42.9)	34.9 ± 0.3 (20.7–46.3)	35.2 ± 0.3 (24.9–42.7)	34.8 ± 0.3 (16.5–43.4)
	Haemoglobin, g/L	11.1 ± 0.1 (7.4–14.4)	10.8 ± 0.1 (6.5–14.6)	10.8 ± 0.1 (7.3–13.9)	10.8 ± 0.1 (8.1–14.3)
	Lymphocytes, 10E6/mL	6.9 ± 0.1 (1–15)	6.8 ± 0.1 (3.6–13.3)	5.55 ± 0.09 (2.1–8.6)	5.56 ± 0.09 (3.5–9.2)
	Granulocytes, 10E6/mL	2.4 ± 0.08 (0.9–7.6)	2.9 ± 0.2 (0.3–16)	2.08 ± 0.05 (0.8–4.5)	2.2 ± 0.2 (0.6–25.9)
	G:L ratio[1]	0.4 ± 0.02 (0.12–1.5)	0.44 ± 0.03 (0.04–2.68)	0.39 ± 0.01 (0.18–1.05)	0.39 ± 0.02 (0.14–3.01)
	Monocytes, 10E6/mL	0.7 ± 0.02 (0.1-2)	0.75 ± 0.03 (0.1–3.3)	0.55 ± 0.01 (0.3-1)	0.59 ± 0.02 (0.3–3.1)
Gene expression	TNF-α, normalised[3]	1.2 ± 0.2 (0.1–6.3)	1.4 ± 0.2 (0.4–5.1)	1.4 ± 0.2 (0.1–5.2)	0.94 ± 0.1 (0.04–3.79)
	IL-10, normalised[3]	1.4 ± 0.3 (0-10.9)	1.6 ± 0.3 (0-9.4)	1.1 ± 0.1 (0.1–3.4)	1 ± 0.1 (0.2-4)
	TLR4, normalised[3]	0.5 ± 0.05 (0.09–1.57)	0.54 ± 0.05 (0.13–1.54)	0.57 ± 0.05 (0.14–1.25)	0.52 ± 0.05 (0.1–1.56)

[1]VH:CD ratio: Villus height to Crypt depth ratio, D:F ratio: d-ROM to FRAP ratio, G:L ratio: Granulocytes to Lymphocytes ratio.

[2]CARRU: Carratelli Unit, 1 CARRU = 0.08 mg H_2O_2/100mL of sample.

[3]Gene expression is expressed as relative abundance of the target gene compared to the house-keeping genes. The normalisation factor was calculated with the GeNorm algorithm [54]

Category	Variable	ENR control	ENR inoculated	MIN control	MIN inoculated
	TLR6, normalised[3]	0.55 ± 0.04 (0.12–1.07)	0.61 ± 0.05 (0.19–1.19)	0.68 ± 0.06 (0.07–1.66)	0.57 ± 0.05 (0.1–1.26)
	NOD2, normalised[3]	1.3 ± 0.2 (0.2–4.5)	1.5 ± 0.2 (0.2–6.5)	1.2 ± 0.2 (0.1–6.2)	1 ± 0.2 (0.1–6.4)
	iNOS, normalised[3]	1 ± 0.1 (0.1–3.4)	1 ± 0.1 (0.1-4)	0.9 ± 0.1 (0.1–4.1)	0.68 ± 0.07 (0.07–2.06)

[1]VH:CD ratio: Villus height to Crypt depth ratio, D:F ratio: d-ROM to FRAP ratio, G:L ratio: Granulocytes to Lymphocytes ratio.

[2]CARRU: Carratelli Unit, 1 CARRU = 0.08 mg H_2O_2/100mL of sample.

[3]Gene expression is expressed as relative abundance of the target gene compared to the house-keeping genes. The normalisation factor was calculated with the GeNorm algorithm [54]

Table 3
Number of observations and p-values associated with the fixed effects selected in the final statistical models.

Variable	Number of observations	H	I	T	S	H × I	H × T	I × T	H × I × T
Shedding									
ETEC inoculum strain	725	> 0.05	#	< 0.001	#	#	0.005	#	#
Total *E. coli*	1572	> 0.05	< 0.001	< 0.001	#	0.001	0.001	< 0.001	< 0.001
Clinical signs									
Days with diarrhoea	113	#	0.004	#	#	#	#	#	#
Histology									
Villus height	113	#	#	#	#	#	#	#	#
Crypt depth	113	#	#	#	#	#	#	#	#
VH:CD ratio	113	#	#	#	#	#	#	#	#
Growth performance									
ADG	579	> 0.05	> 0.05	< 0.001	#	> 0.05	> 0.05	< 0.001	0.001
Biochemistry									
D-lactate	1036	< 0.001	> 0.05	> 0.05	#	#	#	0.032	#
Haptoglobin	918	#	#	0.001	#	#	#	#	#
d-ROM	1038	> 0.05	#	< 0.001	#	#	0.023	#	#
FRAP	1039	#	#	< 0.001	0.001	#	#	#	#
D:F ratio	1038	#	#	< 0.001	#	#	#	#	#
Cortisol	984	> 0.05	> 0.05	< 0.001	< 0.001	#	0.020	0.018	#

H: Housing; I: Inoculation; T: Time; S: Sex. ADG: Average Daily Gain; VH:CD ratio: Villus height to Crypt depth ratio, D:F ratio: d-ROM to FRAP ratio, G:L ratio: Granulocytes to Lymphocytes ratio. #: not included in the final model.

Variable	Number of observations	H	I	T	S	H × I	H × T	I × T	H × I × T
Shedding									
Glucose	1033	#	> 0.05	< 0.001	#	#	#	< 0.001	#
NEFA	1035	> 0.05	> 0.05	< 0.001	#	> 0.05	> 0.05	< 0.001	< 0.001
Creatinine	1028	#	#	< 0.001	0.017	#	#	#	#
Haematology									
Red blood cells	678	> 0.05	> 0.05	< 0.001	#	> 0.05	> 0.05	0.033	0.047
Haematocrit	678	#	#	< 0.001	#	#	#	#	#
Haemoglobin	678	#	#	< 0.001	#	#	#	#	#
Lymphocytes	678	0.001	#	< 0.001	#	#	#	#	#
Granulocytes	678	> 0.05	#	< 0.001	#	#	< 0.001	#	#
G:L ratio	678	> 0.05	#	< 0.001	#	#	< 0.001	#	#
Monocytes	678	0.002	0.002	< 0.001	0.043	#	< 0.001	0.001	#
Gene expression									
TNF-α	170	> 0.05	> 0.05	> 0.05	#	> 0.05	0.043	> 0.05	0.048
IL10	166	0.017	#	< 0.001	#	#	0.001	#	#
TLR4	172	#	#	#	#	#	#	#	#
TLR6	168	#	#	0.003	#	#	#	#	#
NOD2	170	#	#	#	#	#	#	#	#
iNOS	167	#	#	#	#	#	#	#	#

H: Housing; I: Inoculation; T: Time; S: Sex. ADG: Average Daily Gain; VH:CD ratio: Villus height to Crypt depth ratio, D:F ratio: d-ROM to FRAP ratio, G:L ratio: Granulocytes to Lymphocytes ratio. #: not included in the final model.

E. coli shedding

Only inoculated groups were included for ETEC shedding analysis and all inoculated pigs were confirmed negative for ETEC shedding on D-3. Statistical analyses were therefore performed post-inoculation only (D1–D28). ETEC shedding was affected by Housing × Time (P = 0.005, Fig. 2a). In the ENR-inoculated group, ETEC shedding peaked on D3–D7 compared to D9–D28 (all P < 0.001) and decreased on D14–D28 when ETEC shedding became lower than on D1 (all P < 0.050). In the MIN-inoculated group, ETEC shedding peaked on D2–D7 compared to D9–D28 (all P < 0.001) and decreased on D11–D28 when ETEC shedding became lower than on D1 (all P < 0.001). Furthermore, ENR-inoculated shed more ETEC than MIN-inoculated on D4, D5, and D9 (all P < 0.050).

Total *E. coli* shedding was affected by Housing × Inoculation × Time (P < 0.001, Fig. 2b). Control pigs in both housing conditions had few marginal temporal variations. For ENR-inoculated, total *E. coli* shedding peaked between D3–D7 compared to D9–D28 (all P < 0.010, except D7 compared to D9 and D21, P > 0.050) while, for MIN-inoculated, it peaked between D2–D5 compared to D11–D17 (all P < 0.050). Furthermore, ENR-inoculated shed more *E. coli* than MIN-inoculated on D4 and D7 (both P < 0.050). They also shed more total *E. coli* than ENR-control on D-3, D1, D3–D9 (all P < 0.001), and D14 (P = 0.006), and more than MIN-control on D4, D5, and D7 (all P < 0.050).

Clinical signs and performance

The number of days with diarrhoea per piglet ranged from 0 to 5 days and inoculated pigs had more days with diarrhoea compared to control pigs (P < 0.001, Fig. 2c). Rectal temperature post-inoculation was considered abnormal below 38.6°C or above 40.0°C according to 3 standard deviations. Therefore, rectal temperature was abnormal at least once for 6 ENR-inoculated (22%), 2 ENR-control (7%), 4 MIN-inoculated (14%), and 1 MIN-control (4%). According to Fisher's Exact Test, more inoculated pigs had abnormal rectal temperature at least once post-inoculation compared to control pigs (95% confidence interval for odds ratio: 0.923–23.175; P = 0.043).

Regarding histological analyses, there was no effect of Sex, Housing or Inoculation on villus height (VH), crypt depth (CD), or the VH:CD ratio.

The ADG was affected by Housing × Inoculation × Time (P = 0.001, Fig. 3). ADG increased over time for all groups but ENR-inoculated had a lower ADG before the challenge (D-5–D0) compared to the three other treatment groups (all P < 0.050). After the ETEC challenge, MIN-inoculated had a higher ADG compared to MIN-control during the period D9–D16 (P < 0.001).

Biochemistry

D-lactate

ENR pigs had higher D-lactate concentration than MIN pigs (P < 0.001, Fig. 2d). D-lactate was also affected by Inoculation × Time (P = 0.032). For inoculated pigs, D-lactate concentration was higher on D2 (compared to D1, D11, and D21, P < 0.010) and lower on D11 (compared to D3, D7, and D28, P < 0.05), while for control

pigs, D-lactate concentration did not significantly vary over time. Furthermore, inoculated pigs had higher D-lactate than control pigs on D2 ($P = 0.004$) and D28 ($P = 0.046$).

Haptoglobin and metabolic stress

Haptoglobin was only affected by Time ($P = 0.001$), which did not provide information on treatment effect.

The d-ROM level was affected by Housing × Time ($P = 0.023$, Fig. 4a) but the overall kinetics were very comparable with an increase of d-ROM over time in both housing conditions. According to pairwise comparisons, there were no differences between housing conditions on a given day post-inoculation. Females had a higher FRAP than males ($P = 0.001$). FRAP and the d-ROM:FRAP ratio were also affected by Time ($P < 0.001$ Fig. 4b and 4c), which did not provide information on treatment effect.

Females had higher cortisol levels compared to males ($P < 0.001$). Plasma cortisol was also affected by Inoculation × Time ($P = 0.018$, Fig. 4d). Inoculated pigs had lower plasma cortisol concentration than control pigs on D1 ($P = 0.008$), D2 ($P = 0.029$), and D3 ($P = 0.047$). Plasma cortisol was additionally affected by Housing × Time ($P = 0.020$). ENR pigs had higher cortisol concentration than MIN pigs on D28 ($P = 0.026$). The kinetics was however similar between groups, with an increase in cortisol over time.

Energy metabolism

Glucose concentration was affected by Inoculation × Time ($P < 0.001$, Fig. 5a). Control pigs had an increase in glucose on D1 compared to D-3 ($P = 0.007$) while inoculated pigs had lower glucose concentration on D1 compared to D3–D28 (all $P < 0.050$). Glucose concentration remained lower for inoculated pigs compared to control pigs on D1–D7 and on D28 (all $P < 0.050$).

NEFA concentration was affected by Housing × Inoculation × Time ($P < 0.001$, Fig. 5b). For MIN-control, NEFA concentration did not significantly change over time. For ENR-control, besides slightly higher concentrations on D2 compared to D7, and D14–D28 (all $P < 0.050$), NEFA concentrations were relatively stable. On the other hand, inoculated pigs had highly variable NEFA concentrations. For ENR-inoculated, NEFA concentration was elevated on D1 and on D7 compared to D14–D28 (all $P < 0.010$). For MIN-inoculated, NEFA concentration was also elevated on D1 compared to D-3, D2, D3, and D11–D14 (all $P < 0.050$). NEFA concentration in MIN-inoculated was also elevated on D7 and on D28 compared to D2 and D3 (all $P < 0.010$). Additionally, ENR-inoculated had higher NEFA concentrations compared to ENR-control on D7 ($P < 0.001$). MIN-inoculated had higher NEFA concentrations compared to MIN-control on D1 and D21–D28 (all $P < 0.050$) but lower on D2 ($P = 0.044$). MIN-inoculated also had higher NEFA concentrations compared to ENR-control on D7 and on D21–D28 (all $P < 0.050$) but lower on D2 ($P = 0.040$). Finally, MIN-inoculated had higher NEFA concentrations compared to ENR-inoculated on D21–D28 ($P \leq 0.001$).

Females had higher creatinine than males ($P = 0.017$). Creatinine was also affected by Time ($P < 0.001$), which did not provide information on treatment effect.

Haematology

Red blood cells

Red blood cells were affected by Housing × Inoculation × Time (P = 0.047) but the temporal variations were similar between groups, with an increase in red blood cells over time. According to pairwise comparisons, there were no differences between groups on a given day post-inoculation.

Haematocrit and haemoglobin were affected by Time (P < 0.001), mostly increasing over time.

White blood cells

ENR pigs had overall more circulating lymphocytes than MIN pigs (P = 0.001, Fig. 6a). Lymphocytes were also affected by Time (P < 0.001), with a steady increase in number over time.

Granulocytes were affected by Housing × Time (P < 0.001, Fig. 6b). While MIN did not significantly vary over time, ENR pigs had more circulating granulocytes on D-3 and D1 compared to D7–D28 (P ≤ 0.001). Additionally, pairwise comparisons show ENR pigs had more circulating granulocytes than MIN pigs on D1 (P = 0.030).

As a result, the Granulocytes:Lymphocytes (G:L) ratio was also affected by Housing × Time (P < 0.001, Fig. 6c), but the temporal variation was similar between the two housing conditions, with a decrease over time, and pairwise comparisons between housing conditions were not significant on a given day post-inoculation.

Females had fewer circulating monocytes compared to males (P = 0.043). Monocytes were also affected by Housing × Time (P < 0.001, Fig. 6d). Pairwise comparisons suggest ENR pigs had more circulating monocytes than MIN pigs on D-3 (P = 0.008) and D1 (P = 0.009). Monocytes were finally affected by Inoculation × Time (P = 0.001). Pairwise comparisons suggest inoculated pigs had more circulating monocytes than MIN pigs on D-3 (P = 0.002) and D7 (P = 0.041).

Gene expression

TNF-α expression was affected by Housing × Inoculation × Time (P = 0.048). On D1, MIN-control expressed less TNF-α than on D-3 and D11 (both P < 0.050), but, according to pairwise comparisons, there were no differences groups on a given day post-inoculation. Other groups did not significantly vary over time for TNF-α expression. IL-10 expression was affected by Housing × Time (P = 0.001, Fig. 7). ENR pigs expressed less IL-10 on D1 compared to D-3 (P < 0.001) and D11 (P = 0.042), which resulted in a lower expression in ENR than MIN pigs on this day (P = 0.016). IL-10 expression did not significantly vary over time for MIN pigs. TLR6 expression was affected by Time (P = 0.003), with an increase over time. TLR4, NOD2 and iNOS expressions were not affected by Sex, Housing, Inoculation, or Time.

Discussion

This study aimed at investigating the consequences of environmental and social enrichments on the resilience of piglets experimentally challenged with an ETEC strain as a model of PWD. Although specific pathogen free animals are valuable models in experimental research, their controlled rearing conditions

imply specificities in terms of microbiota, immune system, and immune stimulation [22]. Therefore, the present results should be interpreted with caution when extrapolating to commercial production conditions.

Firstly, we investigated the global effect of environmental and social enrichments on our parameters. Notably, enriched pigs had overall more D-lactate than pigs from the minimal housing condition. D-lactate is a metabolite specific to prokaryotic cells and the D-lactate detected in piglets' blood mainly originates from intestinal bacteria during carbohydrate fermentation [23]. Enriched pigs may therefore have a different microbiota that constantly produces more lactate or a higher gut permeability compared to minimally enriched pigs. In the present study, the provision of substrate that piglet could ingest in the enriched environment may have contributed to modulate the composition of their microbiota, as observed when pigs are housed on straw [17, 24]. ENR pigs additionally distinguished from MIN pigs regarding their immune parameters. They showed more circulating lymphocytes in the plasma, and punctually more circulating granulocytes and monocytes. The internal microbiome is expected to influence host immunity development, especially during early life [25]. The external microbial environment may also modulate immune regulation, although results are inconsistent across the literature. Studies have reported that a higher pathogenic exposure (e.g., enriched, out-door or dirty environment) may induce specific immune stimulation or redistribution of blood immune parameters between compartments [26–29] while other studies suggest better homeostatic capacities at the intestinal level [25]. While the immune differences between ENR and MIN pigs might be due to differences in their gut microbiota, they may also reflect differences in the overall level of stress. For example, the greater numbers of all white blood cell populations simultaneously suggest possibly a greater haematopoietic activity in ENR pigs. This has been observed during chronic stress in other species [30]. In pigs, however, no effects or opposite effects were observed when using environmental enrichment [31, 32]. Thus, blood white blood cell count may react differently depending on enrichment protocols. Interestingly, on D1, enriched pigs expressed more IL-10 than ENR-control in their circulating immune cells. IL-10 is an anti-inflammatory cytokine acting as a negative-feedback regulator which inhibits the production of pro-inflammatory cytokines either during infection or to maintain gut microbiota homeostasis [33]. This suggest that ENR pigs might have developed a more inflammatory profile, which contradicts the theory that improved welfare and emotional state would favour an anti-inflammatory profile [34, 35]. It must, however, be bear in mind that changes in immune parameters remain poorly understood, notably in the context of enrichment [36]. Overall, our results provide evidence that the contrasting housing conditions influenced the gut microbiota and circulating immune cells of the piglets.

Regarding piglets' response to the ETEC challenge, all piglets from the inoculated groups shed rifampicin-resistant *E. coli*, indicating that the colonisation with the inoculum strain succeeded. We had designed the ETEC challenge to induce dysbiosis without causing too much harm to the piglets' health. Most of the physiological and clinical measurements confirm this, with the exception of four piglets who were euthanised because of the ETEC inoculation. Shedding peaked around D4, and decreased gradually thereafter. It is worth noting that the inoculated ETEC strain had not completely disappeared from the faeces by the end of the experiment on D28. Inoculated pigs experienced more days with diarrhoea and a higher prevalence of abnormal rectal temperatures (< 38.6°C or > 40.0°C) compared to control pigs. However, clinical expression was variable. Some individuals showed no signs of diarrhoea and few individuals reached abnormal rectal temperature thresholds. This variability is consistent with previous findings suggesting that

PWD can occur without overt clinical signs. It was reported that during PWD epidemics, pigs will sometimes die suddenly without preceding signs of diarrhoea [5] and can even maintain normal rectal temperature during the infection [10]. Moreover, rectal temperature may not be a reliable indicator, as it tends to overestimate core body temperature [37] or be influenced by diarrheic faeces [38]. Inoculated pigs showed elevated D-lactate concentrations on D2 compared with control pigs. An increase in D-lactate in the blood circulation of inoculated groups indicates that more bacterial D-lactate crossed the gut barrier, which is consistent with a decreased intestinal integrity and digestive dysbiosis [23]. ETEC infection can further damage the intestinal barrier, commonly reducing villus height at the ileum, duodenum or jejunum levels [39]. In our samples, intestinal morphology was not affected in inoculated piglets, possibly because the intestinal mucosa had time to regenerate by the end of the experiment, four weeks after inoculation. Interestingly, a comparative histopathological evaluation of pigs with and without PWD conducted by Blirup-Plum et al. (2023) showed most lesions were not significantly associated with diarrhoea, suggesting that histopathological findings have limited predictive value for PWD and for the identification of specific pathogens such as ETEC [40]. Regarding the energy metabolism, inoculated pigs had a glucose decrease on D1 compared to control pigs and MIN-inoculated had increased NEFA on D1 compared to MIN-control. These results may also indicate a brief alteration of the energy metabolism after the ETEC-induced immune activation [41], or due to a reduced feed intake [42]. There was no effect of inoculation on white blood cells subpopulations, haptoglobin or gene expression in immune cells, indicating that the ETEC challenge was not sever enough to induce systemic immune and inflammatory responses, detectable in the plasma or in blood circulating immune cells. Although inflammation can increase oxidative stress [43], d-ROM levels were not influenced by the inoculation status, which may further support the lack of a strong inflammatory response. Cortisol is also expected to rise in response to acute inflammation [44], but inoculated pigs had lower cortisol levels on D1–D3 compared to control pigs. A possible explanation could be the suppression of energetically costly endocrine responses after the initial HPA-axis activation phase. Overall, the disease challenge was mostly characterised with marked ETEC shedding, clinical signs of diarrhoea and abnormal rectal temperature, and additional evidence of a temporary increase in gut permeability and mobilisation of the energy metabolism. Taken together, these results show that the inoculation had short-term and limited impact on piglets' health and physiology, despite the persistence of the ETEC pathogen.

Finally, regarding the influence of environmental enrichment on ETEC resistance and resilience, our results show that ENR-inoculated pigs shed more ETEC and for a longer period than MIN-inoculated pigs, suggesting that ENR pigs were less resistant to the pathogen burden. The enriched environment may have provided more opportunities for pathogen development. Firstly, enrichment materials can promote contamination between pen mates [45]. If bedding material is provided, the regular removal of soiled bedding is therefore necessary to reduce the microbial load on farms [1], which was applied on our experimental facility. Thus, physical enrichment need to be designed depending on sanitary status on farms to provide health benefits through welfare improvement that outweigh sanitary risks [46]. Third, commensal microbiota contributes to the protection of the host against entero-pathogens and microbiota dysbiosis may impair this protection [47]. Early socialisation can also induce microbial transfer between piglets from different litters and therefore alter microbiota. If, as we hypothesised, enrichment modulated the composition of piglets' microbiota, it may have consequences on resistance to the ETEC challenge, although a positive effect of early socialisation on gut health during the weaning transition has been proposed [48]. It is worth noting that

the total *E. coli* shedding of ENR-inoculated pigs, including commensal *E. coli*, became higher than that of MIN-inoculated pigs as a consequence of the ETEC colonisation. Piglets' growth was also affected by the challenge. Although the growth performance did not significantly decrease during the challenge, which is in line with limited clinical signs and fairly moderate immune response, we observed a potential compensatory growth in MIN-inoculated pigs between D9 and D16. Weaner pigs challenged with ETEC typically show lower ADG across studies compared to unchallenged controls over the post-challenge period [39] and there may be a compensatory feed intake after decrease in inoculated groups [49]. As a reminder, ENR-inoculated had a lower ADG before the challenge (D-5–D0) compared to the three other treatment groups. This slower growth is attributed to the rotavirus contamination during batch 2 and was not visible during batch 1 and 3. Overall, environmental and social enrichment appeared to increase the susceptibility of ENR-inoculated pigs during the disease challenge. However, to encompass the concept of disease resilience, we must consider resistance to the effects of the pathogen burden in terms of physiology, production, and behaviour [20, 21]. As we found no evidence of more adverse effects of the ETEC challenge on clinical signs, physiology and performance of ENR-inoculated pigs, we conclude that the disease resilience did not differ between the enriched and minimal housing conditions. It could even be argued that ENR-inoculated pigs had better coping capacities with the consequences of the pathogen burden despite their higher ETEC shedding.

Conclusion

This study evaluated the performance and response to an ETEC challenge in weaned piglets kept within contrasting housing conditions: highly enriched with physical and social enrichments versus minimally enriched. The low differences between inoculated and control piglets show that the health challenge was moderate, while still allowing to highlight differences between housing conditions. Notably, ETEC shedding was higher in the enriched housing condition, without adverse effects on disease resilience (clinical signs, physiology, growth performance). As this study did not assess pathogen transmission, the implications of these differences in faecal shedding for transmission dynamics remain to be determined. These interesting results will further improve our comprehension of the impact of enrichment on piglets' health and welfare but additional studies on the link between enrichments, microbiota and immune response is warranted to better understand the complex mechanisms of immune stimulation versus regulation in these contexts. Finally, these observations need to be confronted to commercial production conditions, subject to more complex environmental factors and multiple pathogens during the weaning transition.

Materials et methods

Ethics declarations

We followed established principles of laboratory animal use and care and the French law on animal experiments. They comply with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The French ethical committee n°16 approved the study design (APAFIS project #47624-2024021917438405 v6), which was planned, conducted and reported in accordance with the ARRIVE guidelines [50].

Animals

The present study was conducted at the research facilities of the Ploufragan-Plouzané-Niort laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES). A total of 119 specific pathogen-free piglets (n = 52 females, n = 67 males) were born in these same facilities and used for the experiment. Piglets were of Large White pure breed and offspring of 12 multiparous sows (range parity: 2–5; mean ± SD: 3.1 ± 1.3). The study was conducted over three batches starting in June 2024, November 2024, and April 2025, respectively. Each piglet received an ear tag for identification and its canines were slightly ground. The animals' tails were not docked and the males were not castrated. We weaned the piglets at 26.5 ± 1.6 days of age and placed them in groups of 10 individuals (Fig. 1), except for one group of 9 piglets, due to an insufficient number of weaned piglets. Consequently, we used 40, 40, and 39 piglets per batch, respectively.

Contrasting housing conditions

Animals were allocated to one of two housing conditions: minimal housing (MIN) or enriched housing (ENR). We randomly assigned sows to either condition and they were moved to the corresponding experimental pens during the last week of gestation. Their piglets were born and remained under the same housing conditions throughout the lactation period. This housing contrast was maintained after weaning: piglets from MIN sows were transferred to MIN post-weaning pens, whereas piglets from ENR mothers were transferred to ENR post-weaning pens. Each post-weaning pen housed 10 piglets, mixing five piglets from each of two litters within the same housing condition.

In the MIN condition, each sow was housed in a 1.0 m² farrowing crate (1.80 × 0.56 m with a 1.25 × 1.00 m rubber floor mat) while the litter had access to a 3.2 m² maternity pen (2.00 × 1.60 m) with metallic slatted floor and an additional PVC nest (0.23 m²; 1.17 × 0.20 m). After weaning, MIN piglets were transferred to 7.0 m² pens (2.85 × 2.45 m) with solid floor. During the maternity and post-weaning phases, we provided two permanent enrichment items to the animals: a rope and a rubber ball.

In the ENR condition, each sow and her litter had access to a 6.0 m² free farrowing pen (2.61 × 2.30 m) with solid floor. ENR sows had access to a mix of straw and hay as nesting material, which was removed after farrowing to prevent the piglets from eating it. Pens were equipped with an adjustable blocking system that allowed us to temporarily restrain the sow one or two days before farrowing until 48 hours after farrowing. We also temporary restrained ENR sows during piglets handling procedures. Early socialisation between two adjacent ENR pens was achieved by opening a sliding door from 9.5 ± 1.6 days of age onwards. At weaning, ENR piglets were transferred to 13.4 m² pens (2.85 × 4.70 m) with solid floor. In ENR pens, we provided the piglets with durable objects of various materials (PVC, rubber, wood, metal) and destructible items (egg cartons, ropes, jute). We rotated these enrichments every two to three days in order to maintain novelty and attractiveness. At all times, the pen contained a combination of durable objects and destructible materials. We also provided wood shavings as litter material and regularly renewed it according to soiling state. During the maternity phase, this litter was spread on the floor and, after weaning, it was provided in a 2.7 m² rooting area delimited with PVC partitions (15 cm high). Every day, except weekends, animal technicians performed

gentle stroking of the ENR sows and piglets. Radio music (Muse M-051 R®, Muse Radio Company) featuring variety genres was also played three times daily for one-hour periods. In post-weaning pens only, we additionally provided a permanent cognitive enrichment in the form of two horizontal PVC cylinders perforated and refilled daily with food pellets. Piglets could rotate these cylinders with their snout in order to make the food pellets fall on the ground.

In both housing conditions, water was provided *ad libitum*. We provided sows with controlled gestating and lactating diets. We distributed food pellets of a commercial starter feed to piglets in maternity pens, starting with small amounts from approximately 5.5 ± 1.9 days of age, and *ad libitum* from 19.5 ± 1.9 days onwards. Artificial lighting was programmed from 7:30 to 19:00 and the room temperature was set to 20°C. All maternity and post-weaning pens had additional heating lamps for the thermal comfort of the piglets.

Enterotoxigenic E. coli challenge

Within each housing condition, we allocated the weaned piglets to either a control or inoculated pen. We balanced the groups for sex and average weight at weaning (Table 1). The experiment took place in a level 2 biosecurity facility to avoid contamination of the pigs with external pathogens and to avoid external dissemination of the ETEC strain. This includes regulated air filtration and compartmentation, compulsory showers, and the use of personal protective equipment for animal keepers and experimenters. Wood shavings were sterilised by dehydration in ovens (between 200 and 300°C), cooled and packaged by the manufacturer. Food pellets were heat-treated at 80°C by the manufacturer. Before the experiment, all pens and enrichments were decontaminated using a vapor-phase hydrogen peroxide system to ensure the piglets remained specific pathogen-free until the disease challenge. Eleven days after weaning (37.5 ± 1.9 days of age), piglets were confronted to a digestive dysbiosis induced by a strain of ETEC selected in our laboratory, namely strain 12-269M This colistin resistant strain was isolated from PWD-suffering piglets and was further made resistant to rifampicin for selection purpose. It was of serotype O-149 F4, with additional resistance to sulfamethoxazole, trimethoprim, tetracycline, and ampicillin, gentamicin, and chloramphenicol. The inoculum was cultivated on Mueller Hinton broth containing colistin (2 mg/L) for 18 h at 37°C, under gentle agitation. Bacterial cells were pelleted by centrifugation (5180 × g, 5 min) and resuspended in peptone-buffered broth to achieve a final concentration of 10^7 colony-forming units (CFU)/mL. We administered 10 mL of this inoculum orally to each animal of the inoculated group. Piglets were priorly genotyped for susceptibility to F4 ETEC adhesion (5.5 ± 1.9 days of age), using the Muc4 Polymerase Chain Reaction (PCR) proposed by Rasschaert et al. [51], so that only F4-susceptible pigs were included in inoculated groups (Table 1). Control pigs received 10 mL of sterile peptone-buffered broth orally.

Table 1
Body weight (mean ± SEM) at weaning and inoculation, susceptibility to F4 ETEC adhesion and sex distribution across experimental groups.

Weight, kg	**ENR control**	**ENR inoculated**	**MIN control**	**MIN inoculated**
Weaning (age 28 days)	7.0 ± 0.4	6.7 ± 0.3	6.8 ± 0.3	7.2 ± 0.2
Inoculation (age 39 days)	12.9 ± 0.4	12.0 ± 0.5	13.4 ± 0.4	13.3 ± 0.3
Susceptibility to F4 ETEC adhesion				
Resistant	14	0	12	0
Susceptible	16	30	16	30
Unknown	0	0	1	0
Sex				
Number of females	14	14	12	12
Number of males	16	16	17	18

Sample collection and assays

The day of inoculation is designated as D0 (Fig. 1). Piglets' physiological parameters were monitored until 28 days post-inoculation (D28). Average daily gain (ADG) was calculated for each piglet using the weight measurements on D-13 (two days before weaning), D-5, D0, D9, D16, and D28. On weekdays, clinical observations consisted in looking for general clinical signs, including the visual observation of faecal consistency (hard, normal, soft, diarrhoea, watery diarrhoea) and taking rectal temperatures. We proposed 1 mL of grenadine syrup to each piglet after any session of invasive procedures (rectal temperature and blood sampling) to associate a positive event after these manipulations.

Faecal samples and bacterial counts

Individual faecal samples were collected from pigs on D-3, D1, D2, D3, D4, D5, D7, D9, D11, D14, D17, D21, D24, and D28. If not spontaneous, the collectors could gently stimulate defecation using a rectal thermometer. Approximately 2 g of faeces were collected and diluted to 1:9 in buffered peptone water supplemented with 20% glycerol using a gravimetric diluter (DiluFlow elite®, Interscience), then homogenised in a digital mixer (MiniMix®100, Interscience). The dilution was then stored at -70°C. Using an automatic plater (easySpiral Dilute®, Interscience), 100µL of decimal dilutions (10^{-3} to 10^{-8}) were plated onto MacConkey media for total *E. coli* shedding or onto MacConkey media supplemented with rifampicin (250 mg/L) for shedding of the inoculated ETEC strain, ETEC shedding hereafter. The Petri dishes were incubated at 37°C for 18 to 24 hours before reading with an automatic colony counter (Scan®1200, Interscience).

Blood samples

An experimented technician collected blood samples from the jugular vein of each piglet using vacutainer tubes, while another maintained the animal with a nose sling. Sampling took place outside the pen and was abandoned if the total duration of the procedure, including trapping, exceeded 4 minutes. Depending on sampling day, plasma was obtained using a 4 mL EDTA tube for blood cell counts (D-3, D1, D7, D14, D21 and D28), and/or a 4 mL heparin tube for biochemistry analyses (D-3, D1, D2, D3, D7, D11, D14, D21, and D28). For half of the pigs, we collected two additional 8 mL heparin tubes for gene expression analysis (D-3, D1 and D11), balancing for treatment, sex and litter. Blood sampling schedule was designed in accordance with limit volumes and recovery periods applied to multiple sampling as described in Diehl et al. (2001), considering a mean blood volume of 65 mL/kg for pigs.

Biochemistry

The 4 mL heparinised tubes collected on D-3, D1, D2, D3, D7, D11, D14, D21, and 28 were centrifuged for 15 minutes at 2,000 g and then aliquoted into Eppendorf tubes before being stored at -70°C for haptoglobin, ferric reducing ability of plasma (FRAP), and derivatives of Reactive Oxygen Metabolites (d-ROM) analyses and at -20°C for glucose, non-esterified fatty acids (NEFA), creatinine and cortisol analyses. We used commercial kits and a sequential analyser (Gallery Plus®, Thermo Fisher Scientific) to assess the acute phase protein haptoglobin (Tridelta Development Ltd), d-ROM (H&D srl), glucose, NEFA, and creatinine (Thermo Fisher Scientific). FRAP was evaluated as described before [53]. Plasma cortisol was assessed using the AIA cortisol kit (Tosoh Europe). All samples were analysed in duplicates and the intra-assay CV were below 5%.

Haematology

The 4 mL EDTA tubes collected on D-3, D1, D7, D14, D21 and D28 were analysed immediately to obtain a complete blood cell counts on an automated haematology analyser (MS9.5 with M-Sampler®, Melet Schloesing Laboratories).

Gene expression in immune blood cells

The two 8 mL heparinised tubes collected on D-3, D1 and D11 were immediately pooled for each pig in order to collect peripheral blood mononuclear cells (PBMCs). PBMCs were isolated using Leucosep® tubes by centrifugation at 800 g for 15 minutes with the brake off. Briefly, cells were subjected to red blood cells lysis, washed in Roswell Park Memorial Institute medium and live PBMCs were counted on an automatic cell counter (LUNA® Fluorescence Cell Counter, Thermo Fisher Scientific). Three Eppendorf tubes per pig containing each $1{,}10 \times 10^7$ PBMCs were vortexed vigorously with 350 µL of lysis buffer LBP (Macherey-Nagel) and stored at -70°C until gene expression analysis. Ribonucleic acid was extracted from the thawed PBMCs using a kit (NucleoSpin RNA Plus, Macherey-Nagel). When samples did not yield enough RNA, they were concentrated using a kit (RNA Clean-Up XS, Macherey-Nagel). Inverse transcription was performed

(Reliance Select cDNA Synthesis Kit, Bio-Rad) using a thermoregulator (PTC-100, Bio-Rad). Resulting complementary DNA was diluted to a concentration of 0.4 ng/µL, and stored at -70°C until final analysis. We considered gene expression for tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10), respectively, an inflammatory and an anti-inflammatory cytokine), toll-like receptors 4 and 6 (TLR4 and TLR6), the nucleotide-binding oligomerisation domain 2 (NOD2), which are pathogen recognition receptors involved in *E. coli* recognition, and inducible nitric oxide synthase (iNOS), which is necessary to microbial killing of bacteria by macrophages (Supplementary material 1). Reverse Transcription quantitative PCR (RT-qPCR) was performed on a real-time PCR system (StepOnePlus®96 well, Thermo Fisher Scientific). The relative abundance of the target genes compared to the house-keeping genes was estimated using a normalisation factor calculated with the GeNorm algorithm [54].

Euthanasia, intestine sampling and histology

Piglets were euthanised between D29 and D32. We first anaesthetised animals maintained with a nose sling *via* an injection into the auricular vein (Zoletil 100; 5 mg/kg body weight of tiletamine and 5 mg/kg of zolazepam), followed by jugular vein exsanguination. We collected a 10-cm intestinal segment at the jejunum-ileum junction during autopsy and stored it in 10% buffered formaldehyde until histological analysis. The histological analysis was performed by an external laboratory (Labocéa, Ploufragan, France). Tissues were dewaxed and dehydrated, while transverse sections were stained with Hematoxyline-Eosine-Saffron and mounted on glass slides. The slides were visualised on a microscope (Leica® DM 2500, Leica Microsystems). Villus height and crypt depth were measured with Archimed Software (version 12.3.11) on images acquired at 50× magnification using a numerical camera (SONY® XCD U100CR, Sony Corporation). Five villi and crypts were measured per pig in order to calculate mean villus height and crypt depth.

Statistical analyses

Analyses were performed using the R programming language [55] and RStudio development environment [56]. Individual piglet served as the statistical unit.

Linear mixed-effect models

Data from blood and faecal sampling and from ADG were analysed as repeated measures using linear mixed effect models with the function *lmer()* from the R package *lme4* [57]. The piglet and sow were included as random effects. Fixed effects initially included sex, and the interaction between housing (ENR or MIN), inoculation status (inoculated or control) and day post-inoculation (D-3 to D28). For ETEC shedding, inoculation status was not included in the model, so fixed effects initially included sex, and the interaction between housing and day post-inoculation (D1 to D28). For each pig, we calculated the number of days with diarrhoea (soft, diarrhoea or watery diarrhoea) after inoculation between D1 and D28. Rectal temperature was considered abnormal outside a range of 3 standard deviations and occurrence of abnormal rectal temperature was analysed using Fisher's exact test due to low frequencies. Euthanised pigs were excluded from clinical signs analyses as these analyses involve an overview of the entire post-inoculation period (D1

to D28). For the number of days with diarrhoea and for histological analyses, there was only one datapoint per pig, so only the sow was included as a random effect and the interaction between sex, housing, and inoculation was initially included as a fixed effect.

An automatic bidirectional selection of fixed effects was applied using the function *step()* from the core R package *stats* [55]. We tested for multicollinearity using the variance inflation factor (VIF < 5) with the function *vif()* from the R package *car* [58]. Weight was not included as a covariate due to its collinearity with Time (VIF > 5). We performed graphical inspection of model residuals and applied transformations when necessary (Supplementary material 2) to achieve a better approximation of normal distribution and homoscedasticity, using the optimal normalizing transformation algorithm provided by the R package *bestNormalize* [59]. The significance of fixed effects was evaluated using analysis of variance with the *Anova()* function implemented in the R package *car*. Multiple comparisons for the interactions were performed using the R package *emmeans* [60] and they were accommodated using Tukey's HSD test. For all analyses, differences were considered significant when $P < 0.05$. In this paper, we present the p-values of biologically meaningful effects. This is to simplify the large number of contrasts arising from the high number of sampling days. The complete list of p-values for the pairwise comparisons is provided as Supplementary material 2.

Impact of uncontrolled factors

Despite strict biosecurity measures, traces of pathogen contamination were detected during batch 2. Following weaning, faeces from inoculated pigs were diarrhoeic and tested positive for rotavirus by RT-PCR. However, clinical signs of diarrhoea had resolved by the day of inoculation (D0). In the faeces of control pigs (7 ENR-control, 10 MIN-control), ETEC from the inoculated strain was detected on D-3 (10E2–10E6 CFU/mL). Between D2–D5 only a couple of pigs (2–6 pigs) remained contaminated (10E2–10E3 CFU/mL) and ETEC had completely disappeared from the faeces of control pigs from D7 onwards. A sensitivity analysis was performed by refitting the linear models with only batch 1 and batch 3. The results suggest our models were robust despite the reduced statistical power, with few marginal differences. Therefore, pigs from batch 2 remained included in the final analysis.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Author Contribution

S.A., E.M., F.P., G.L.D., Fl.T., N.R. and S.P. conceived and designed the study. F.P. and G.L.D. provided the animals while L.L.D. prepared the inoculum for the study. E.M., N.R. and S.P. supervised the project. S.A. and S.P. conducted the experimental work, with support from E.M., F.P., L.L.D., G.L.D. and Fr.T. Afterwards, S.A. proceeded with data curation and anlysis. S.A. wrote the original draft of the manuscript. S.A., E.M., N.R. and S.P. contributed substantially to manuscript review and editing, with contributions from F.P., L.L.D., G.L.D., Fl.T. and Fr.T. All authors reviewed and approved the final manuscript.

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Data Availability

The datasets analysed for the present study are available from the corresponding author upon reasonable request.

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Figures

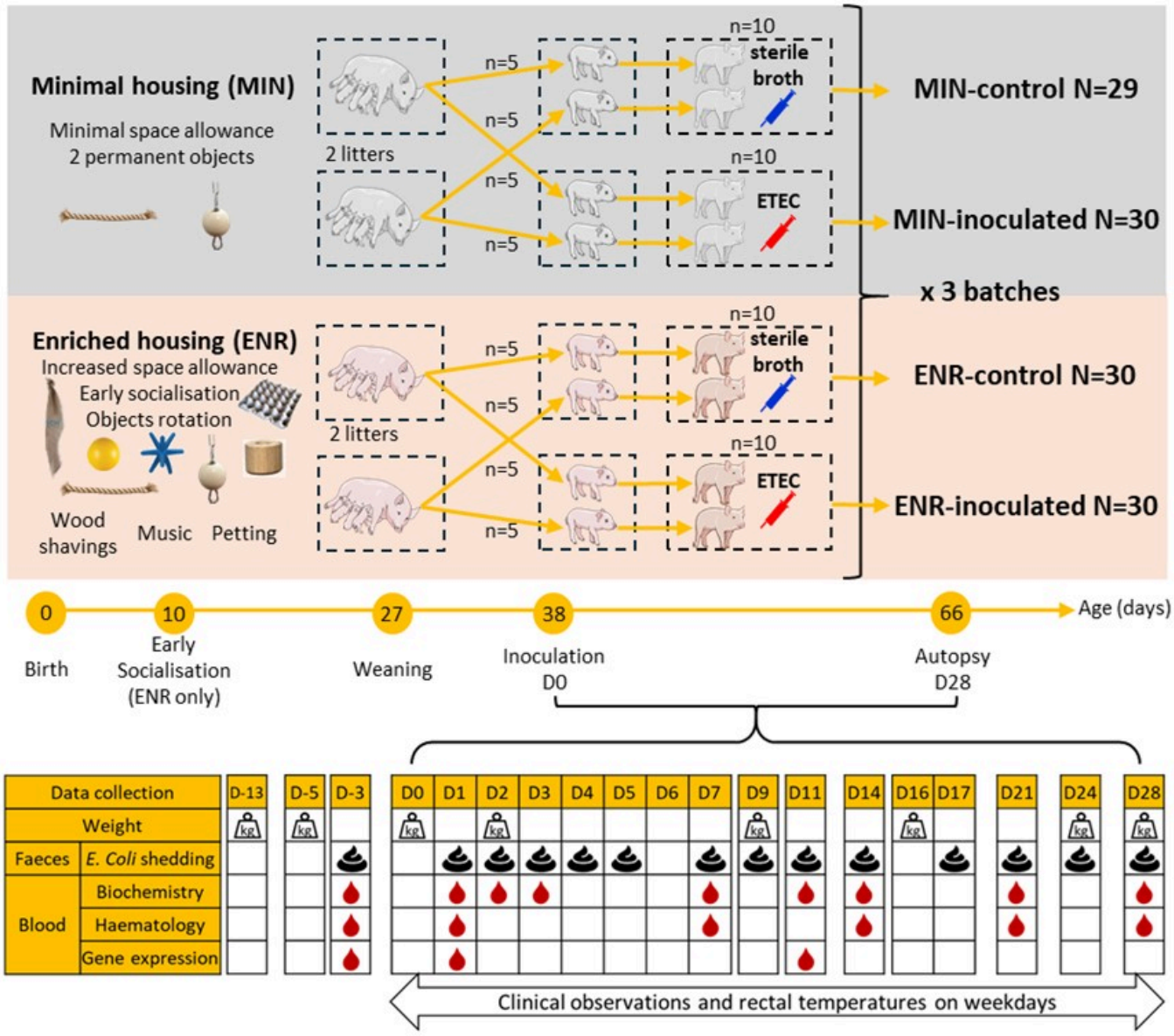


Figure 1.

Figure 1

Experimental design and chronology of data collection during the enterotoxigenic *E. coli*challenge.

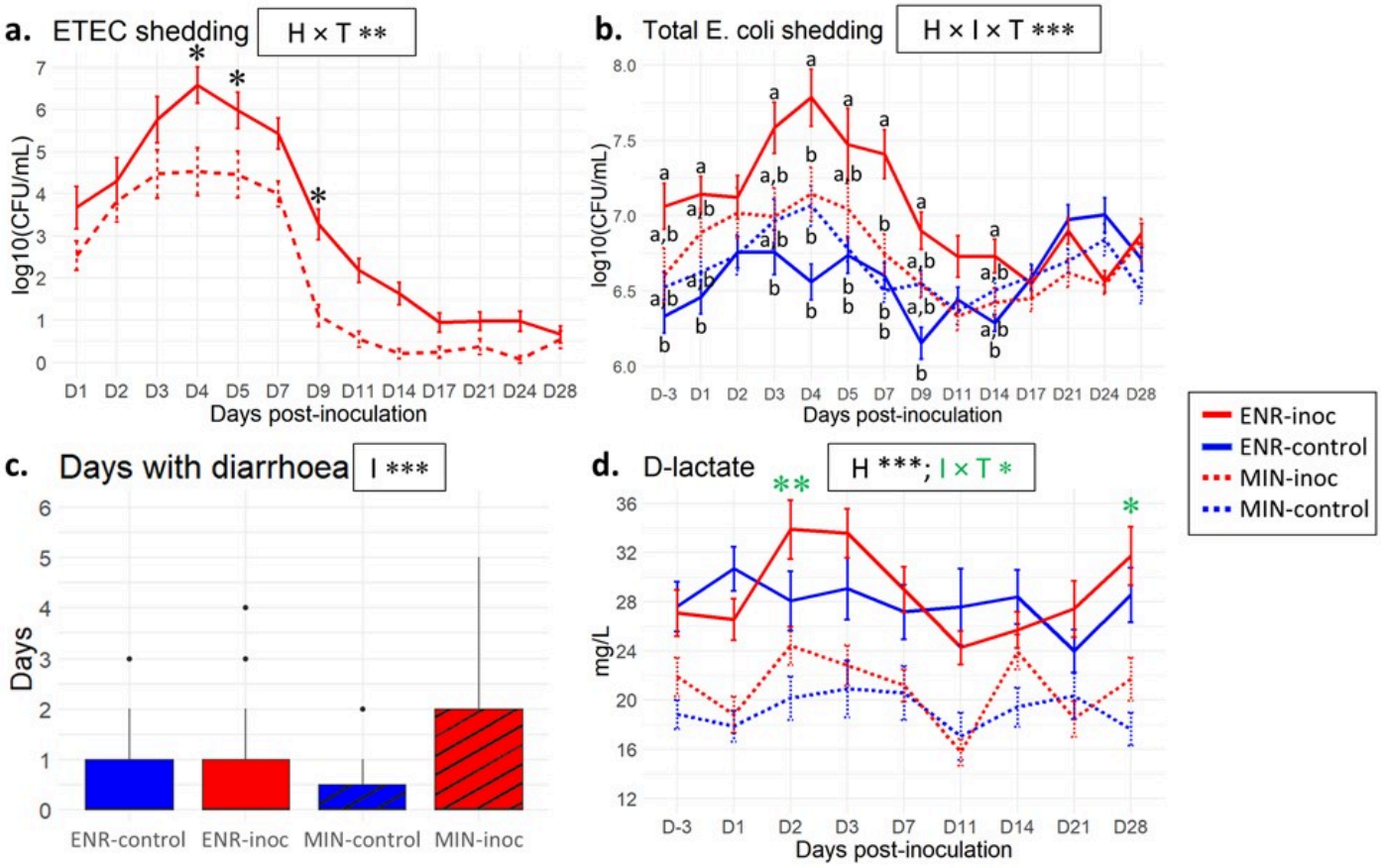


Figure 2.

Figure 2

Piglets' faecal excretion and digestive health. Means and SEM of enterotoxigenic *E. coli*(ETEC) shedding in the faeces (**a**), total *E. coli* shedding in the faeces (**b**), boxplots of the duration of diarrhoea (**c**), and D-lactate plasma concentrations (**d**). ENR: pigs housed in enriched pens. MIN: pigs housed in minimal pens. The effects of housing (H), inoculation (I), time (T), and their interactions are presented in a square box as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between either the two housing conditions (in black) or the two inoculation statuses (in green) are also indicated as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between the four treatment groups (housing × inoculation) are indicated by the lack of a common letter (a, b).

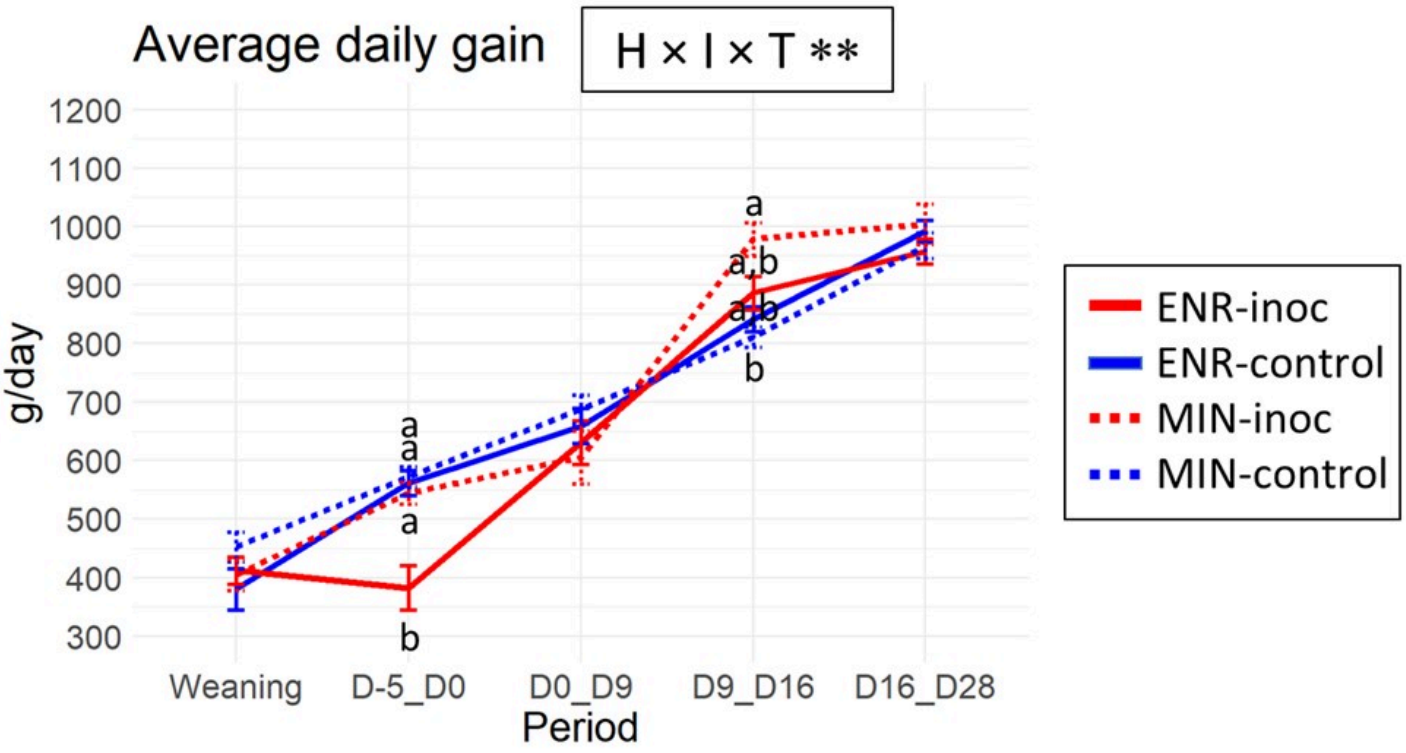


Figure 3.

Figure 3

Piglets' growth performance. Means and SEM of average daily gain at weaning (between D-13 and D-5), between D-5 and D0, between D0 and D9, between D9 and D16, and between D16 and D28 post-inoculation. ENR: pigs housed in enriched pens. MIN: pigs housed in minimal pens. The effects of housing (H), inoculation (I), time (T), and their interactions are presented in a square box as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between the four treatment groups (housing × inoculation) are indicated by the lack of a common letter (a, b).

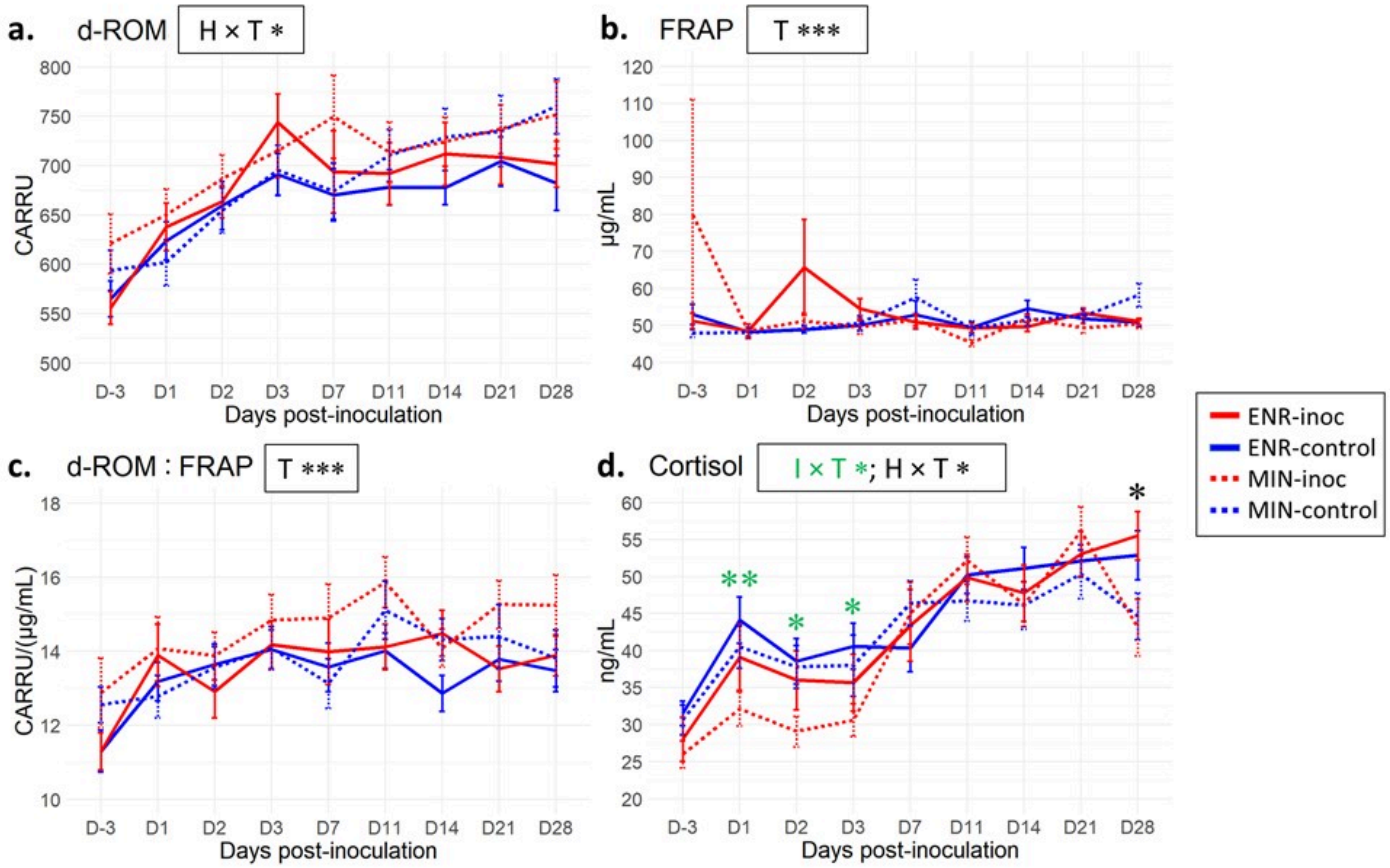


Figure 4.

Figure 4

Piglets' metabolic stress. Means and SEM of plasma derivatives of Reactive Oxygen metabolites (d-ROM) (**a**), ferric reducing ability of plasma (FRAP) (**b**), the ratio between d-ROM and FRAP (**c**), and cortisol plasma concentrations (**d**). ENR: pigs housed in enriched pens. MIN: pigs housed in minimal pens. The effects of housing (H), inoculation (I), time (T), and their interactions are presented in a square box as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between either the two housing conditions (in black) or the two inoculation statuses (in green) are also indicated as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

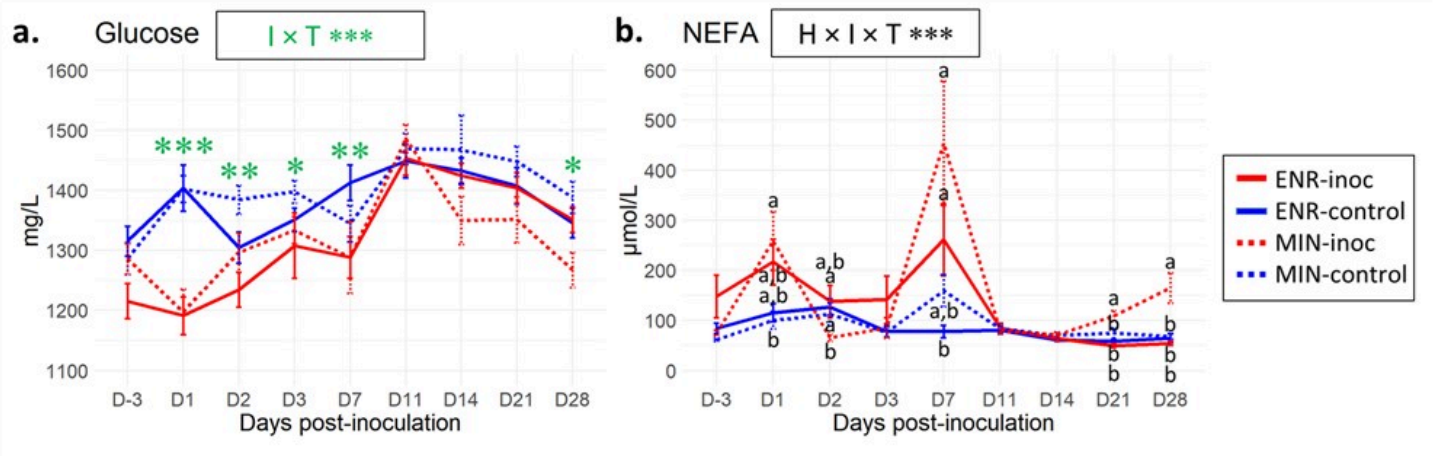


Figure 5.

Figure 5

Piglets' energy metabolism. Means and SEM of glucose plasma concentrations (**a**), non-esterified fatty acids (NEFA) plasma concentrations (**b**). The effects of housing (H), inoculation (I), time (T), and their interactions are presented in a square box as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between the two inoculation statuses are also indicated in green as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between the four treatment groups (housing × inoculation) are indicated by the lack of a common letter (a, b).

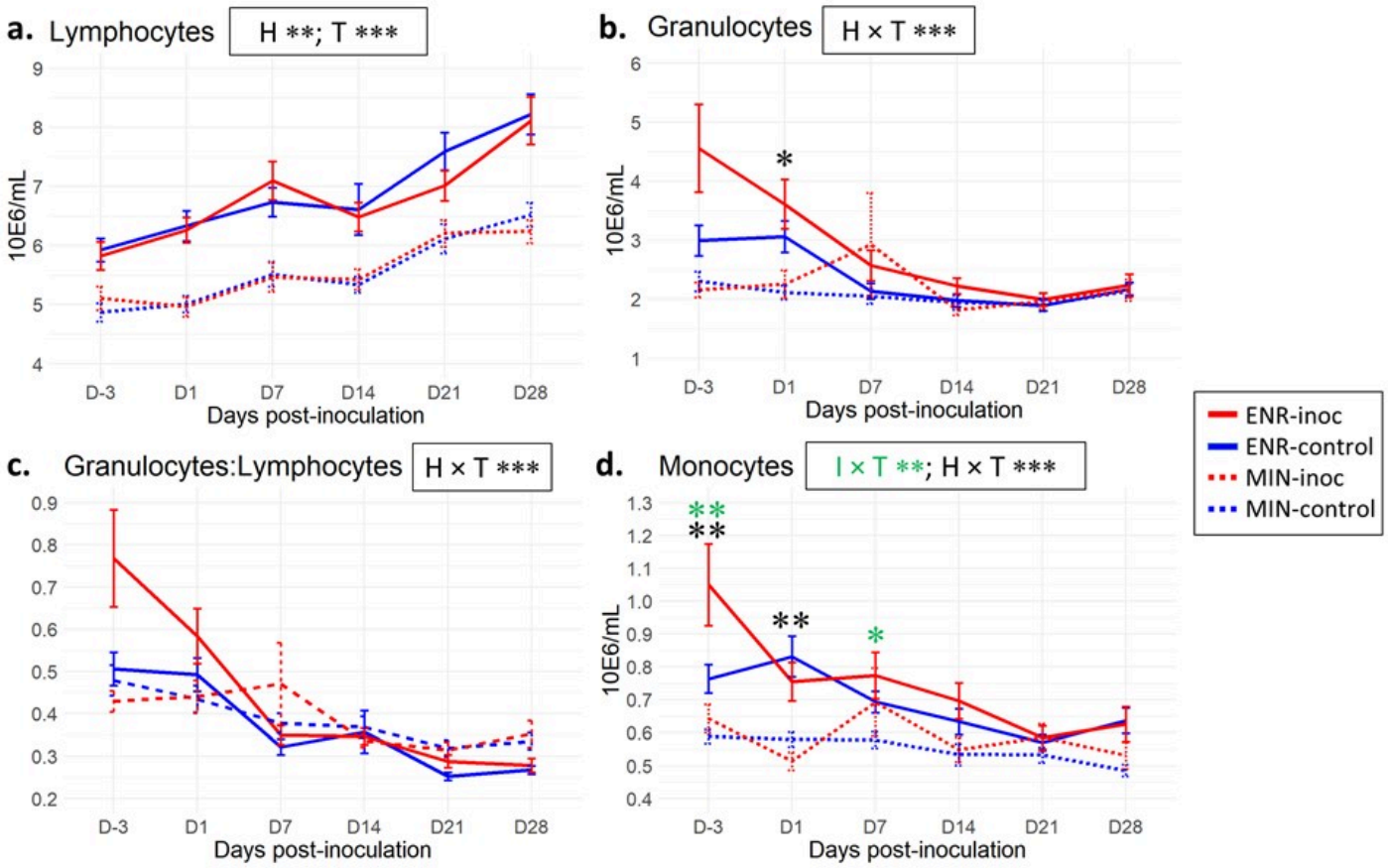


Figure 6.

Figure 6

Piglets' white blood cell counts. Means and SEM of the number of blood circulating lymphocytes (**a**), number of blood circulating granulocytes (**b**), the ratio between granulocytes and lymphocytes (**c**), and number of blood circulating monocytes (**d**). ENR: pigs housed in enriched pens. MIN: pigs housed in minimal pens. The effects of housing (H), inoculation (I), time (T), and their interactions are presented in a square box as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between either the two housing conditions (in black) or the two inoculation statuses (in green) are also indicated as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

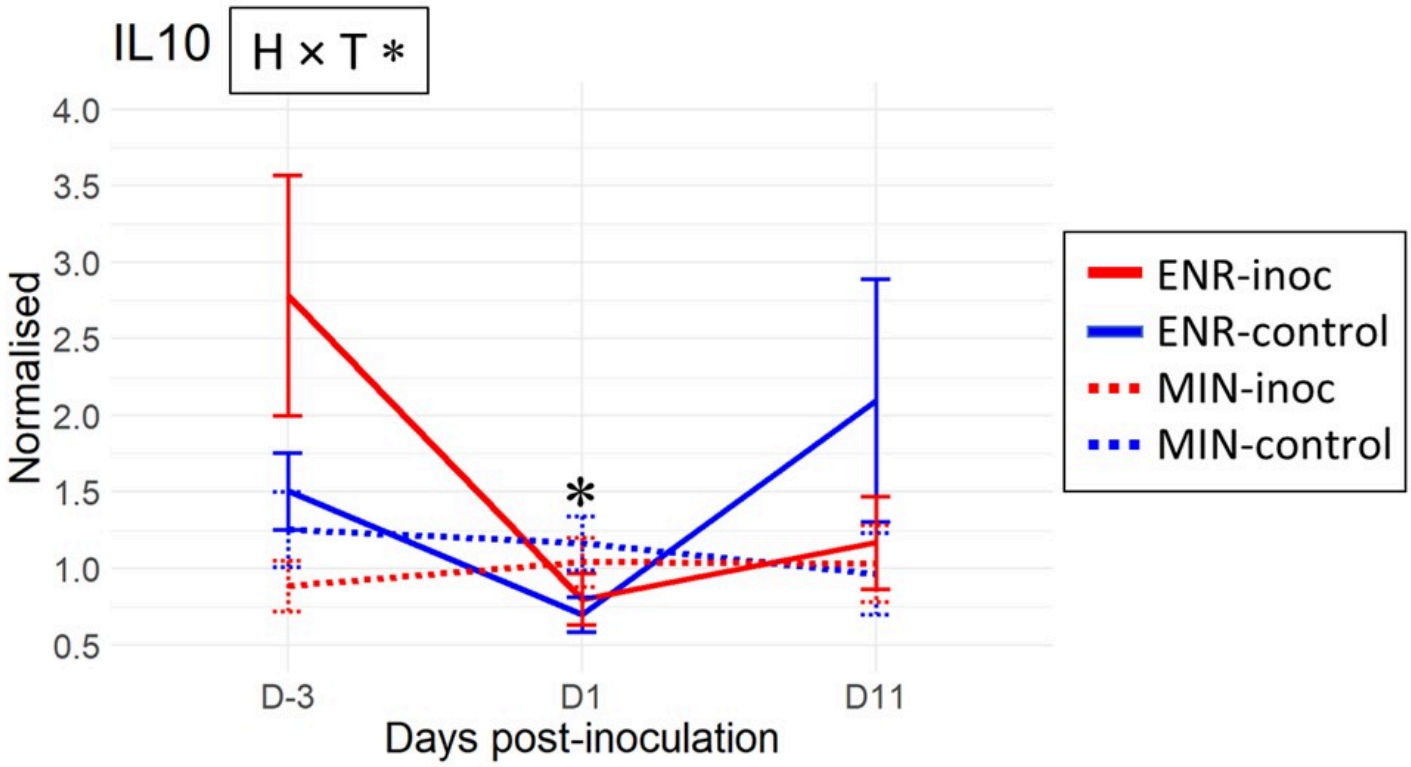


Figure 7.

Figure 7

Gene expression in peripheral blood mononuclear cells. Means and SEM of normalised interleukin-10 (IL-10) expression. ENR: pigs housed in enriched pens. MIN: pigs housed in minimal pens. The effects of housing (H), inoculation (I), time (T), and their interactions are presented in a square box as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant pairwise comparisons between the two housing conditions are also indicated as ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial1.docx
- Supplementarymaterial2.xlsx