HEAT TOLERANCE IN LACTATING SOWS: DIETARY STRATEGIES, METABOLIC BIOMARKERS AND MICROBIOME SIGNATURE

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By

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Executive Summary

Environmental hyperthermia is a main welfare and economic problem in pig production particularly given the rise in heat wave episodes and the hyperprolificacy of modern sows. This project aimed to identify effective nutritional strategies to mitigate the impact of heat on performance, metabolic biomarkers and microbiome signatures of heat tolerance (HT) in lactating sows. Dietary strategies including the use of cooling agents, appetite enhancers and low crude protein (LP) diets to improve performance and welfare during environmental hyperthermia were evaluated using 2 climate control rooms at The University of Queensland (Gatton, QLD, Australia) in Experiment (Exp) 1 and 2. In addition, the impact of heat stress (HS) in lactating sows' performance, physiology, plasma metabolome, plasma and liver proteome and faecal microbiome was investigated. Experiment 3 was performed to corroborate the findings of Exp 1 and 2 in a commercial setting at JBS Pork Australia, Corowa, NSW, during the winter and summer season of 2023 and 2024.

Rectal (RT), vaginal diurnal (VT), vaginal nocturnal (VTN), eye (ET) and ear (EaT) temperatures and respiration rate (RR) were significantly ($P<0.05$) increased in lactating sows during the heating days of the climate control rooms when compared to TN conditions, confirming that the animals were under HS in Exp 1 and 2. Sows fed the LP diet showed lower ($P<0.05$) VT (Exp 1; under HS) and RT (Exp 2; under both TN and HS conditions), when compared to the standard protein (SP) treatment. In Exp 2, the feed intake (FI) of sows was significantly ($P<0.05$) reduced when comparing TN to HS conditions. However, a significant reduction in FI when comparing TN to day 3 of HS was observed in the SP, but not the LP group indicating a better capacity to cope with HS in the later. The abundance of 23 liver proteins related to AA, lipid and ATP/carbohydrate metabolism as well as stress/immune response, cytoskeleton organization, and programmed cell death were found to have a strong negative correlation ($r<-0.7$, $P<0.05$) with feed intake reduction under HS in Exp 2. The faecal microbiome analysis showed 5 features (Anaerovoracaceae, Muribaculaceae, *Treponema*, *Cryptobacteroides* and Bacteroidales) positively correlated (FDR<0.05) with body temperatures. In addition, five bacterial metabolic pathways related to vitamin B_{12} metabolism were enhanced in the LP sows (LDA>2).

Lactating sows showed reduced FI ($P<0.001$), piglets' weaning weight ($P<0.01$) and backfat loss ($P<0.01$) as well as increased RT ($P<0.01$) in summer when compared to winter during Exp 3. The commercial trial corroborated the positive effect of the LP diet on body temperatures, reducing the average RT of sows ($P<0.05$). However, improvements in FI were not illustrated. Although reduced piglets' birth weights ($P<0.05$) were identified in LP when compared to SP sows, no differences on weaning weights were observed between treatments. A total of 66, 52 and 27 blood metabolites were identified to be differentially abundant ($P<0.05$) between seasons and diets (within summer and winter), respectively. During summer, a reduction in the abundance of lipids and AA was observed as well as an increase in steroid hormones. Sows offered the SP diet had a higher abundance of lipids, cholesterol, bile acids and pro-inflammatory molecules, such as arachidonic and arachidic acids, but lower concentrations of AA vs. the LP group in summer ($P<0.05$). Tryptophan (Trp) was identified in the metabolome of HS sows in Exp 2 and 3, whereas pregnanolone and arachidonic acid had several derivates/precursors described as differentially abundant between seasons in Exp 3, suggesting a biomarker potential for HS.

In conclusion, the use of LP diets lowered internal body temperatures and RR as well as increased ADFI in lactating sows. Low protein diets reduced the blood levels of pro-inflammatory molecules, lipids and steroidal hormones and increased alpha AA and gut microbiota vitamin B_{12} synthesis in lactating sows under hot weather conditions, improving their metabolic capacity to cope with environmental hyperthermia. Arachidonic acid, Trp and pregnenolone, have the potential to be used as metabolic indices of HS in lactating sows.

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1. Introduction

Environmental hyperthermia is a main welfare and economic problem for the swine industry. Pigs experience heat stress (HS) when environmental temperatures exceed their physiological compensatory mechanisms to maintain body temperature (Liu et al., 2022). Heat stress episodes are expected to increase in frequency and severity in the future as rises in temperature (by approx. 2°C) have been forecasted by the Australian Bureau of Meteorology (2024). Among pigs, gestating and lactating sows are particularly vulnerable to high ambient temperatures due to their intense metabolism (Williams et al., 2013). Moreover, the hyperprolificacy of modern sows has led to an increase in metabolic heat production associated with the physiological strain of supporting large litters, making modern genetics highly susceptible to environmental hyperthermia (Adi et al., 2022). Decreased feed intake, lower milk production and reduced piglet growth, compromised gut integrity and delayed oestrus are among the most common negative impacts of HS in lactating sows (Black et al., 1993; Prunier et al., 1997; Messias de Bragan et al., 1998; Pearce et al., 2014; Liu et al., 2016). This reduced reproductive performance is a direct consequence of 4 main physiological changes produced by the increase in body temperature:

1- **Hypoxia and cell lysis.** This is a consequence of lack of oxygen in key productive organs due to the redirection of blood flow away from internal organs towards the skin for heat dissipation. The sustained insufficient blood flow in mammary glands and gastrointestinal tract (GIT) may lead to decreased milk production/piglet growth and altered intestinal function and increased endo-toxaemia risk in the GIT (Lambert et al., 2009; Ni et al., 2020).

2- **Altered oxidative stress/antioxidant pathway.** Prolific sows experience basal oxidative DNA damage induced by the increased production of reactive oxygen species (ROS) during late gestation and lactation stages, which can lead to cell death and/or diminished normal cell function in different tissues (Berchieri-Ronchi et al., 2011; Toy et al., 2009). Heat stress further promotes the production of ROS and impairs the compensatory capacity of dietary and endogen antioxidants by reducing glutathione/oxidized glutathione ratio and depleting glutathione peroxidase, which is the main body enzyme that neutralizes ROS. Elevated oxidative stress is often related to reproductive and health disorders, impairing milk production and altering intestinal absorption capacity (i.e., decreasing villus/crypt ratio and barrier function, which can increase hindgut fermentation and body heat production, further exacerbating HS symptoms).

3- **Respiratory alkalosis.** Hyperventilation depletes blood CO_2 and the bicarbonate buffering system, triggering alkalosis. Respiratory alkalosis can disturb various biochemical and physiological process, such as decreasing calcium ionization and increasing lactate production, leading to reduce milk viscosity (essential for adhesion of milk proteins to the piglet's intestinal lining) as well as impaired fatty acid beta-oxidation and mitochondrial

function due to acetyl-CoA build up (Liu et al., 2018). The reduction in glycolysis, gluconeogenesis and citric acid production, as well as mitochondrial oxidation, due to high lactate levels, increases the effects of oxidation reactions resulting in tissue damage and inflammation (Relman, 1972).

4- **Changes in the microbiota.** The increase of undigested dietary nutrients (e.g. fibre and proteins) in the hindgut can cause shifts in the rate of bacterial heat production which, in turn, may increase metabolic heat production and contribute to the onset of HS (Armstrong et al., 2019).

Heat tolerance (HT) has been defined as the capacity to cope with environment hyperthermia, which can be determined by evaluating the maintenance of body temperature (e.g., rectal and skin temperatures) and other physiological parameters, such as heart and respiratory rate. In addition, previous studies indicate that changes in plasma levels of certain metabolites such as glucose and amino acids (AA) (e.g., Glu, Asn, Arg, Lys and Pro) can be used as predictors of HT in growing pigs (Dou et al., 2017). However, changes in blood biomarkers following HS have not been extensively studied in lactating sows. Lactating sows have distinctive metabolic/hormonal signatures and nutritional requirements compared to growing and fattening pigs. Moreover, sows experience significant metabolic changes as they move from an anabolic to a catabolic metabolism during late gestation and early lactation (Hedemann et al., 2012). This is particularly evident in modern genetic lines as they cannot fully compensate for the increased metabolic demands associated with a higher milk production via high feed intake alone (Strathe et al., 2017). Thus, there is a need to better understand the metabolic changes of lactating sows exposed to environmental hyperthermia and their relationship with HT.

Some dietary interventions that have been tested in sows with relative success in reducing or protecting against HS are, the use of citrulline and its precursors (Pro and Glu) as hyperthermic protectors and redox homeostasis regulators (Phang et al., 2010, Phang 2019), betaine as an osmoprotectant (Ratiyanto et al., 2009), creatine to reduce oxidative stress (Santacruz et al., 2017), and dextrose to improve weaning to oestrus interval and litter growth (Choi et al., 2017; Plush et al., 2019). However, much remains unknown on the use of supplements and/or dietary modifications to promote HT in lactating sows, particularly regarding the modulation of hindgut fermentation and the gut microbiota internal heat production (more recently defined as one of the key contributing factors to the onset of HS in sows (Armstrong et al., 2019)).

The overall aims of this project were to 1) develop dietary strategies to improve lactating sows' compensatory mechanisms to cope with environmental high temperatures and 2) identify metabolic and microbiome markers that may explain the individual variation in HT during lactation. It was hypothesised that:

1- The reduction of dietary crude protein (coupled with the supplementation of synthetic AA) will reduce hindgut fermentation and, in consequence, the

internal heat production of lactating sows, improving HT during environmental hyperthermia.

2- The use of flavour enhancers and essential oils will increase feed and water intakes leading to increase sow and the litter's performance under environmental hyperthermia.

3- The high individual variation regarding sow HT is partly related to acquired specific microbiome profiles and/or innate metabolic pathways that enhance the capacity to cope with the metabolic effects of HS during lactation.

2. Experiment 1

All procedures and animals used in Exp 1 were approved by The University of Queensland Animal Ethics Committee (certificate number: 2020/AE000340).

2.1. Methodology

2.1.1. Animals and housing

A total of 16 gestating sows (Large White; 273.25 ± 6.47 kg body weight) between parities 2 and 8 were sourced from the UQ Piggery, The University of Queensland (Gatton campus), and moved to the Queensland Animal Science Precinct (QASP) one week prior to farrowing. Within QASP, sows were randomly allocated to the farrowing pens of the environmentally controlled rooms 143 and 145. Each pen was equipped with a fully slatted plastic floor, a farrowing cage of 2.1 X 2.1, a creep area with a matt floor and two independent nipple drinkers. One of the nipples provided water while the other provided an experimental treatment (water + natural cooling polyols) on demand.

2.1.2. Experimental design

The experiment arrangement is summarized in Table 1. The experimental design consisted of one week of adaptation before farrowing followed by four feeding periods, and three 48-h washout periods between each feeding period. Periods 1 to 4 consisted of 6 days each (3 days in thermoneutral (TN) and 3 days in HS). At any given time, there was one room (8 sows) with a TN environment and one room (8 sows) with a HS environment. During each feeding period sows received 1 of 4 dietary treatments (see section "Diets"). Thus, by the end of the experiment, each lactating sow received all 4 dietary treatments in both climate environments, resulting in 16 replicates for each treatment and temperature program (sows were used as paired controls). During the washout periods, sows were given a mixture of all experimental diets. The washout period of 2 days that followed each feeding period aimed at clearing the gastrointestinal tract and the circulatory system of previous feeds and active compounds, avoiding potential carryover effects. Both rooms were maintained at 20°C during the adaptation period (until farrowing). The farrowing was induced via Lutalyse® injection (1 ml) the day before due date, at 8:00 am and 15:00 pm.

After farrowing, each room started its own temperature program: 3 days of HS followed by 3 days of TN in room 143 and vice-versa in room 145 (Table 1). The HS program consisted of a baseline temperature of 24°C and of 2°C increments every hour starting at 8:00 am until reaching 33°C at 12:30 pm. The maximum temperature was then maintained for 2 hours before being reduced by 2°C every hour until back to the baseline at 24°C. Humidity was maintained at approx. 60% (61.6 ± 0.18 %) in both rooms during the experiment. The TN room maintained a steady environmental temperature of 20°C. The HS-TN/TN-HS cycle was repeated 4 times in each room, thus at any given time there was one room with a TN and one room with a HS environment. Overall, each sow in lactation underwent 4 cycles of HS and 4 cycles of TN conditions and received each dietary treatment once, following a Latin Square Design. This is a complete factorial arrangement of 4 (dietary treatments) x 2 (environmental temperatures) factors that allowed for 16 replicates per treatment per each environmental temperature.

Table 1. Feeding periods (heat stress –HS- or thermoneutral –TN-) followed by washout periods applied in sows within the temperature control rooms 143 and 145 of the Queensland Animal Science Precinct (UQ) during the last week of gestation and subsequent 32 days of lactation.

		Room 143								**Room 145**							
		Sow number															
Period*		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
		Adaptation (TN) Last week gestation/farrowing (batch 1)															
Lactation	1	HS	HS	HS	HS	HS	HS	HS	HS	Adaptation (TN) Last week of gestation/farrowing (batch 2)							
		TN	TN	TN	TN	TN	TN	TN	TN								
		Washout (2 days)															
	2	HS	HS	HS	HS	HS	HS	HS	HS	TN	TN	TN	TN	TN	TN	TN	TN
		TN	TN	TN	TN	TN	TN	TN	TN	HS	HS	HS	HS	HS	HS	HS	HS
		Washout (2 days)															
	3	HS	HS	HS	HS	HS	HS	HS	HS	TN	TN	TN	TN	TN	TN	TN	TN
		TN	TN	TN	TN	TN	TN	TN	TN	HS	HS	HS	HS	HS	HS	HS	HS
		Washout (2 days)															
	4	HS	HS	HS	HS	HS	HS	HS	HS	TN	TN	TN	TN	TN	TN	TN	TN
		TN	TN	TN	TN	TN	TN	TN	TN	HS	HS	HS	HS	HS	HS	HS	HS
		Washout (2 days)															
	5									TN	TN	TN	TN	TN	TN	TN	TN
										HS	HS	HS	HS	HS	HS	HS	HS

* Each feeding period consisted of 3 days of HS followed by 3 days of TN (or vice versa).

2.1.3. Diets

The experimental diets are described in Table 2. Four dietary interventions to promote HT were tested following a factorial design with two main effects: Protein

level (Standard (19%) versus Low (16%)) and umami feed flavour (Y/N) -750 ppm- plus cooling agents (0.005 % menthol and eucalyptus oil) solubilised in water (Y/N). During each feeding period, the 4 dietary treatments (standard protein no flavour (SPNF), standard protein flavour (SPF), low protein no flavour (LPNF) and low protein flavour (LPF)) were allocated to one sow in each room (2 sows/treatment/room). Thus, a total of 16 replicates per treatment were obtained at the end of the experiment. All sow diets were formulated to cover the minimum nutritional requirements based on the NRC (2012).

Table 2. Composition of diets (as fed basis) for Exp. 1.

	SP[1]	LP[2]
Ingredients	%	%
Rolled wheat	65.53	73.39
Rolled barley	10.00	10.06
Canola meal	5.00	-
Soyabean meal	9.35	-
Soyabean full fat	5.40	3.77
Soycomil	-	2.01
Canola oil	1.00	1.51
Dextrose	-	4.02
Limestone	1.10	1.16
Monodicalcium phosphate	1.35	1.66
Salt	0.30	0.30
Choline chloride	-	0.14
Lysine HCL	0.41	0.73
DL-Methionine	-	0.12
Threonine	0.11	0.28
Tryptophan	-	0.05
L-Valine	0.12	0.30
L-Isoleucine	-	0.17
Mycosorb	0.05	0.05
Bentonite	0.08	0.08
Vitamin & mineral premix	0.2	0.2
Calculated energy and nutrients		
Dry matter, %	89.74	90.28
DE[3], MJ/kg	14.00	14.08
Protein, %	19.19	15.68
Lys, %	1.13	1.09
Met, %	0.30	0.35
Trp, %	0.26	0.25
Thr, %	0.76	0.73
Val, %	0.98	0.95
Ile, %	0.73	0.70
A[4]Lys, %	1.00	1.00
AMet, %	0.26	0.31
ATrp, %	0.22	0.22
AThr, %	0.65	0.65
AVal, %	0.85	0.85

AIle, %	0.64	0.64
ALY/DE[4], %/MJ	0.07	0.07
M+C/LYS, g/g	0.59	0.59
Crude Fibre, %	3.00	2.20
Fat, %	3.80	3.90
Total Calcium, %	0.75	0.76
Total Phosphorus, %	0.67	0.66
Calcium/phosphorus, g/g	1.12	1.15
Analysed composition		
Moisture, %	7.64	7.41
Ash, %	5.20	6.44
Crude Protein, %	18.26	16.40
Crude Fat, %	2.64	2.71
Neutral detergent fibre, %	48.03	46.27

[1] standard protein; [2] low protein; [3] digestible energy; [4] available AA (absorbed in the small intestine); [5] Premix provided per kilogram of diet (as-fed basis): Vitamin A, 10000 IU; Vitamin D3, 1800 IU; Vitamin E, 100 IU; Biotin, 0.3 mg; Folic, 2.5 mg; Choline, 1401.49 mg; Vitamin B12, 0.04 mg.

2.1.4. Performance and physiology recordings

Sows' feed intake was recorded every day, whereas their body weight was measured at the start and end of the experiment. In addition, the piglets' average daily gain (ADG) was recorded at the start and end of every feeding period. Physiological parameters including respiration rate (RR), ear (EaT), eye (ET), rectal (RT) and vaginal temperatures (VT) were measured during TN and HS conditions (day 1 and 3 of at approx. 1:00 pm) in every feeding period. The RR was measured by visually counting the flank movements of the sows over a period of 15s and multiplying the value by 4 to obtain the breaths per min. Rectal temperatures were measured via the use of a digital thermometer, whereas VT were recorded every hour via the use of temperature sensors (Thermochron iButton®, RS Components Pty Limited, Australia) loaded in an intravaginal blank CIDR device placed at the entrance of the vagina. However, in this report only the recordings obtained between 12:00 and 1:00 pm (VT) and between 11:00 pm and 12:00 am (VTN) are included. Surface temperatures were taken with a thermal imaging camera (FLIR Systems, USA) pointing at the centre of the eye and the base of the ear in a close distance of approx. 30 cm. Temperatures were recorded by trained personnel (n=4) to minimize variations in the data collection.

2.1.5. Statistical analysis

The effect of day and dietary treatments on sows' performance and physiological parameters was analysed using a linear mixed model in RStudio. The model considered "Period", "Room", "Diet", "Temperature", and the interaction of the last 3 as fixed effects as well as "Sow" and the interaction between "Sow" and "Period" as random effects followed by a Tukey post hoc test for treatment comparison. Similarly, to determine the time effect of environmental hyperthermia on the sow's performance a linear mixed model including "Diet", "Period", "Room"

and “Temperature” and the interaction of the last 3 as fixed effects as well as “Sow” and the interaction between “Sow” and “Period” as random effects was used followed by a Tukey post hoc test for period comparison. The models did not include the factor “cooling agents” as their consumption was close to nil throughout the experiment. The correlation between temperature recordings was performed via Pearson’s correlation. The number of samples (n) refers to the number of sows used. Following animal welfare principles, 2 sows were removed from the trial resulting in a total of n=14 sows used in the statistical analysis. Results were considered statistically significant at $P<0.05$.

2.2. Results

2.2.1. Performance and physiological parameters

2.2.1.1. Temperature effect

The effects of acute (AHS) and chronic heat stress (CHS) on lactating sows’ performance and physiological parameters are described in Figure 2. All body temperatures measured (rectal (RT), vaginal (VT), eye (ET) and ear (EaT)) increased ($P<0.05$) in the sows under HS when compared to TN conditions. Similarly, the RR of the sows increased when comparing TN to AHS or CHS ($P<0.001$). However, in contrast to the body temperatures, the RR decreased between AHS and CHS ($P<0.05$) suggesting an adaptive process in the sows. The effect of HS on sows’ feed intake is shown in Figure 3, A. The average daily feed intake (ADFI) in sows under TN was significantly higher than HS ($P < 0.05$). The effect of HS on the piglets’ growth was not recorded in this experiment as litter weight measurements were performed at the end of every feeding period rather than temperature cycles.

The correlation matrix for temperature recordings of sows under HS is shown in Figure 4. All temperatures measured (RT, VT, VTN, ET and EaT) were positively correlated with one another ($P<0.001$), suggesting that either ET or EaT could be used for a quick and less invasive measurement of body temperature in sows to determine HS with a moderate/reasonable amount of precision ($R^2 = 0.52 – 0.67$).

2.2.1.2. Diet and essential oils effect

The effect of the diet on physiological and performance parameters is illustrated in Figure 2 and 3, respectively. The vaginal temperature (VT) of sows fed the LP diet decreased by day 3 when compared to day 1, but not in the SP group. Feed intake was not significantly altered ($P>0.05$) by the dietary treatments under either TN or HS condition. However, a significant increase ($P<0.05$) in the piglet’s ADG from sows fed diets with the flavour was observed. In addition, a significant interaction ($P<0.01$) between the dietary protein level and the flavour was identified in the piglet’s ADG. Piglets from sows fed the SPNF diet showed significantly lower ($P<0.01$) ADG compared to the other 3 treatments.

The intake of the water treatment with the essential oils (mixture of 0.005% menthol and eucalyptus oil) was negligible, suggesting that the doses tested were above the acceptance threshold for pigs. Additional dose-response studies may need

to be conducted to ascertain the most effective concentration of these cooling agents in lactating sows under HS.

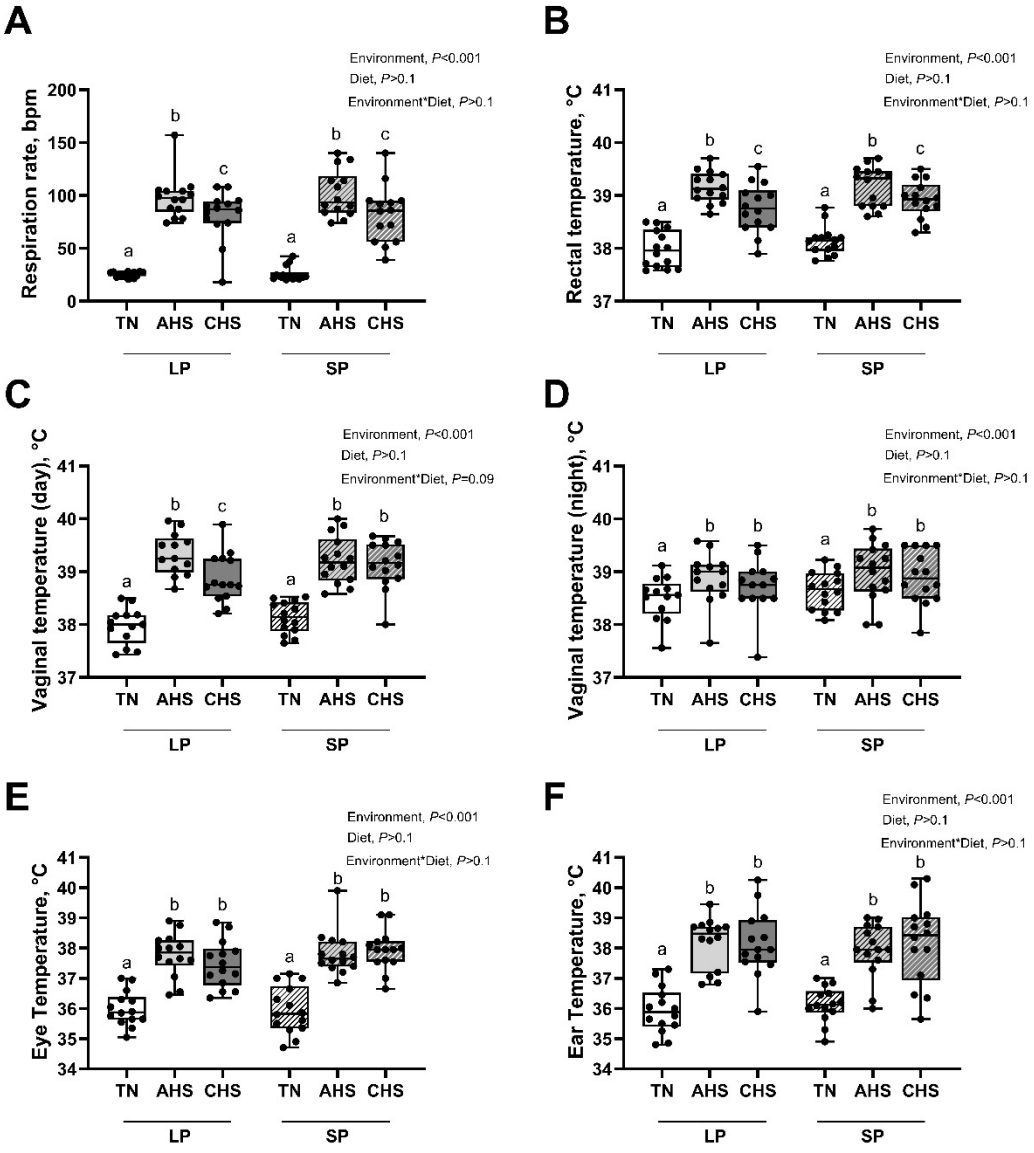


Figure 2. Physiological parameters (respiration rate (A), rectal temperature (B), diurnal (C) and nocturnal vaginal temperature (D)), eye temperature (E) and ear temperature (F)) of lactating sows fed a low (LP) or standard protein diet (SP) under thermoneutral (TN), acute (AHS = 1 day) and chronic heat stress (CHS = 3 days) (environment effect). n=14, each data point within the boxplot represents an individual sow. Different letters (a, b, c) indicate significant differences (P<0.05) within the same treatment. *= P < 0.05, ** = P < 0.01, *** = P < 0.001 across treatments.

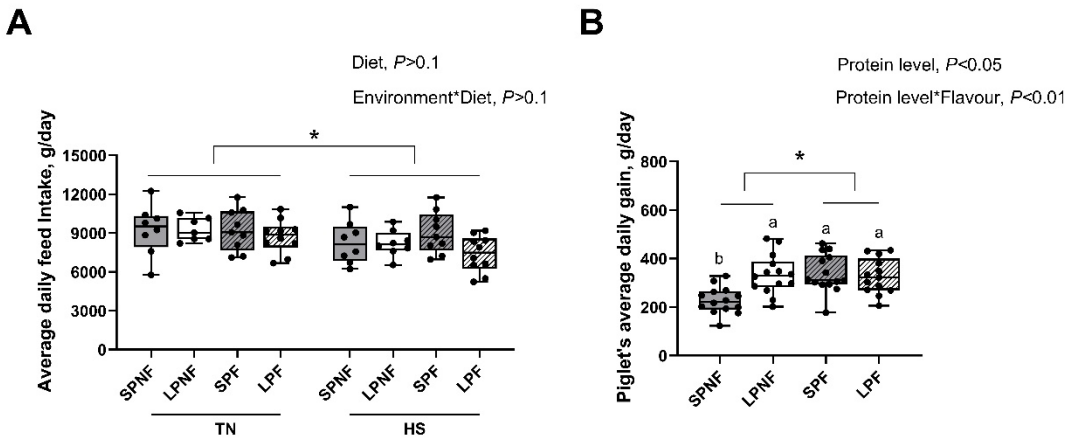


Figure 3. The average daily feed intake of lactating sows (A) and piglets' average daily gain (ADG) (B) when sows were fed a standard (SP) or low (LP) crude protein diet with (F) or without (NF) umami flavour under thermoneutral (TN) and heat stress (HS) conditions. n=14, each data point within the boxplot represents an individual sow. ADG data is the average of the feeding periods (TN + HS). * = P<0.05. Different letters (a, b) indicate significant differences (P<0.05).

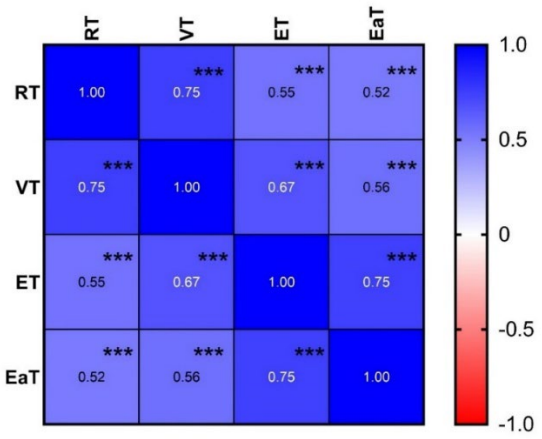


Fig 4: Correlation matrix for temperatures (rectal, vaginal (diurnal), eye and ear) of lactating sows exposed to heat stress. Correlation coefficients (r) are described in each square. *= P<0.05, ** = P<0.01, *** = P<0.001.

2.2.1.3. Period effect

The effect of HS across the 4 experimental periods is illustrated in Fig. 5. Both RT and VT significantly increased (P<0.05) from periods 1 to 2 and then steadily decreased (P<0.01) from period 2 to 4. A similar pattern was also described for VTN and RR. However, significant differences between period 2 (peak temperatures) and period 4 were not found for VTN, while the RR of sows was significantly different across all periods (P<0.05). In contrast, temperature recordings for eyes and ears were not significantly different between periods. In particular, RR and RT were significantly different across periods irrespective of TN or HS conditions, but the changes over time were more pronounced in the later weeks (P<0.05). Due to missing data in Period 1 and 2, the effect of HS on feed intake across periods has been omitted from the results for this experiment.

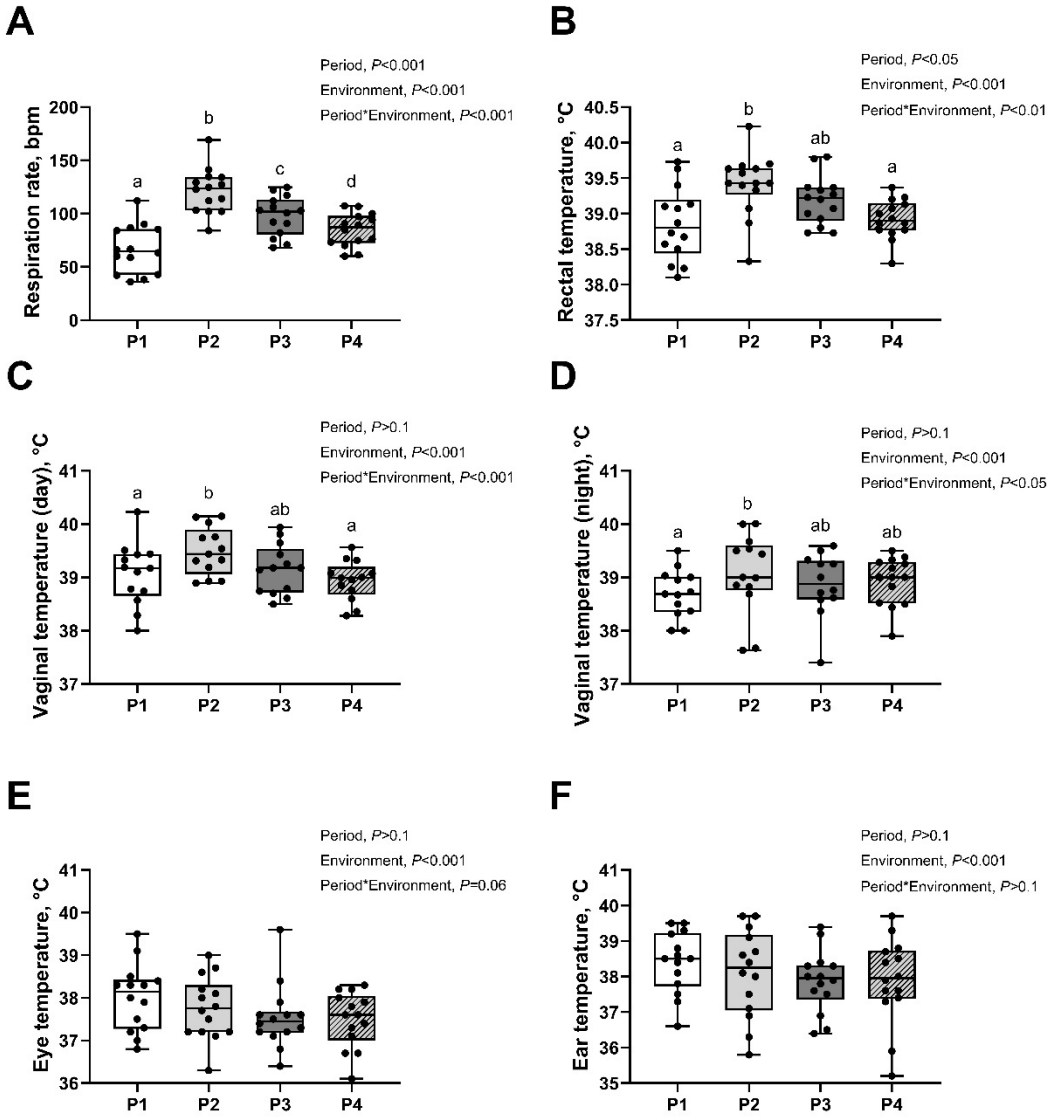


Figure 5. Physiological parameters (respiration rate (A), rectal temperature (B), diurnal (C) and nocturnal vaginal temperature (D)), eye (E) and ear temperatures (F)) of lactating sows under heat stress (environment effect) across 4 consecutive feeding periods. n=14, each data point within the boxplot represents an individual sow. Different letters (a, b, c) indicate significant differences (P<0.05).

3. Experiment 2

All procedures and animals used in Exp 2 were approved by The University of Queensland Animal Ethics Committee (certificate number: 2020/AE000340).

3.1. Methodology

3.1.1. Animals and housing

Fifteen gestating sows (Large White; 308.40 ± 7.84 kg body weight) between parities 2 to 9 were sourced from the UQ Piggery, The University of Queensland (Gatton Campus), and moved to QASP one week prior to farrowing. The rooms and pen conditions used were the same as those previously described in Exp 1, with the only difference that sows had access to only 1 nipple drinker as no multiple water treatments were offered (cooling natural polyols) based on previous results.

3.1.2. Experimental design

The experiment arrangement is the same as that described in Exp 1 (Table 1) with some minor changes. In brief, the HS program in Exp 2 consisted of a baseline temperature of 27°C (rather than 24°C) and 2°C increments every hour starting at 9:30 am until reaching 33°C at 12:30 pm. The maximum temperature was then maintained for 2 hours before being reduced by 2°C every hour until back to baseline at 27°C. These changes in baseline temperatures were implemented to mimic more extreme HS conditions that could ensure an impact not only on feed intake but, also on milk production in sows (Black et al., 1993; Bjerg et al., 2020).

3.1.3. Diets

The dietary formulas used in Exp 2 were the same as in Exp 1 but without the inclusion of flavours and with some minor modifications (Table 3). Two dietary interventions to promote HT (Standard protein (SP) versus Low protein (LP) level) were tested. The 2 dietary treatments were allocated to sows in each room during each feeding period using a Latin Square Design (4 sows/treatment/room). Thus, a total of 30 replicates per treatment were obtained at the end of the experiment. Diets were formulated based on the NRC (2012) nutritional requirements for lactating sows.

Table 3. Composition of diets (as fed basis) for Exp. 2.

	SP[1]	LP[2]
Ingredients	%	%
Rolled wheat	60.81	70.67
Rolled barley	10.00	10.00
Canola meal	5.00	-
Soyabean meal	15.20	-
Soyabean full fat	4.40	6.77
Soycomil	-	2.01
Canola oil	1.00	1.00
Dextrose	-	4.02
Limestone	1.10	1.15
Monodicalcium phosphate	1.35	1.58
Salt	0.30	0.30
Choline chloride	-	0.13
Lysine HCL	0.31	0.72
DL-Methionine	-	0.16
Threonine	0.08	0.29
Tryptophan	-	0.07

L-Valine	0.11	0.34
L-Isoleucine	-	0.20
Mycosorb	-	0.34
Bentonite	-	0.53
Vitamin & mineral premix	0.05	0.05
Calculated energy and nutrients		
Dry matter, %	89.68	90.36
DE[3], MJ/kg	14.00	14.00
Protein, %	19.26	15.21
Lys, %	1.13	1.08
Met, %	0.29	0.37
Trp, %	0.26	0.24
Thr, %	0.76	0.72
Val, %	0.98	0.95
Ile, %	0.76	0.70
A[4]Lys, %	1.00	1.01
AMet, %	0.26	0.32
ATrp, %	0.22	0.22
AThr, %	0.65	0.65
AVal, %	0.85	0.85
AIle, %	0.67	0.65
ALY/DE, %/MJ	0.07	0.07
M+C/LYS, g/g	0.58	0.59
Fat, %	3.81	3.87
Fibre, %	3.11	2.24
Analysed composition		
Moisture, %	7.03	6.85
Ash, %	6.28	7.32
Crude Protein, %	20.57	17.88
Crude Fat, %	3.00	3.48
Neutral detergent fibre, %	47.00	44.93

[1] standard protein; [2] low protein; [3] digestible energy; [4] available AA absorbed from the small intestine.
[5] Premix provided per kilogram of diet (as-fed basis): Vitamin A, 10000 IU; Vitamin D3, 1800 IU; Vitamin E, 100 IU; Biotin, 0.3 mg; Folic, 2.5 mg; Choline, 1401.49 mg; Vitamin B12, 0.04 mg.

3.1.4. Performance and physiology recordings and sample collection

Physiological and performance parameters were recorded as previously detailed in Exp 1. In addition, piglets' ADG was recorded at the start and end of every TN and HS program as to indirectly determine the impact of the environmental temperature on milk production. Sample collection is summarized in Figure 1. Blood and faecal samples were obtained from all sows at TN (day 3 at 1:00 pm) and HS (day 3 at 1:00 pm) conditions in every feeding period following the recording of physiological parameters. Blood was collected from the mammary vein using EDTA vacutainers following Scollo et al. 2019 protocol. Blood samples were centrifuged at 3,000 g for 10 min at 4°C within 1 hour of collection and immediately stored at -80°C until metabolomics analysis. To minimize the stress of the sows during the procedure, a topic anaesthetic ointment was applied before puncture (Emla skin cream (5%

lidocaine) was applied 10 min before blood collection). Faecal samples (approx. 1g) were collected as soon as deposited by the sow and stored at -80°C until microbiome analysis. If no fresh faecal samples were available in the pen at the time of collection, 2 fingers were carefully inserted in the rectum to collect faecal samples close to the anus of the sow. At the end of the experiment, liver samples (1 g approx. from each lobe) were collected from the slaughtered sows at the abattoir (Highchester, Jimbomba) using scalpels and scissors, placed immediately in liquid nitrogen for transport to the UQ, St Lucia Campus, and stored at -80°C until proteomics analysis.

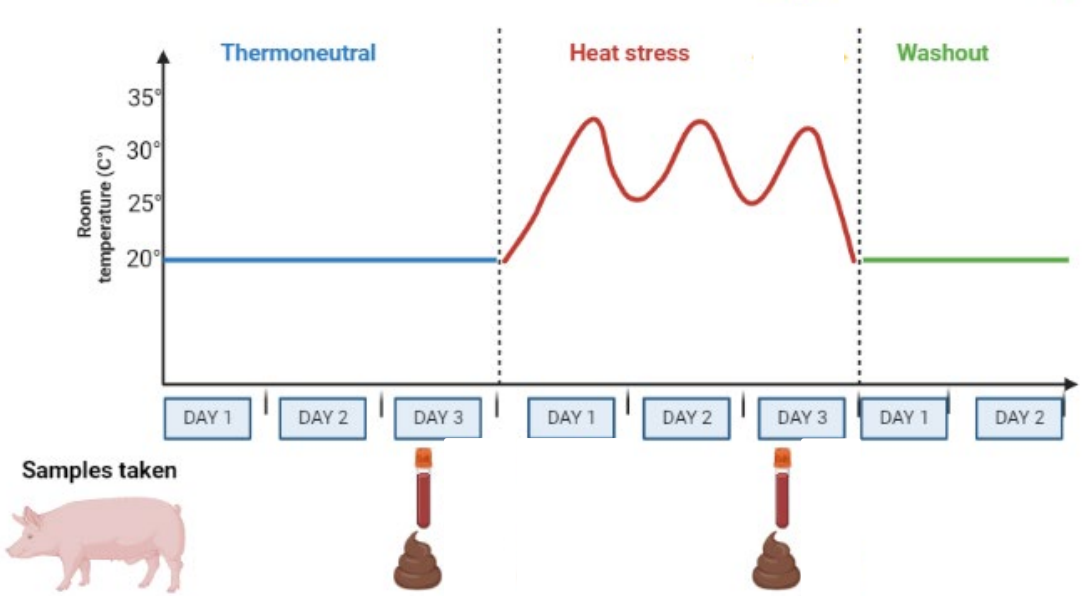


Figure 1: Diagram summarizing the thermal program applied and samples collected from lactating sows. Sows were exposed to 4 feeding periods (heat stress (HS) or thermoneutral (TN) room ambient), and three 48-h washout periods between each feeding period. Feeding periods consisted of 6 days each (3 in TN and 3 in HS). The HS and TN conditions were alternated between 2 rooms so that at any given time there was one group of sows under TN and the other group under HS. Sample collection for the analysis of biomarkers of interest, including blood and faeces, was performed on day 3 of TN and HS (around 1:00 pm) in every feeding period. Figure produced using BioRender (Ontario, Canada)

3.1.5. Omics: sample preparation and mass spectrometry

Plasma and liver samples (mixture of all 5 lobes) were prepared following the FASP method for the proteome analysis. In short, samples were denatured by Guanidine (6M, 50 mM Tris-HCl, pH=8.5) (liver samples homogenized first with Guanidine prior to denaturation), reduced by Dithiothreitol (40 mM), alkylated (20 mM Acrylamide) and cleaned by using Molecular Weight Cut Off tubes (30kDa) to remove detergents via centrifugation at 14,000g for 40 min. Trypsin in pH = 7.0 Ammonium Acetate buffer was applied overnight for the digestion of large proteins. Digested samples were then desalted by ZipTip before LC-MS analysis. The obtained peptides were analysed by Zeno time-of-flight (ZenoTOF) in SWATH mode followed by data-independent acquisition neural network (DIA-NN) for library free identification

(searching against the porcine proteome; UniProt, Proteome UP000008227) and quantified by MSstats for differential protein characterization.

For the metabolome analysis, plasma samples were mixed with an 80% methanol solution and stored at 4°C for 20 min before being centrifuged at 14,000g for 10 min at 4°C to allow for protein precipitation. The supernatant was then transferred into Eppendorf tubes and completely evaporated using a vacuum centrifuge at 14,000 *g* for 30 min (at room temperature). Metabolites were resuspended by adding 80% methanol solution before LC-MS analysis. The eluted molecules from the LC separation were analysed by time-of-flight Mass Spectrometry (TOF-MS) in SWATH mode and then the metabolites identified by referring to HMDB (https://hmdb.ca/), METLIN (https://metlin.scripps.edu/), and MassBank (https://massbank.eu/MassBank/) databases using the MS-DIAL software (5.1 version). The differential metabolites were identified by matching formula, retention times (Rt), accurate mass spectral data with the above databases and reported references.

3.1.6. Microbiome analysis

Bacterial DNA extraction of the sows' faecal samples was performed using the QIAamp PowerFecal Pro DNA kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Full-length sequencing of the 16S rRNA gene was conducted by the Australian Genome Research Facility using the PacBio platform. The resulting HiFi (highly accurate long reads) reads were processed with the Nextflow workflow. Data were imported and analysed using Quantitative Insights Into Microbial Ecology 2 (QIIME2). Each sample was processed with the Divisive Amplicon Denoising Algorithm 2 (DADA2) workflow in QIIME2, applying an expected error threshold of 2, a sequence length range of 1000-1600 bp and the pseudo-pooling method. Only features with a minimum frequency of 5 across all samples were retained. Taxonomy was assigned using a Naïve-Bayesian classifier with the Silva (138.1), RefSeq (16S_6-11-20) and GTDB (r207, bac120, arc53, ssu) databases, applying a minimum confidence level of 0.8. The feature table was rarefied to a sequencing depth of 5700 reads per sample in QIIME2 for downstream analysis. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) was employed to predict the MetaCyc metabolic pathways.

3.1.7. Statistical analysis

The effect of day and dietary treatments on sows' performance and physiological parameters was analysed using a linear mixed model in RStudio. The model considered "Period, "Room", "Diet", "Temperature", and the interaction of the last 3 as fixed effects as well as "Sow" and the interaction between "Sow" and "Period" as random effects followed by a Tukey post hoc test for treatment comparison. Similarly, to determine the time effect of environmental hyperthermia on the sow's performance a linear mixed model including "Diet", "Period", "Room" and "Temperature" and the interaction of the last 3 as fixed effects as well as "Sow" and the interaction between "Sow" and "Period" as random effects was used followed by a Tukey post hoc test for period comparison. The correlation between temperature recordings was performed via Pearson's correlation. The number of

samples (n) refers to the number of pigs used. Following animal welfare principles, 4 sows needed to be removed from the trial resulting in a total of n=11 sows being used (included in the statistical analysis). Results were considered statistically significant at $P<0.05$.

Proteome differential analysis was performed using the DIANN-PeakView-MSstats pipeline. False discovery rate analysis using DIANN was performed on all searches with limits of 99 % identification confidence and 1 % local false discovery rate. The search results were used as ion libraries for SWATH analyses. Peptide abundance was measured using PeakView software with standard settings, summing the integrated areas of up to six fragment ions per peptide. Protein abundance was measured using the sum of the abundances of up to six peptides per protein. Normalization was performed to the total protein abundance in each sample. Statistical analyses for SWATH proteomics were performed using MSstats in R.

Metaboanalyst 6.0 was applied for the metabolome data analysis. Data normalization was performed using "Normalization by sum", "Log transformation" and "Pareto scaling" before proceeding with the multivariate statistical analysis involving partial least squares discriminant analysis (PLS-DA), cross-validation test, volcano plot and hierarchical clustering dendrogram analysis (HCA). Metabolites with a variable importance (VIP) value > 1.0 and P-value < 0.05 in the PLS-DA were considered as potential biomarkers for distinguishing different groups (HS vs. TN and SP vs. LP diet under HS). Metabolites identified as potential biomarkers (VIP> 1.0 and P-value < 0.05) were then run through the same mixed model used for performance and physiological parameters to account for the complexity of the experimental design.

PERmutational Multivariate Analysis of Variance (PERMANOVA) was used to determine the impact of individual sows, diet and environment on faecal microbial community composition. Alpha diversity indices of the microbial communities were analyzed using the same linear mixed model applied for performance followed by a Tukey post hoc test. Sow-adjusted Principal Coordinates Analysis (aPCoA) was performed to visualize ß-diversity across groups, and differences in ordination scores were tested with the Wilcoxon test. Linear discriminant analysis Effect Size (LEfSe) was utilised to identify significant microbial features and metabolic pathways between groups, selecting those with an LDA score greater than 2. Spearman's correlation was applied to examine relationships between differential microbial features and physiological or performance metrics, with multiple p-values adjusted using the false discovery rate (FDR) method. Diversity analyses were performed in Quantitative Insights Into Microbial Ecology 2 (QIIME2), while all other analyses were conducted in RStudio. Results were considered statistically significant at an $FDR<0.05$, unless otherwise stated.

3.2. Results

3.2.1. Performance and physiological parameters

3.2.1.1. Temperature effect

The impact of AHS and CHS on the performance and physiological parameters of lactating sows is shown in Figure 6. Environmental hyperthermia significantly reduced ADFI ($P<0.05$) and increased body temperatures ($P<0.001$) and RR ($P<0.001$) in sows when compared to TN conditions. In addition, piglets tended ($P=0.060$) to grow less (ADG) in HS compared to TN, indicating that sows under environmental hyperthermia most likely experienced a drop in milk yield.

The correlation matrix for temperature recordings of sows under HS is illustrated in Figure 7. Like Experiment 1, all temperatures measured (RT, VT, VTN, ET and EaT) were positively correlated with one another ($P<0.001$), suggesting that either EaT or ET could be used to accurately determine HS in lactating sows. However, EaT was identified as the parameter with the lowest correlation with the internal body temperatures (RT and VT).

3.2.1.2. Diet effect

The ADFI of LP sows during CHS was not significantly different ($P>0.05$) than TN, as opposed to the SP group. In addition, sows fed the LP diet showed reduced RT when compared to the SP treatment across all environmental conditions ($P<0.01$). No differences between the diets were observed for the other parameters.

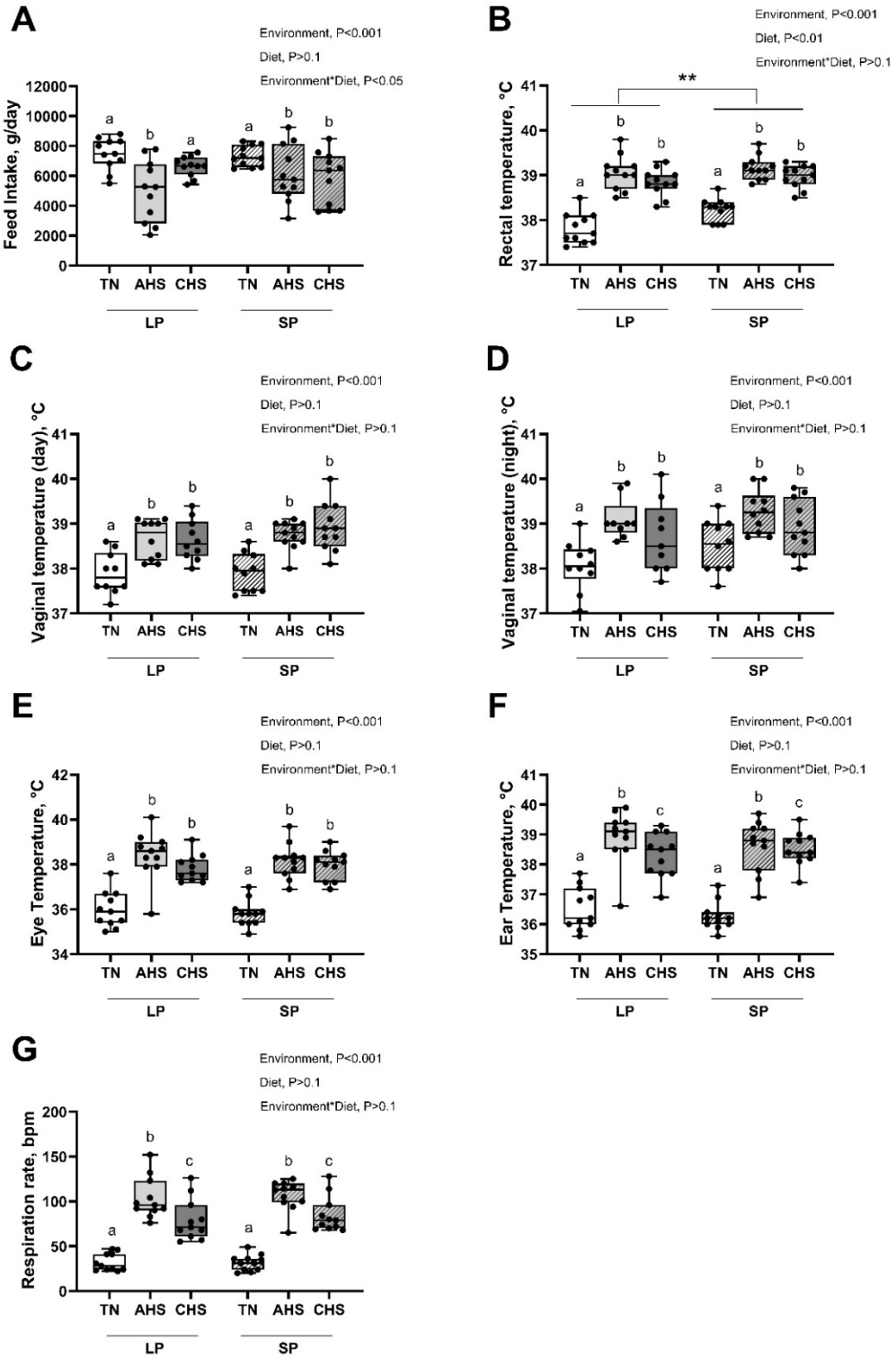


Figure 6. Physiological parameters (respiration rate (A), rectal temperature (B), diurnal (C) and nocturnal vaginal temperature (D)), eye temperature (E) and ear temperature (F)) and feed intake of lactating sows fed a low (LP) or standard protein (SP) diet under thermoneutral (TN), acute (AHS = 1 day) and chronic heat stress (CHS = 3 days) conditions (environment effect). n=11, each data point within the boxplot represents an individual sow. Different letters (a, b, c) indicate significant differences (P<0.05) within the same dietary treatment. *= P<0.05, ** = P<0.01, *** = P<0.001 across dietary treatments.

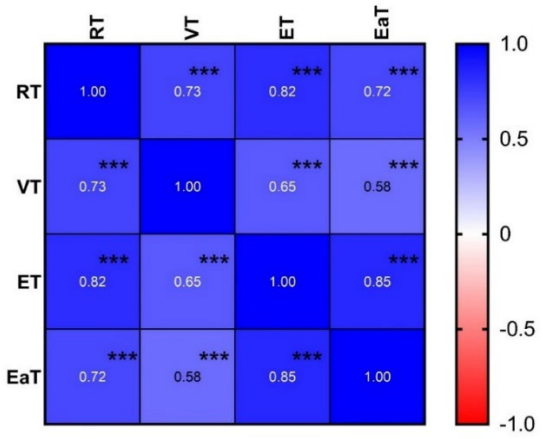


Figure 7: Correlation matrix for temperatures (rectal, vaginal (diurnal), eye and ear) of lactating sows exposed to heat stress. Correlation coefficients (r) are described in each square. *= P<0.05, ** = P<0.01, *** = P<0.001.

3.2.1.3. Period effect

The effect of period on the sows' performance and physiological parameters is illustrated in Figure 8. Sows' feed intake increased from periods 1 to 4 under HS and TN conditions. The sows' ADFI was significantly different between period 1 and 3 (P>0.05) as well as periods 1 and 4 (P<0.01). In contrast, most physiological parameters (RT, VT, RR and EaT) significantly decreased (P<0.05) with time (from period 1 to 4). For RT and VT, significant differences (P<0.05) were observed when comparing Period 1 to 3 and 4, whereas changes (P<0.01) in RR and EaT were observed starting from periods 4 and 2, respectively. The sows' VTN was the only parameter that did not show significant changes across periods. In particular, RR (P<0.01) and RT (P=0.08) reduction over time (differences between periods) was more pronounced in HS than thermoneutral conditions.

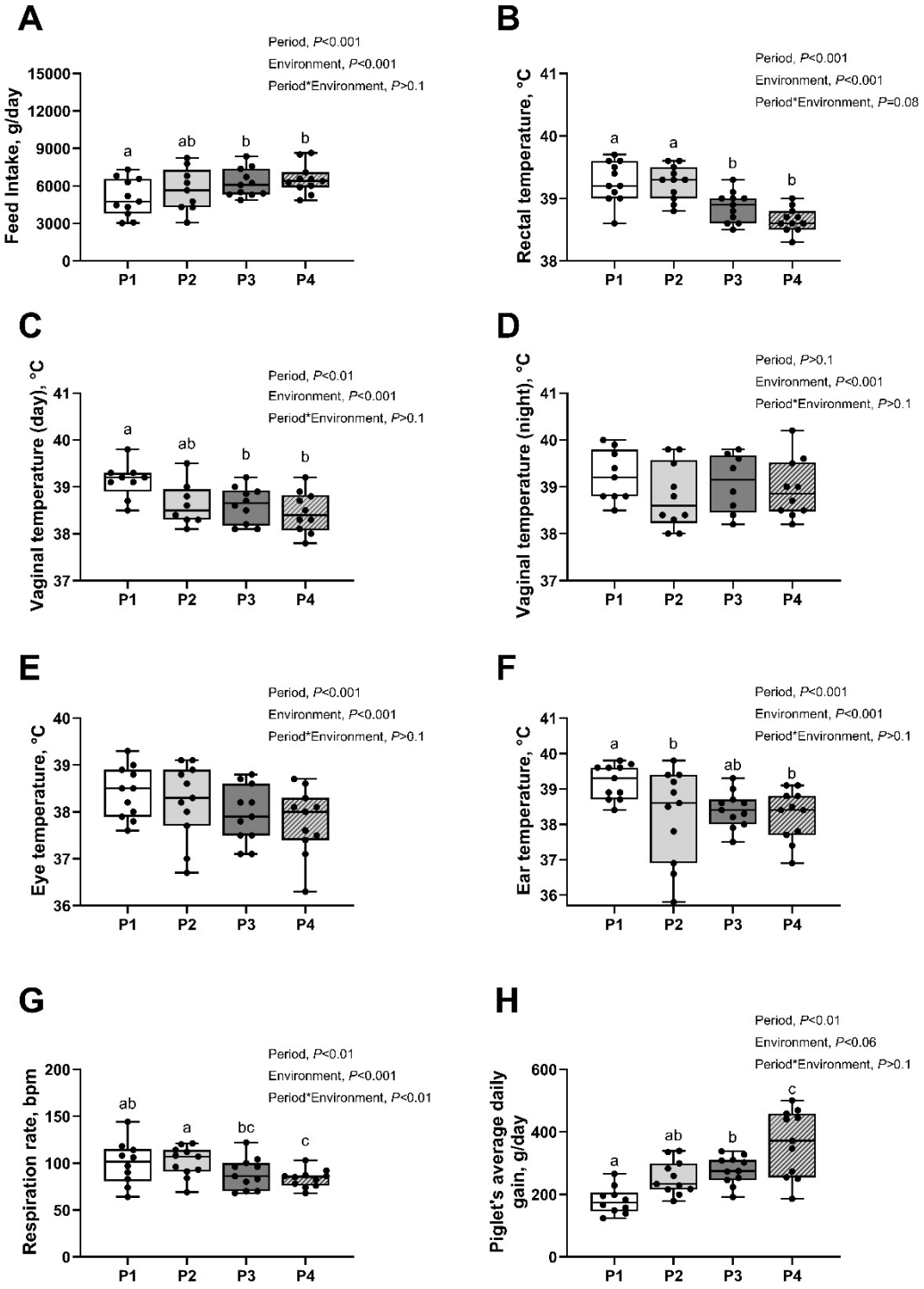


Figure 8: Physiological parameters (respiration rate (A), rectal temperature (B), diurnal (C) and nocturnal vaginal temperature (D)), eye temperature (E) and ear temperature (F)) and feed intake of lactating sows under heat stress (environment effect) across 4 consecutive feeding periods. n=11, each data point within the boxplot represents an individual sow. Different letters (a, b, c) indicate significant differences (P<0.05).

3.2.2. Plasma and liver proteome

The PLS-DA score plot did not show clear differences in the plasma proteome of lactating sows exposed to HS or TN conditions (Figure 9). Similarly, no clear clusters in the plasma proteome were identified when comparing dietary treatments (SP vs. LP diet) under HS (Figure 10). A more in-depth analysis on the top VIP proteins identified via the use of the mixed model applied for the performance and physiological parameters showed 7 proteins to be differentially abundant ($P<0.05$) between environmental conditions. Six of the proteins were related to immune response and inflammation (granulin precursor, complement C8 gamma chain, macrophage stimulating 1 and Ig-like domain-containing proteins) and 1 to carbohydrate and ATP metabolism (maltase-glucoamylase). These results suggest that the plasma proteome is not a good indicator of metabolic changes in lactating sows under cyclic HS.

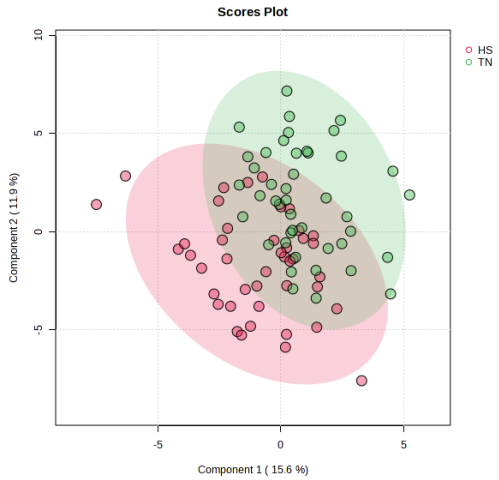


Figure 9. PLS-DA score plot model of lactating sows' plasma proteome during thermoneutral (TN) and heat stress (HS) conditions across 4 feeding periods. Each data point within the models represents an individual sow.

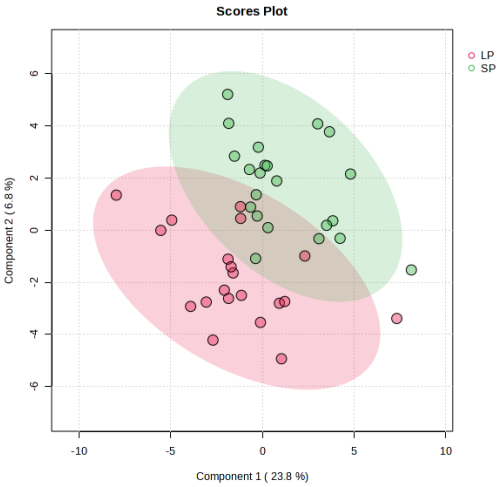
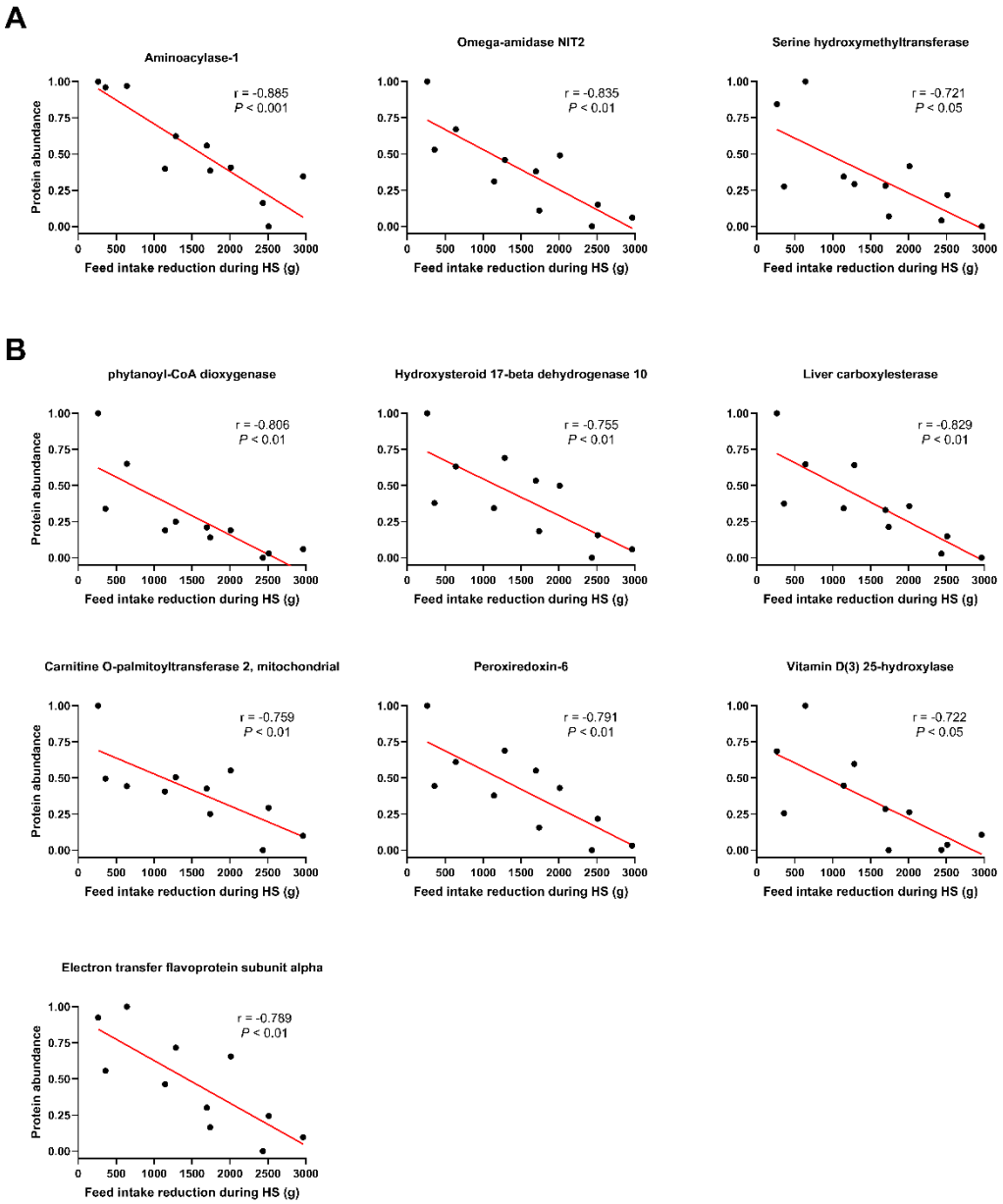


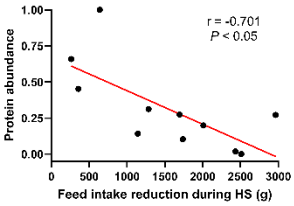
Figure 10. PLS-DA score plot model of lactating sows' plasma proteome fed a standard (SP) vs low protein (LP) diet under heat stress conditions across 2 feeding periods. Each data point within the models represents an individual sow.

The abundance of 92 liver proteins were discovered to be dependent on feed intake levels in sows under cyclic HS following PLS regression analysis. The proteins that were identified to have a strong correlation with feed intake reduction (r>-0.7, P<0.05) during HS are shown in Figure 11. Three proteins were involved in AA metabolism, 7 in lipid metabolism, 4 in ATP/carbohydrate metabolism, 4 in stress/immune response, 3 in cytoskeleton organization and 2 in programmed cell death.

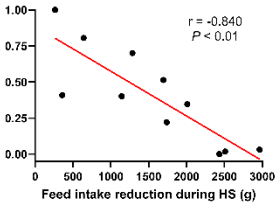


C

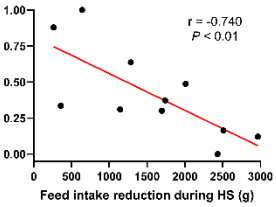
NADH dehydrogenase 1 alpha subcomplex subunit 5



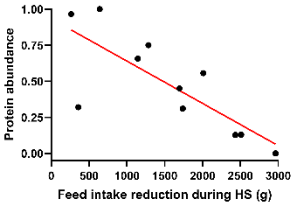
Succinate dehydrogenase iron-sulfur subunit, mitochondrial



Isocitrate dehydrogenase [NADP]

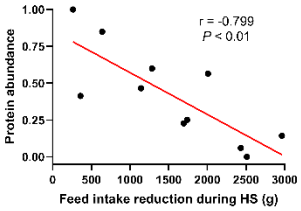


DLST of 2-oxoglutarate dehydrogenase complex, mitochondrial

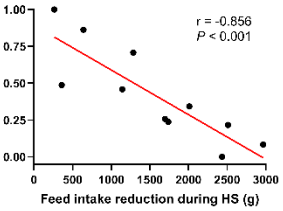


D

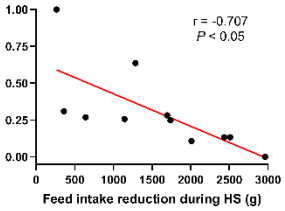
IgG heavy chain



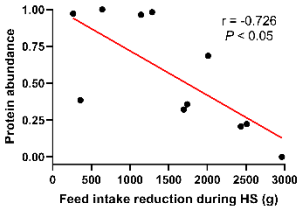
Complement C3



Ig-like domain-containing protein

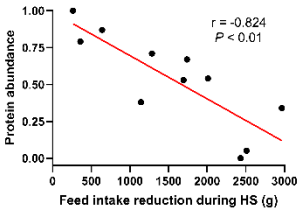


Alpha-1-acid glycoprotein

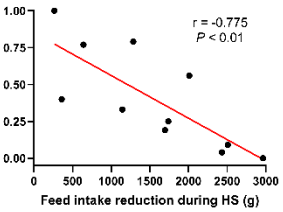


E

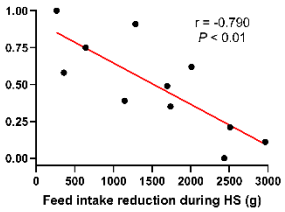
Coronin



Plectin isoform 1a



Profilin



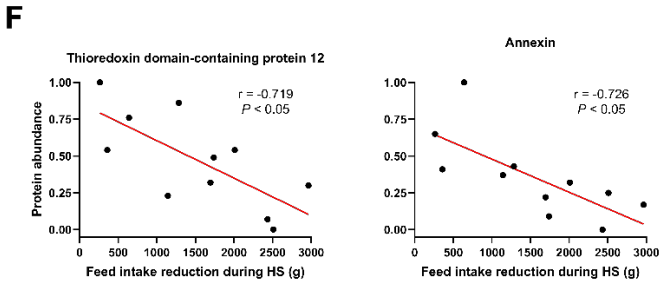


Figure 11. Correlation of liver proteins abundance (normalized) related to AA metabolism (A), lipid metabolism (B), ATP/carbohydrate metabolism (C), immune response (D), cytoskeleton organization (E) and cell death (F) with levels of feed intake reduction (difference between HS and TN feed intake) in sows under cyclic heat stress. n=11, each data point within the boxplot represents an individual sow. Significant differences at P<0.05.

3.2.3. Plasma metabolome

The PLS-DA score plot did not show apparent differences in the plasma metabolome of lactating sows when comparing environmental conditions (Figure 12). Nonetheless, 2 clusters were observed when comparing dietary treatments (SP vs. LP diet) under HS and TN (Figure 13). The initial analysis (volcano plot) showed 12 plasma metabolites that were differentially abundant, of which 9 and 3 were found to be up and downregulated, respectively, when comparing the LP to the SP treatment under HS (Figure 14, A). Similarly, the comparison between LP and SP under TN conditions illustrated 5 plasma metabolites that were differentially abundant, of which 1 and 4 were shown to be up and downregulated (Figure 14, B). To account for the complexity of the experimental design (sows exposed to different periods, environmental temperatures and diets), a mixed model (same as the one applied for performance and physiological parameters) was then used on the metabolites identified with a VIP>1 from the PLS regression analysis. The mixed model revealed 11 and 19 metabolites with P<0.05 when comparing diets in summer and winter, respectively (Table 5 and 6). The metabolites identified as differently abundant between diets were mainly related to AA metabolism (lower abundance in SP) in both HS and TN.

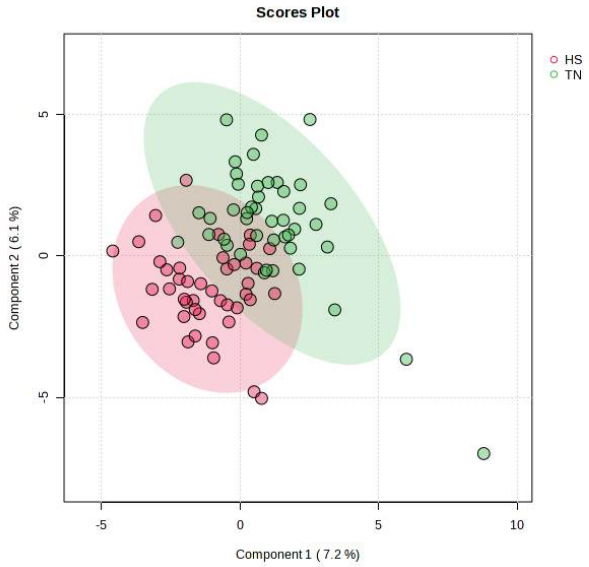


Figure 12. PLS-DA score plot model of lactating sows' plasma metabolome under heat stress (HS) and thermoneutral (TN) conditions. Each data point within the models represents an individual sow.

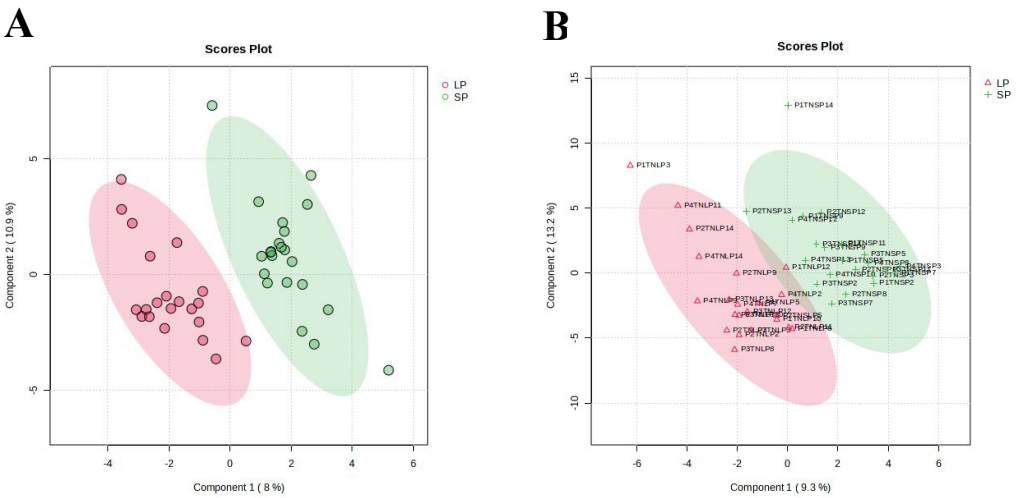


Figure 13. PLS-DA score plot models of lactating sows' plasma metabolome fed a standard (SP) vs low protein (LP) diet under heat stress (A) and thermoneutral conditions (B). Each data point within the models represents an individual sow.

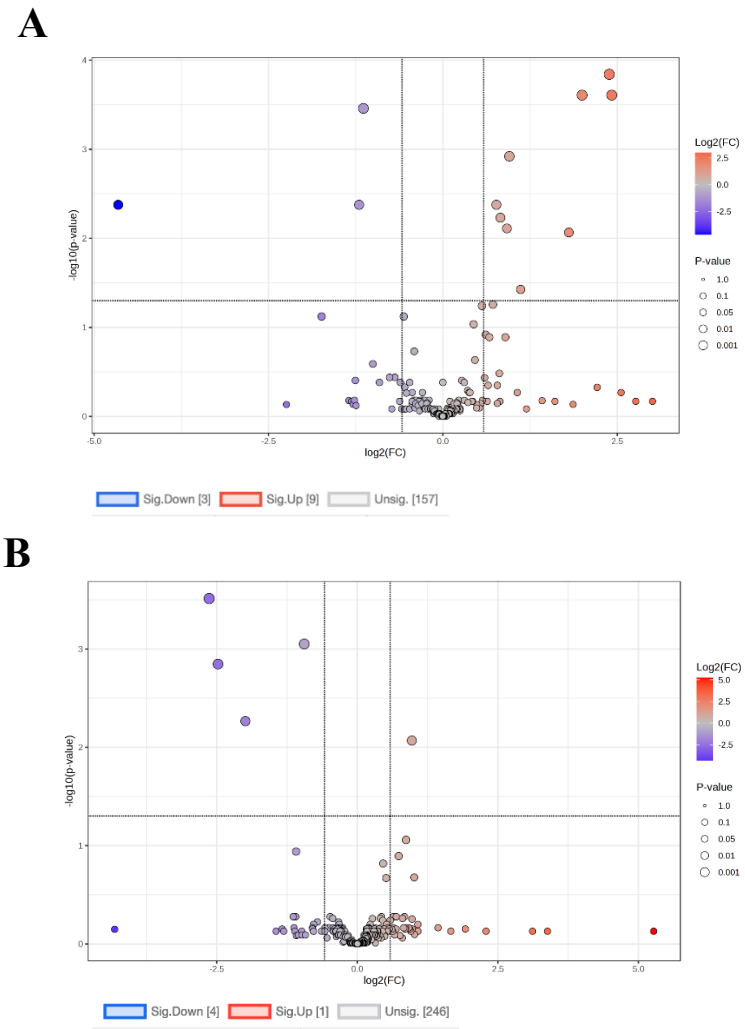


Figure 14. Volcano plots of the plasma metabolites differentially abundant in lactating sows fed a standard (SP) vs low protein (LP) diet under heat stress (A) and TN conditions (B). Log10 normalized for each metabolite was represented by different colors: high (red), low (blue), or average (white).

Table 5. Differentially abundant plasma metabolites identified between low and standard protein diets in lactating sows under heat stress.

Compound name	Sub Class	log2fold^	*P* value
Hydroxyphenyl lactate_3_4	lactic acids	0.95061	<0.001
Indolepropionic_acid_3	propionic acids	0.48495	0.074
Catechol	catechols	1.8989	<0.001
Kaurenic_acid	diterpenes	-0.01978	0.070
Phenylalanine	alpha amino acids	0.74827	<0.001
L_5_Oxoproline	amino acid derivatives	0.65028	0.010
Nicotinamide	nicotinic acids	-1.2296	0.050
Pantothenate	pantothenic acids	0.78543	<0.001
Salicylic_acid	benzoic acids	0.96059	<0.001
Tryptophan	alpha amino acids	1.0099	0.040
Deoxycholic_acid	bile acids, alcohols and derivatives	0.5186	0.099

^degree of change in the relative abundance of plasma metabolites when comparing a low to a standard protein diet in heat stress (negative and positive numbers show down and upregulation, respectively). Significant difference at P<0.05.

Table 6. Differentially abundant plasma metabolites identified between low and standard protein diets in lactating sows under thermoneutral.

Compound name	Sub Class	log2fold^	*P* value
4_Aminoisoxazolidin_3_one	oxazolidines	-0.26016	0.015
3_Hydroxy puerarin	isoflavonoids	-1.9905	0.001
Hydroxyphenyl lactate_3_4	lactic acids	-0.35873	0.024
butylparaben	benzoic acids	0.45789	0.007
Catechol	catechols	-1.097	0.025
Cyclohexanamine	aliphatic amines	0.73537	0.015
Hippuric acid	hippuric acids	-0.31178	0.003
Indoline	indoles (aromatic heterocycles)	-0.33346	0.004
Isatin	indoles (aromatic heterocycles)	0.82224	0.034
L_Phenylalanine	alpha amino acids	-0.43698	0.005
L_Arginine	alpha amino acids	0.51101	0.012
L_Histidine	alpha amino acids	0.40783	0.014
L_Methionine	alpha amino acids	0.86541	<0.001
L_Tyrosine	alpha amino acids	-0.94548	<0.001
N_Methylisoleucine	alpha amino acids	0.96879	<0.001
Nicotinamide	nicotinic acids	-0.37494	0.049
Glycohyodeoxycholic acid	bile acids, alcohols and derivatives	-0.71054	0.017
0_Hydroxyhippuric acid	hippuric acids	-1.0904	<0.001
Suberosin	coumaric acids and derivatives	-0.29696	0.006

^degree of change in the relative abundance of plasma metabolites when comparing a low to a standard protein diet in thermoneutral (negative and positive numbers show down and upregulation, respectively). Significant difference at P<0.05.

3.2.4. Microbiome

The fecal microbiota composition (phylum and genus level) of lactating sows exposed to cyclic HS is illustrated in Figure 15. The most abundant bacteria belonged to the *Lactobacillus*, *Terrisporobacter*, *Romboutsia*, *Clostridium* and *Limosilactobacillu* genera. In addition, differences (P<0.05) in the alpha (within sample) diversity indices were observed between environmental conditions (HS vs. TN) and feeding periods (Period 2 vs. 3). Specifically, the HS group and period 3 exhibited higher phylogenetic diversity (i.e., microbial richness and evolutionary complexity) than their TN counterpart. Moreover, individual animals were found to have a significant influence on microbial communities, impacting alpha and beta (between samples) diversity, accounting for approx. 41% of the variation. Following adjustment for the "sow" effect, using unweighted UniFrac distance, differences (P<0.05) in the microbial communities were identified for the diet, period and environment effects. However, such differences were not detected with the weighted UniFrac method that accounts for microbial abundance, suggesting that the differences in microbial communities observed were associated with low abundant features (abundance below 1%).

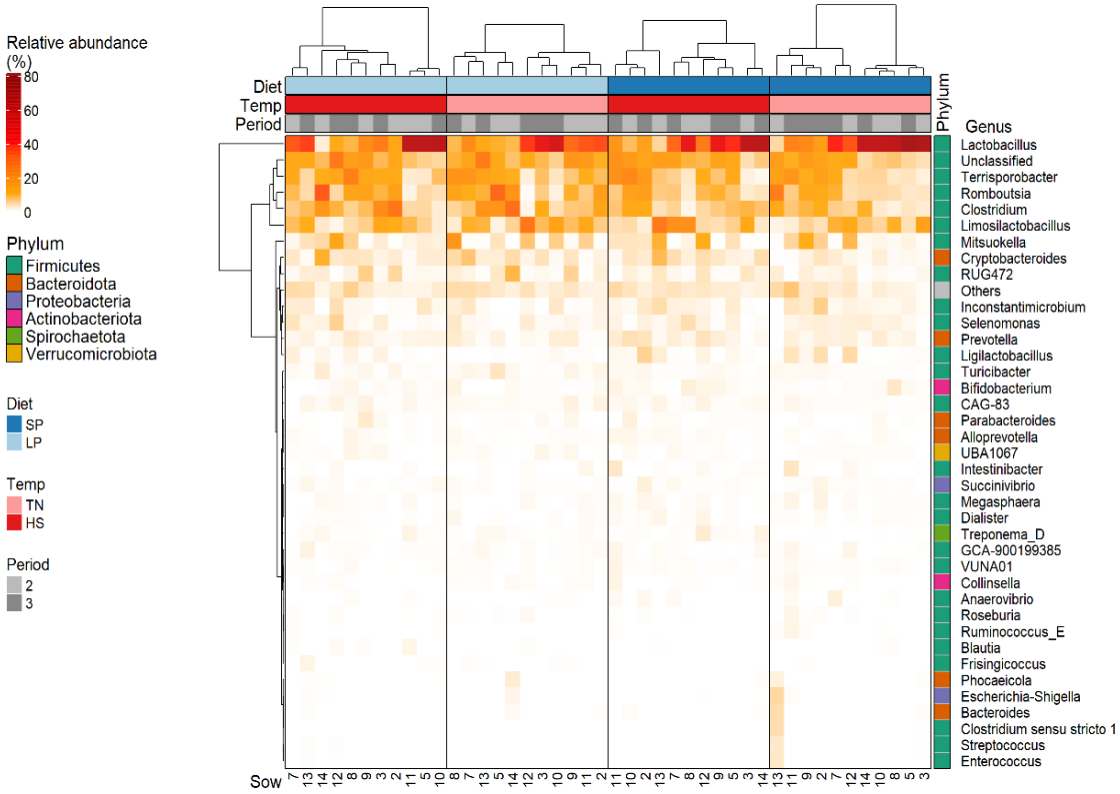


Figure 15. Relative abundances of fecal microbiota at the genus level in lactating sows across different temperatures (thermoneutral (TN) vs. heat stress (HS)), diets (low (LP) vs. standard protein (SP)), and lactating periods (Period 2 and 3). The genera included were those with a relative abundance of above 1%.

Using Linear discriminant analysis Effect Size (LEfSe), 56, 51 and 30 microbial features were identified as significantly different (P<0.05; LDA score >2) between lactating periods, diets, and environmental temperatures, respectively. Among these identified features, only 15 were correlated (FDR<0.1) with physiological or performance metrics, including ADFI, RR and body temperatures (Figure 16). In particular, 5 features belonging to Anaerovoracaceae, Muribaculaceae, *Treponema*, *Cryptobacteroides*, and Bacteroidales showed a strong positive correlation with body temperatures. In addition, five metabolic pathways in the microbiome exhibited significant differences (LDA>2) between diets, including pathways involved in vitamin B_{12} metabolism, while only one distinct pathway (LDA>2) was identified for temperatures and period related to TCA cycle and heme synthesis, respectively (Figure 17).

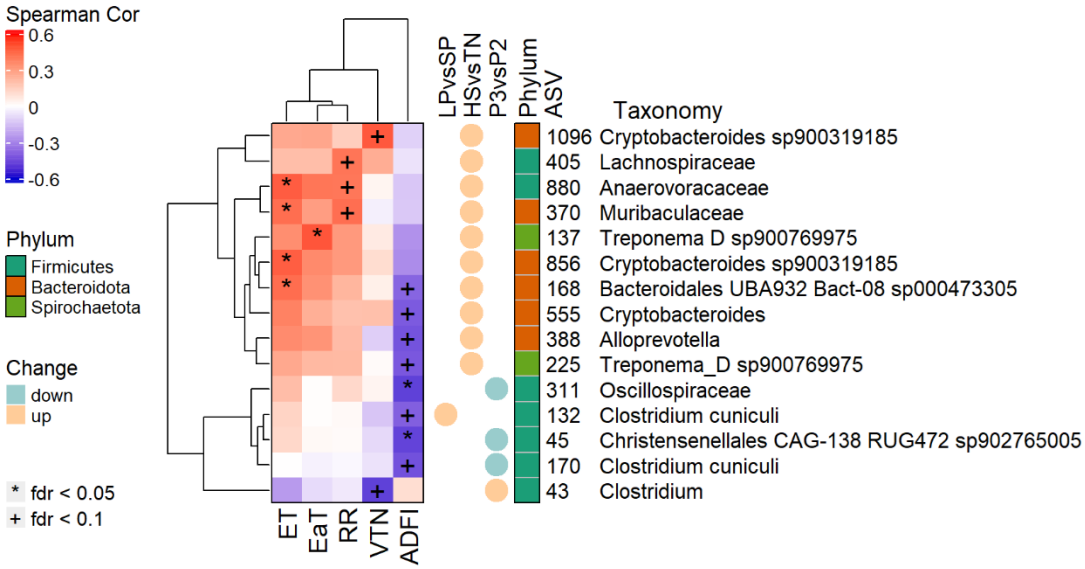


Figure 16. Heatmap depicting the correlations between identified differential microbial features and physiological/performance parameters with an FDR<0.1. Colored circles indicate the factors (diets (low (LP) vs. standard protein (SP), environment (heat stress (HS) vs. thermoneutral (TN)) or feeding periods (Period 3 (P3) vs. 2 (P2)) in which the microbial features showed differences. Green circles represent microbial features that were downregulated in the first group compared to the second group, while orange circles indicate upregulation.

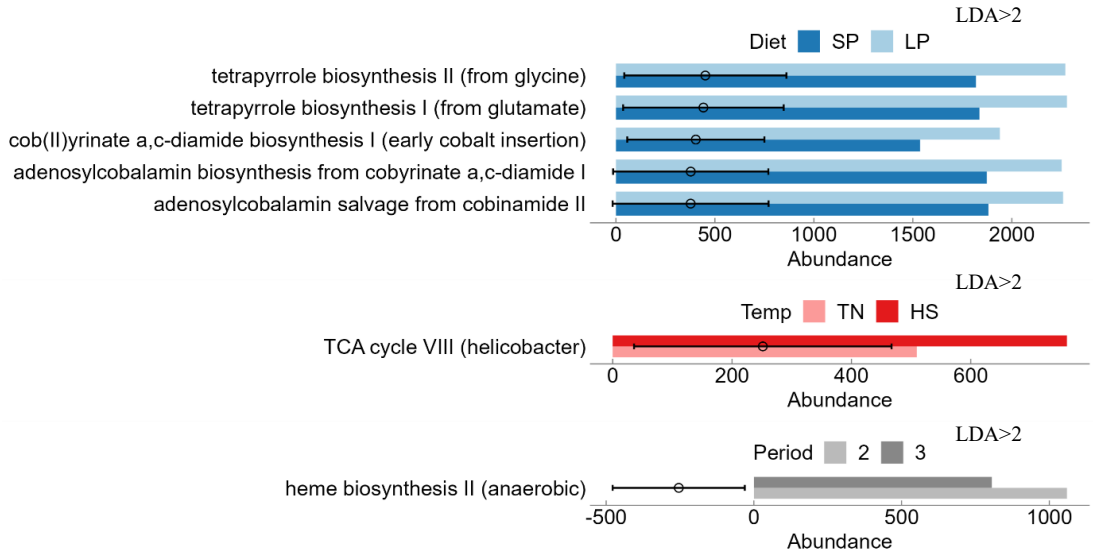


Figure 17. Differential metabolic pathways identified between diets (low (LP) vs. standard protein (SP)), environmental conditions (thermoneutral (TN) vs. heat stress (HS)) and feeding periods (Period 2 (P2) vs. 3 (P3)) in the microbiome of lactating sows, analyzed using Linear discriminant analysis Effect Size (LefSe, LDA>2.0). Bars indicate the pathway abundance in each group. The empty black circles represent the mean difference between groups, while the black lines denote the 95% confidence interval of the difference.

4. Experiment 3

All procedures and animals used in Exp 3 were approved by the Rivalea (Australia) Pty Ltd Animal Ethics Committee (certificate number: 23-026).

4.1. Methodology

A commercial study was conducted in the gestating and lactating facilities of JBS Pork Australia Pty Ltd (Corowa, NSW, Australia) during winter/early spring (August to October 2023) and the following summer (November 2023 to March 2024).

4.1.1. Winter phase

4.1.1.1. Animals, housing and diet

This batch of sows was selected primarily to evaluate physiological and metabolic changes across seasons, serving as a control group for the HS/summer conditions.
A group of multiparous Large White X Landrace sows (n=56) between parity 2 and 3 were selected based on weight, previous reproductive (no previous medication during lactation and the number of weaned pigs during previous lactations) and lactating performance (e.g., no agalactia, etc.) as well as their proximity to their farrowing date. Sows were individually housed in farrowing stalls starting approx. 1 week prior to their farrowing date. The selected sows were randomly assigned to 1 of 2 dietary treatments (as mash feed, restricted feeding during pre-farrowing and *ad libitum* access during lactation): a standard commercial lactation diet (14.8 MJ

DE/kg, 16.5% CP) or low crude protein diet (14.8 MJ DE/kg, 13.5% CP). Diets were formulated to be iso-caloric, have similar levels of crude fibre and essential AA, but contain different concentrations of CP (Table 4). All formulations cover the nutritional requirements of lactating sows based on the NRC (2012). Sows' feed intake (disappearance) during gestation and lactation, body weight and P2 back fat at the entry to the farrowing house and at the end of lactation were measured. Moreover, the number of days to remate, number of piglets born alive, mummies, still born, total born, birth weights, number of piglets weaned, litter weight at weaning and mortalities during lactation were recorded.

4.1.1.2. Parameters recording and sampling

Temperature and humidity in the farrowing house were recorded every hour using an automatic logger for the duration of the experiment. In addition, the sows' RT were measured weekly (at the same day and time every week: 12:00-2:00 pm) during lactation. At the peak of lactation (day 14 to 18 post-farrowing) blood samples (~2 ml) were collected. Samples and measurements taken during winter were used as control (to compare with the summer trial). Details regarding the procedures performed for sampling and the recording of physiological parameters are described in detail in the "Summer phase" section below.

4.1.2. Summer phase

4.1.2.1. Animals, housing and diet

A total of 400 multiparous, between parity 2 and 4, Large White X Landrace sows (from 5 batches of approx. 80 sows each, inclusive of the winter phase sows) were used in the experiment. Sows were individually housed in farrowing stalls starting approx. 1 week prior to their farrowing date. Sows were randomly assigned to 1 of 2 dietary treatments (same treatments described in the "winter trial") during the pre-farrowing and lactation period (Table 4). Temperature and humidity in the farrowing house were recorded every hour using an automatic logger during the experiment.

Table 4. Composition of diets (as fed basis) for Exp. 3.

	SP[1] Winter	LP[2] Winter	SP Summer	LP Summer
Ingredients	%	%	%	%
Rolled wheat	49.48	48.23	47.94	50.32
Barley	13.33	20.00	13.33	15
Millmix	10.00	14.83	10.00	15
Canola meal	10.00	-	11.00	-
Soybean meal	3.00	-	3.00	1.83
Full fat soy	-	2.5	-	2.5
Meat meal	4.33	-	5.30	-
Blood meal	-	1.00	-	1.00
Soycomil	-	2.5	-	2.5
Fish oil (semi refined)	0.40	0.40	0.40	0.4
Tallow mixer	5.67	5.5	5.67	5.67
Betaine	0.1	0.1	0.4	0.4
Salt	0.33	0.33	0.33	0.33
Limestone	1.03	1.13	1.00	1.47

Magnesium sulphate	0.4	0.4	0.4	0.4
Dicalcium phosphate	-	0.67	-	-
Monocalcium phosphate	-	-	-	0.60
Krave AP flavour	0.03	0.03	0.03	0.03
Phytase (Quantum Blue 5G)	0.02	0.02	0.02	0.02
Lysine	0.33	0.47	0.29	0.43
DL-Methionine	-	0.13	-	0.11
Tryptophan	0.01	0.04	0.02	0.03
Threonine	0.04	0.17	0.04	0.15
Valine	-	0.02	-	0.02
Rivalea mineral blend[5]	0.20	0.20	0.20	0.20
Pig reproduction blend	0.05	0.05	0.05	0.05
Endox	0.02	0.02	0.02	0.02
Lysoforte booster	0.05	0.05	0.05	0.05
Presan	0.10	0.10	0.10	0.10
Vitamin blend[5]	0.12	0.12	0.12	0.12
Arbocel	0.83	0.83	0.83	0.83
Revelate	0.01	0.01	0.01	0.01
Fysal MP	0.10	0.10	0.10	0.10
Colour-grits (red/blue)	0.10	0.10	0.10	0.10
Calculated energy and nutrients				
Dry matter, %	9.69	9.81	9.91	10.20
DE[3], MJ/Kg	14.80	14.84	14.70	14.73
Protein, %	16.52	13.88	16.51	13.59
Lys, %	0.95	0.95	0.95	0.95
Met, %	0.28	0.35	0.30	0.35
Trp, %	0.24	0.24	0.25	0.24
Thr, %	0.65	0.67	0.67	0.66
Val, %	0.77	0.71	0.78	0.71
ALys[4], %	0.81	0.85	0.81	0.85
AMet, %	0.24	0.29	0.25	0.31
ATrp, %	0.21	0.21	0.20	0.20
AThr, %	0.54	0.55	0.51	0.55
AVal, %	0.58	0.58	0.60	0.58
ALY/DE, %/MJ	0.055	0.058	0.055	0.058
M+C/LYS, g/g	0.61	0.62	0.66	0.65
Fat, %	8.05	7.82	8.16	7.95
Fibre, %	4.16	3.66	3.76	3.6
Ash, %	5.25	4.97	5.55	5.35
Analysed composition				
Moisture, %	8.48	8.96	7.78	7.77
Ash, %	4.01	4.37	4.49	4.18
Crude protein, %	16.74	14.06	16.40	14.31
Crude fat, %	8.59	8.35	7.84	7.37
Neutral detergent fibre, %	9.68	11.35	9.84	12.34

[1] standard protein; [2] low protein; [3] digestible energy; [4] available lysine; [5] Provided per kilogram of diet (as-fed basis): Copper, 8 ppm; manganese, 30 ppm; zinc, 60 ppm; iron, 80 ppm; iodine, 2 ppm; selenium, 0.3 ppm; chromium, 400 ppm; Vitamin A, 15000 IU; Vitamin D3, 3300 IU; Vitamin E, 90 IU; Vitamin K, 1 mg; Vitamin B1, 1.5 mg; Vitamin B2, 5 mg; Vitamin B6, 3 mg; Vitamin B12, 0.01 mg; Niacin, 30 mg; Pantothenic acid, 15 mg; Biotin, 0.3 mg; Folic, 5 mg; Vitamin C, 100 mg.

4.1.2.2 Performance and reproductive measurements

Feed intake (disappearance), body weight (at start and end of trial/weaning), P2 back fat (at start and end of trial/weaning), days to remate after weaning, number of piglets born alive, mummies, still-born, total born, birth weights, number of

piglets weaned, litter weight at weaning and mortalities during lactation (average length of 27.20 + 0.17 days) were recorded in all sows.

4.1.2.3. Physiology recordings

Sow temperatures were recorded 3 times a week (every Monday, Wednesday and Friday) between 12:00 and 2:00 pm in the sows followed from the winter trial (n=41) The RR of the sows (number of breaths per min) was determined by visually counting the flank movements of the sows during the same days/time as the temperatures. Skin temperatures were measured via the use of temperature record pointers. Measurements were taken at approximately 30 cm away from the target (eye or ear). The users ensured that the eye or the back of the ear was in the middle of the camera/image before taking the picture. Rectal temperatures of the animals were determined by placing a digital thermometer in the rectum. To minimize variability in measurements, no more than 3 personnel recorded the physiological parameters.

4.1.2.4. Blood sampling

Blood samples were collected from the jugular vein of the winter sows where possible (n=41). The procedure was performed during the peak of lactation (day 14 to 28), between 12:00 and 2:00 pm, after recording the physiological parameters. Animal were restrained with a snare and securely contained before sample collection. The blood was retrieved using EDTA vacutainers centrifugated at 3000 rpm for 10 min at 4°C. Plasma samples (stored at -20°C in Rivalea) were then transported in dry ice to UQ, St Lucia Campus, for metabolomics analysis.

4.1.2.5. Piglet management

All the routinary procedures performed at the farm were followed throughout the experiment (e.g., vaccination, iron injection, docking of tails, etc.). Any adverse event (e.g., diarrhea, scours, meningitis, etc.) and antibiotic treatment provided were recorded as well as fostering events within dietary treatments. Minimal fostering was practiced.

4.1.2.6. Metabolomics: sample preparation and mass spectrometry

Procedure for sample preparation and data processing were performed as previously described in section 2.2.5. In brief, plasma samples were mixed with an 80% methanol solution, stored at -20°C and centrifuged (14,000 *g* at 4°C for 10 min) to allow for protein precipitation. The supernatant was then collected, transferred into new Eppendorf tubes and evaporated using a vacuum centrifuge (14,000 *g* at room temperature for 30 min). The dry pellet (metabolites) was then resuspended using an 80% methanol solution before LC-MS analysis. Metabolites were analyzed using TOF-MS in SWATH mode and then identified by referring to HMDB (https://hmdb.ca/), METLIN (https://metlin.scripps.edu/), and MassBank (https://massbank.eu/MassBank/) databases in MS-DIAL.

4.1.2.7. Statistical analysis

The effect of the "season" and the diet on sows' performance and physiological parameters was analysed using a linear mixed model in RStudio. The model considered "Season", "Diet", and their interaction as fixed effects and "Replicate" and "Sow" as random effects, when considering the subgroup of animals followed from winter to summer (n=41). Tukey post hoc test was used when contrasting interactions. When comparing the diet effect within season (summer, n=400; winter, n=56) the "Season" and "Sow" effects were dropped from the model. For the sows' performance parameters, the "Initial weight" (weight recorded during gestation/entrance to the farrowing shed) was considered as a co-variate in the mixed model. To compare the piglet's performance (i.e., weight gain) the same model described above was used with the inclusion of the piglet's birth weight as co-variate. For the physiological parameters that were collected (in the subgroup of animals followed from winter, n=41) only during the summer (i.e., RR, ET and EaT), the "Season" and "Sow" effects were dropped from the model.

The analysis of the metabolomics data was performed as described in section 2.2.7. In short, Metaboanalyst 6.0 (https://www.metaboanalyst.ca/) was used to normalize the data acquired from the LC-MS and performed a multivariate analysis involving PLSDA, Cross-validation test, volcano plot and HCA. Metabolites with variable importance (VIP) values > 1.0 and P-value < 0.05 were considered as potential biomarkers for distinguishing different groups (summer (S) vs. winter (W) season and SP vs LP diet in summer or winter). Cross-validation tests were performed on the corresponding PLSDA mode to confirm the quality of the model. The application of a mixed model was not needed in this experiment as the multivariate statistical model allow for a clear distinction of groups/clusters. Metabolite enrichment results found with a P-value < 0.1, following HCA are included in this report.

4.2. Results

4.2.1. Performance, reproductive and physiological parameters

The performance and reproductive parameters of sows during summer (n=400) and winter (n=56) are described in Table 7. No significant differences were observed in the summer or winter cohort for performance and reproductive parameters when comparing diets. The results described in the sections below correspond to the subgroup of sows that were successfully followed from winter to summer (n=41), which performance, reproductive and physiological parameters were evaluated in both seasons.

Table 7. Performance of lactating sows and their litter during summer (n=400) and winter (n=56).

	Dietary Treatment Summer				Dietary Treatment Winter			
Parameters	SP[1]	LP[2]	SEM[3]	*P*-value	SP	LP	SEM	*P*-value
Initial^ weight, kg	273.30	275.35	3.19	0.404	262.35	253.90	6.97	0.201
Initial P2 backfat, mm	17.90	17.80	0.85	0.932	18.04	17.82	0.86	0.751
Weight at weaning, kg	247.12	246.52	1.18	0.328	235.71	233.90	3.98	0.654
P2 backfat at weaning, mm	17.80	17.30	0.65	0.270	16.51	16.96	0.69	0.480
Estimated weight loss, kg	-27.60	-28.70	1.12	0.428	-21.44	-23.25	3.98	0.653
P2 backfat change, mm	-0.174	-0.442	1.01	0.409	-1.75	-0.71	0.73	0.176
ADFI[4], kg	5.82	5.80	0.15	0.805	7.60	7.95	0.38	0.115
Weaning-remate interval, d	4.43	4.33	0.11	0.272	4.50	5.05	0.40	0.306
Piglets born alive	13.90	13.70	0.22	0.492	13.00	13.61	0.60	0.387
Stillbirths	0.89	0.86	0.08	0.761	0.92	0.48	0.20	0.119
Mummified piglets	0.26	0.25	0.04	0.935	0.35	0.39	0.14	0.804
Total piglets born	15.20	15.10	0.26	0.712	14.27	14.64	0.70	0.615
Litter's mortality rate, %	15.90	15.60	0.97	0.772	16.05	19.43	2.75	0.373
Piglets' birth weight, kg	1.50	1.50	0.02	0.988	1.45	1.37	0.05	0.209
Piglets' weaning weight, kg	7.43	7.35	0.21	0.472	8.37	8.45	0.35	0.803
Piglets' ADG[5], kg	0.25	0.24	0.01	0.123	0.25	0.26	0.01	0.420
Piglets weaned	10.53	11.00	0.48	0.469	10.62	10.22	0.32	0.362

[1] standard protein diet; [2] low protein diet; [3] standard error of the mean; [4] average daily feed intake; [5] average daily weight gain; ^parameters measured at the entrance to the farrowing shed.

4.2.1.1 Season effect

The average shed temperature in winter and summer was 20.2°C (peak temperature of 22.8°C) and 25.9°C (peak temperature of 29.3°C), respectively, indicating that temperatures were not as high during the summer as compared to previous years (for more details refer to Table 8). Nonetheless, an effect of season on the sows' performance was still observed. Sows lost more P2 backfat ($P<0.01$) in winter than in summer (Figure 18). In addition, feed intake during lactation was reduced ($P<0.001$) in summer when compared to winter (Figure 19). Moreover, sows' RT was increased ($P<0.01$) during the first 2 weeks at the farrowing house in summer compared to winter, but not in subsequent weeks (3 to 5)) (Figure 20). The total number of piglets born ($P=0.073$) and litter number ($P<0.001$) in summer were higher than that of winter, likely related to their higher parity number and weight during the summer trial (Figure 21). However, the opposite (winter>summer) was observed for the piglets' weight at weaning ($P<0.01$). Surface temperatures and RT were moderately correlated ($P<0.05$) in all weeks during the summer season, except for eye and rectal measurements on week 2 (Figure 22).

Table 8. Weekly temperatures and relative humidity at Rivalea's farrowing sheds during Experiment 3.

	Week 1	Week 2	Week 3	Week 4	Week 5
Winter Season					
Temperature, ˚C	19.4±0.2^	19.9±0.3	20.6±0.4	20.5±0.3	18.8±0.6
Relative humidity, %	50.0±2.0	46.9±0.7	44.0±3.3	38.0±1.4	45.4±3.5
Summer Season					
Temperature, ˚C	25.8±0.3	26.7±0.2	26.0±0.4	25.1±0.4	26.3±0.6
Relative Humidity, %	29.1±1.8	32.2±1.9	34.7±2.7	35.8±1.2	32.9±1.9

^Standard error of the mean.

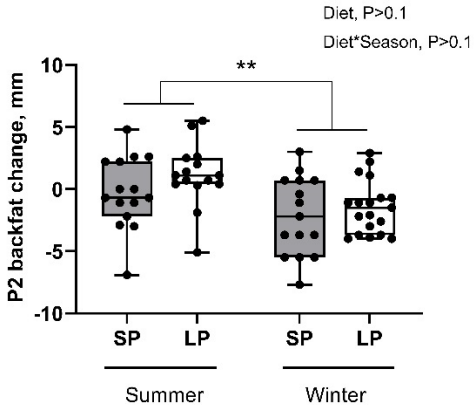


Figure 18. P2 backfat loss during winter and summer season in lactating sows fed a standard (SP, n=18) or low protein (LP, n=23) diet. Each data point within the boxplot represents an individual sow. Significant differences at P<0.05, ** = P<0.01.

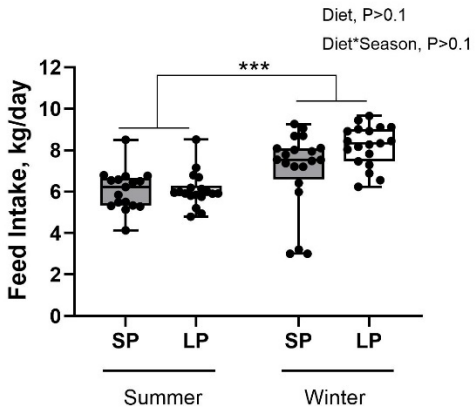


Figure 19. Lactation feed intake during winter and summer seasons in sows fed a standard (SP, n=18) or low protein (LP, n=23) diet. Each data point within the boxplot represents an individual sow. *= P<0.05, ** = P<0.01, *** = P<0.001.

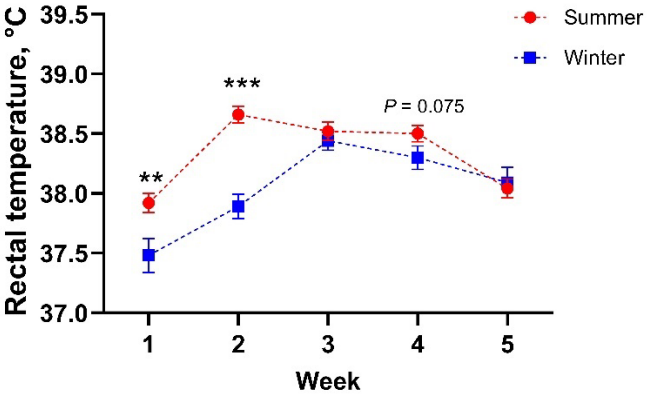


Figure 20. Winter and summer rectal temperatures of sows during late gestation (week 1) and lactation (week 2 to 5). *= P<0.05, ** = P<0.01, *** = P<0.001.

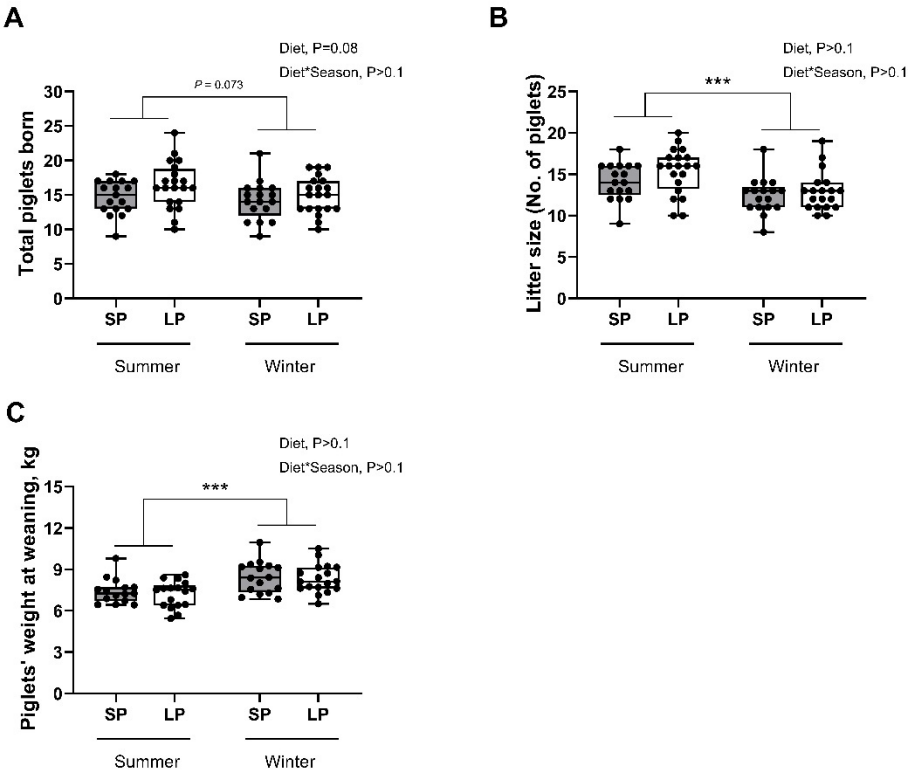


Figure 21. Piglets' performance (total born (including stillbirths and mummies) (A), litter size (B) and piglets' weight at weaning (C)) during winter and summer seasons from lactating sows fed a standard (SP, n=18) or low protein (LP, n=23) diet. n=23. Each data point within the boxplot represents an individual sow. *= P<0.05, ** = P<0.01, *** = P<0.001.

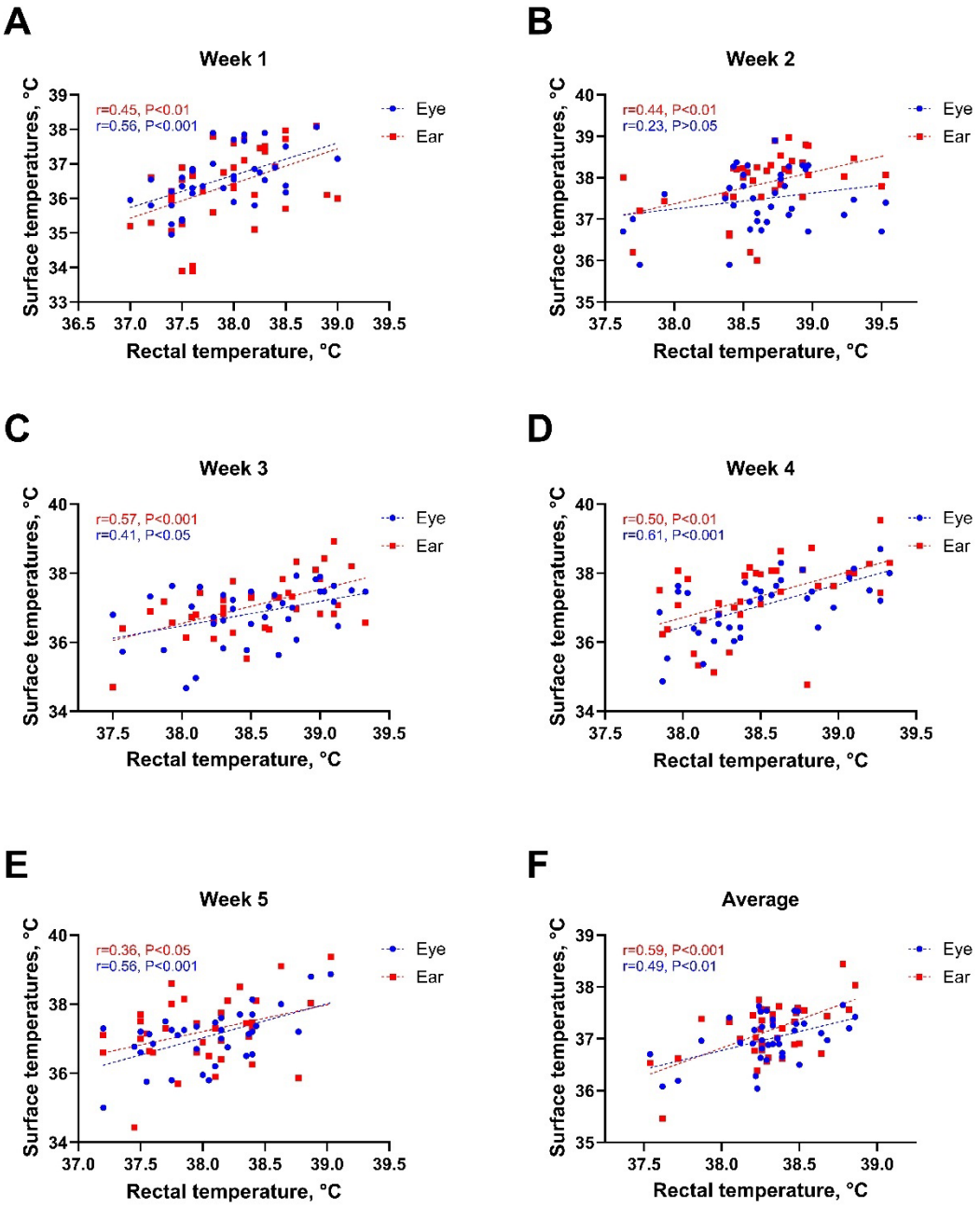


Figure 22. Correlation of surface (eye and ear) and rectal temperatures during summer in sows from the last week of gestation (week 1) to weaning (week 5). n=41, each data point within the boxplot represents an individual sow. *= P<0.05, ** = P<0.01, *** = P<0.001.

4.2.1.2. Diet effect

Sows fed the LP diet had lower eye (P<0.01) and ear (P=0.073) temperatures during the second week of summer at the farrowing house as compared to the SP group (Figure 23, A and B). Moreover, sows fed the LP diet showed higher and lower RR during week 2 (P=0.054) and 3 (P<0.01) during the summer trial, respectively, in comparison to the SP sows (Figure 23, C). Although RT were consistently lower in

LP compared to SP sows across all weeks when considering both summer and winter, these differences were only numerical when comparing the treatments in week 1 to 4. Nonetheless, the LP diet significantly (P<0.05) reduced RT in week 5 (Figure 23, D). Moreover, the average RT of the sows across the 5 weeks at the farrowing house was significantly lower in the LP vs. the SP diet (P<0.05) when considering both the summer and winter trials (Figure 24). In addition, sows fed the LP diet had piglets with lower average birth weight (P<0.05) when compared to the SP diet (Figure 25, A). However, no dietary differences were observed for weights at weaning (Figure 25, B). It is important to mention that the lactation length of the LP group was higher than the SP treatment (P<0.05, approx. 1 day longer), which could have contributed to the lack of differences observed in the piglets' weaning weight between diets.

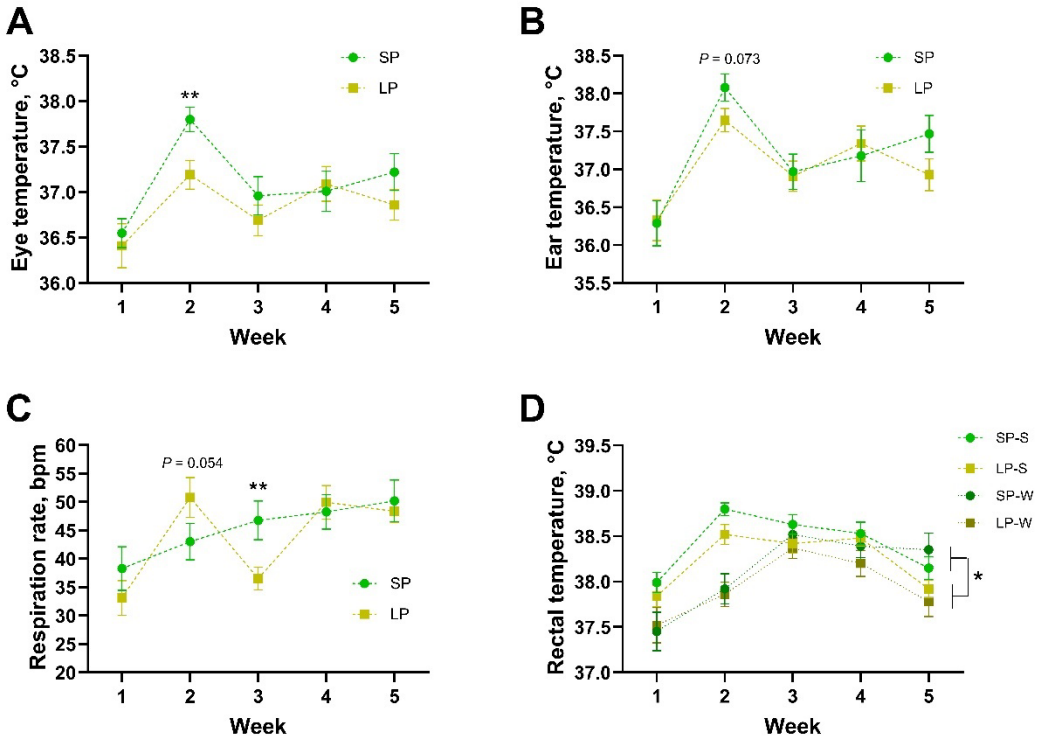


Figure 23. Physiological parameters (eye temperature (A), ear temperature (B), respiration rate (C) and rectal temperature (D)) of sows during late gestation (week 1) and lactation (week 2 to 5) fed a standard (SP, n=18) or low protein diet (LP, n=23). All physiological parameters were recorded during summer except for rectal temperatures, which were recorded during summer (S) and winter (W). * = P<0.05, ** = P<0.01.

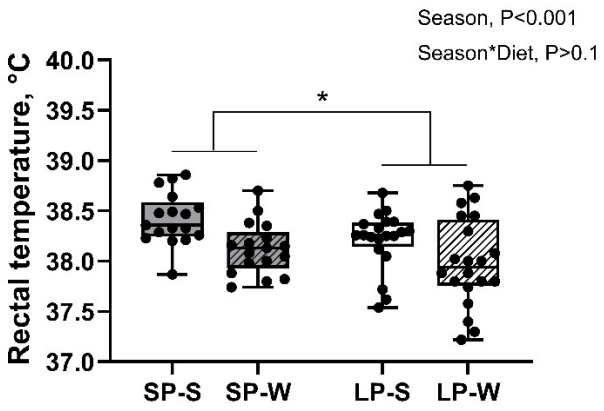


Figure 24. Average rectal temperature of sows across 5 weeks at farrowing stalls during winter (W) and summer (S) seasons. n=41, each data point within the boxplot represents an individual sow. * = P<0.05.

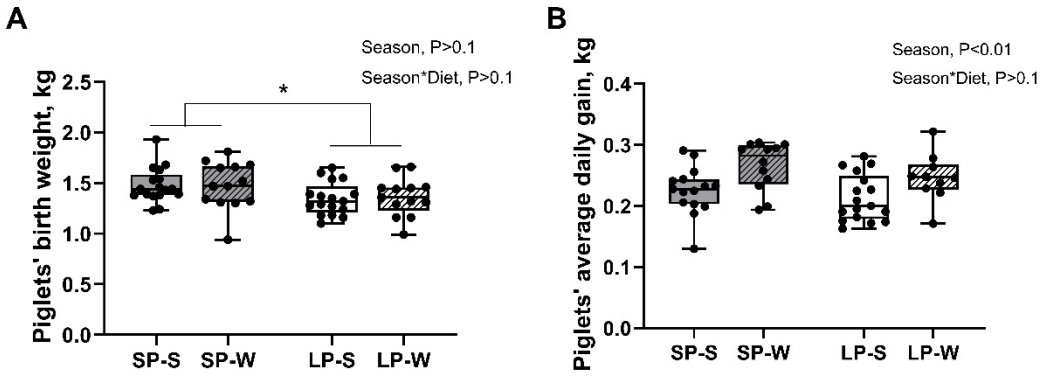


Figure 25. Piglets' weight at birth (A) and average daily gain (B) from sows fed a standard (SP, n=18) or low protein (LP, n=23) diet during summer (S) and winter (W) seasons. Each data point within the boxplot represents an individual sow. * = P<0.05.

4.2.2. Plasma metabolome

4.2.2.1. Season effect

The PLS-DA score plot showed a clear separation between the summer and winter plasma metabolome of lactating sows, suggesting a strong seasonal effect (Fig. 26). A total of 225 and 334 plasma metabolites were found to be up and downregulated, respectively, when comparing seasons (Figure 27). Among the plasma metabolites identified to be differentially abundant, 66 had a P value below 0.05 and were successfully annotated (Table 9). The metabolites listed were primarily associated with lipid and AA metabolism (lower abundance in summer) as well as steroid hormones (higher abundance in summer). Metabolic pathways altered between summer and winter are described in Table 10.

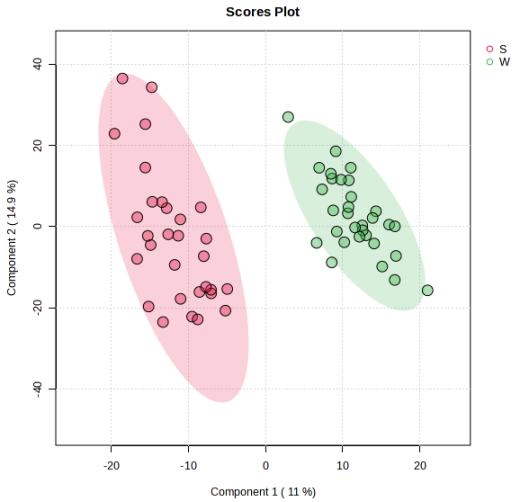


Figure 26. PLS-DA score plot models of lactating sows' plasma metabolome during summer (S) and winter (W). Each data point within the models represents an individual sow.

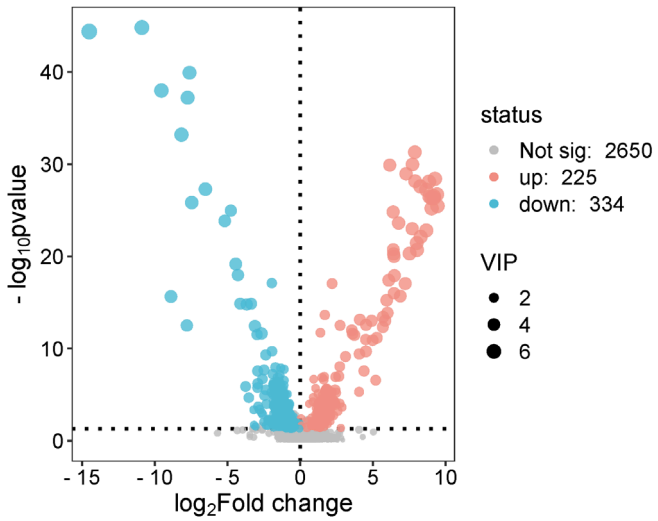


Figure 27. Volcano plot of the plasma metabolites differentially abundant between summer and winter season in lactating sows. -log10 p values are represented by different colors: high (red), low (blue), or average (grey).

Table 9. Differentially abundant plasma metabolites identified between summer and winter seasons in lactating sows.

Compound name	Sub Class	log2fold^	*P* value
Aspartyl-Threonine	dipeptides	-9.5459	8.33E-36
Glycylhydroxyproline	dipeptides	-6.5185	1.10E-25
L-trans-alpha-Amino-2-carboxycyclopropaneacetic acid	alpha amino acids	-5.1900	1.73E-22
Cinnamic acid	cinnamic acids	-1.9526	6.06E-16
5-Hydroxy-L-tryptophan	serotonins	-1.9418	8.68E-09
LysoPC(14:0/0:0)	lysophospholipids	-1.7142	4.51E-07
Enterolactone	dibenzylbutyrolactone lignans	-1.4046	2.73E-06
Leucyl-Tryptophan	dipeptides	1.6518	4.14E-06
LysoPC(P-18:1(9Z)/0:0)	lysophospholipids	0.9233	7.40E-06
Austalide B	xanthenes	-1.5557	1.52E-05
LysoPC(15:0/0:0)	lysophospholipids	-1.4124	1.66E-05
1-Hydroxy-2,12,15-heneicosatrien-4-one	long-chain fatty alcohols	1.7792	6.41E-05
LysoPC(P-16:0/0:0)	lysophospholipids	1.7338	8.23E-05
LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	lysophospholipids	-1.6481	8.62E-05
3,4-Methylenesebacic acid	medium-chain fatty acids	-1.2112	9.93E-05
LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	lysophospholipids	-1.1227	1.83E-04
LysoPE(0:0/18:1(11Z))	lysophospholipids	-0.9992	2.16E-04
LysoPA(20:3(5Z,8Z,11Z)/0:0)	lysophosphatidic acids	-1.1396	2.59E-04
LysoPE(0:0/18:3(6Z,9Z,12Z))	lysophospholipids	-1.3507	4.60E-04
LysoPE(18:1(11Z)/0:0)	lysophospholipids	-1.2404	5.32E-04
LysoPC(17:0/0:0)	lysophospholipids	-1.3234	7.66E-04
3b,17b-Dihydroxyetiocholane	androgens and derivatives	1.3396	0.001
Goshuyic acid	long-chain fatty acids	1.5753	0.001
Daidzein	isoflavanols	-1.5590	0.001
Pregnanediol	gluco/mineralocorticoids, progestogins and derivatives	1.6965	0.001
LysoPE(0:0/20:1(11Z))	lysophospholipids	-1.2230	0.001
LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	lysophospholipids	-1.1288	0.002
Indole-3-propionic acid	propionic acids	-1.2217	0.002
LysoPC(18:0/0:0)	lysophospholipids	-0.9337	0.002
LysoPC(O-18:0/0:0)	lysophospholipids	1.9904	0.002
Alloepipregnanolone	gluco/mineralocorticoids, progestogins and derivative	1.5419	0.002
LysoPC(P-18:0/0:0)	lysophospholipids	1.8425	0.002
10Z-Nonadecenoic acid	long-chain fatty acids	1.6219	0.003
LysoPE(P-16:0/0:0)	lysophospholipids	1.0843	0.004
LysoPE(0:0/22:0)	lysophospholipids	-1.7109	0.004
Sinapic acid	hydroxycinnamic acids.	-1.0897	0.006
PE(P-18:0/20:1(11Z))	phosphatidylethanolamines	2.5885	0.007
20alpha-Dihydroprogesterone	gluco/mineralocorticoids, progestogins and derivatives.	1.3665	0.007

Hippuric acid	hippuric acids	-0.9075	0.008
4-Hydroxyretinoic acid	retinoids	1.1619	0.009
LysoPE(0:0/20:2(11Z,14Z))	lysophospholipids	-1.0105	0.001
LysoPE(20:4(8Z,11Z,14Z,17Z)/0:0)	lysophospholipids	-0.8103	0.001
All-trans-13,14-dihydroretinol	retinoids	1.7934	0.001
Pregnenolone	gluco/mineralocorticoids, progestogins and derivatives.	1.3466	0.001
8,9-Epoxyeicosatrienoic acid	long-chain fatty acids	1.2692	0.002
Cholesteryl acetate	cholesteryl esters	-0.7241	0.002
2-Aminoheptanoate	alpha-amino acids	-0.8907	0.002
Oleic acid	long-chain fatty acids	1.1236	0.003
PE(15:0/14:1(9Z))	phosphatidylethanolamines.	1.3635	0.003
Perillic acid	menthane monoterpenoids	1.5118	0.003
PC(14:0/18:1(9Z))	phosphatidylcholines	-1.5262	0.003
8-Dehydrocholesterol	cholesterols and derivatives	-1.0089	0.003
Arachidonic acid	long-chain fatty acids.	1.1670	0.003
PC(18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	phosphatidylcholines.	-1.0205	0.004
24-Hydroxycholesterol	bile acids, alcohols and derivatives.	-0.9664	0.004
LysoPE(22:4(7Z,10Z,13Z,16Z)/0:0)	lysophospholipids	-1.2310	0.004
Indoleacrylic acid	indoles (aromatic heterocycles)	-0.7151	0.005
LysoPC(20:2(11Z,14Z)/0:0)	lysophospholipids	-0.7637	0.005
PS(16:0/14:1(9Z))	phosphatidylserines	1.4838	0.009
Heptadecanoic acid	long-chain fatty acids.	0.9735	0.010
Cholesterol glucuronide	cholesterols and derivatives	-0.7287	0.013
L-Tryptophan	alpha-amino acids	-0.6840	0.019
PC(14:0/16:0)	phosphatidylcholines	-0.5723	0.019
PC(16:1(9Z)/20:0)	phosphatidylcholines	0.9436	0.026
N-Acetylgalactosamine	n-acyl-alpha-hexosamines	-1.4261	0.030
5,6-Epoxy-8,11,14-eicosatrienoic acid	epoxyeicosatrienoic acids	-0.6992	0.049

^degree of change in the relative abundance of plasma metabolites when comparing summer to winter (negative and positive numbers show down and upregulation, respectively). Significant difference at $P<0.05$.

Table 10. Metabolic pathways altered between summer and winter season in lactating sows.

Metabolic Pathway	Total compounds	Hits[1]	-log10(p)	*P* value
Arachidonic acid metabolism	44	4	2.8753	0.0013
Glycerophospholipid metabolism	36	3	2.1219	0.0075
Retinol metabolism	17	2	1.7996	0.0158
Linoleic acid metabolism	5	1	1.2423	0.0572
Biosynthesis of unsaturated fatty acids	36	2	1.1908	0.0644
Tryptophan metabolism	41	2	1.0912	0.0810

[1]Number of metabolites identify (from the total compounds) as part of the metabolic pathway

4.2.2.2. Diet effect

When analyzing the diet effect in the plasma metabolome of lactating sows in summer and winter, well defined clusters were identified in the PLS-DA models, suggesting that the diet had a significant impact in the metabolism of the animals in both seasons (Figure 28). A total of 129 and 13 plasma metabolites were identified to be up and downregulated, respectively, when comparing the SP vs. the LP diet in summer (Figure 29A). Among the metabolites described, 52 were annotated with a $P<0.05$ (Table 11). In contrast, 57 and 65 metabolites were identified to be up and downregulated, respectively, when comparing the SP vs. the LP diet in winter (Figure 29B). A total of 27 metabolites were successfully annotated with a $P<0.05$ during the colder months (Table 12). Sows offered a SP diet had a higher number of plasma lipids and cholesterol metabolites upregulated when compared to the LP diet in both summer and winter. Moreover, higher levels of serotonin and bile acids, but lower levels of corticosterone were found in the SP diet when compared to the LP group in winter. Metabolic pathways altered between diets in summer, and winter are described in Tables 13 and 14, respectively.

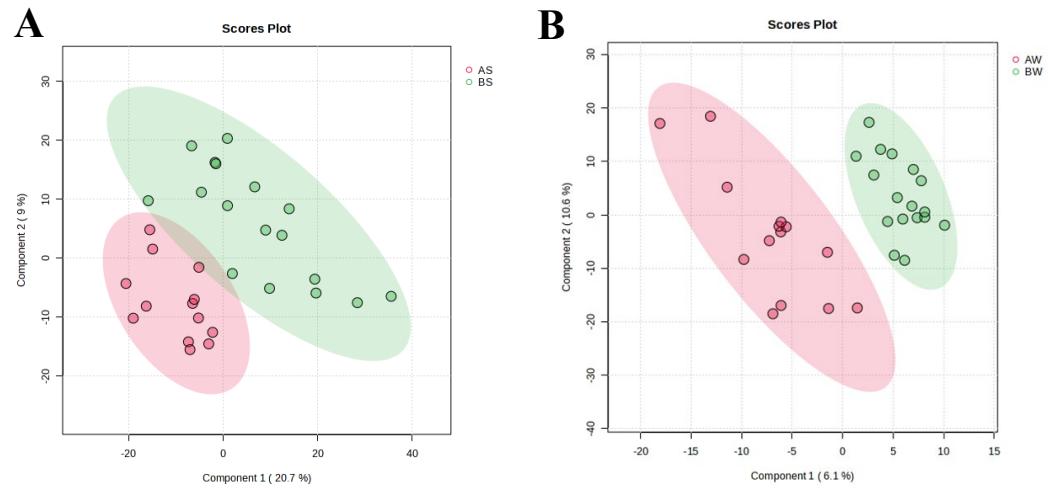


Figure 28. PLS-DA score plots of lactating sows' plasma metabolome fed a standard (labelled as A in the graph legend) or low protein (labelled as B in the graph legend) diet during summer (S) and winter (W). Each data point within the models represents an individual sow.

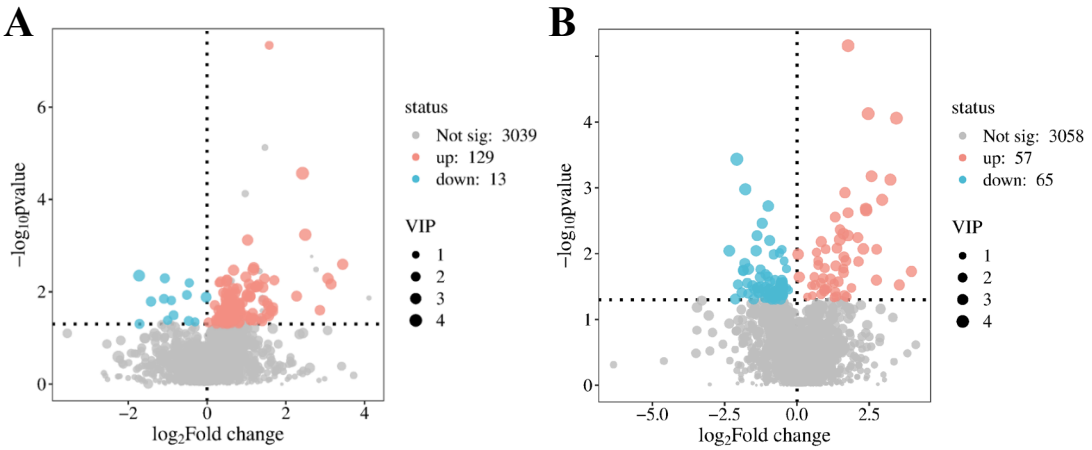


Figure 29. Volcano plot of the plasma metabolites differentially abundant in lactating sows fed a standard vs. low protein diet during summer (A) and winter (B). -log10 p values are represented by different colors: high (red), low (blue), or average (grey).

Table 11. Differentially abundant plasma metabolites identified in lactating sows fed a standard vs. low protein diet during summer.

Compound name	Sub Class	log2fold^	*P* value
Vinyl caffeate	coumaric acids and derivatives	5.6764	6.67E-09
Sinapic acid	hydroxycinnamic acids	5.2382	9.41E-08
3,4-Dimethoxycinnamic acid	coumaric acids and derivatives	-4.8438	1.25E-04
3,4,5-Trimethoxycinnamic acid	coumaric acids and derivatives	-4.3306	2.82E-04
Indole-3-carboxaldehyde	indoles (aromatic heterocycles)	-4.0621	3.91E-04
2,4'-Dimethoxyphloretate	phenylpropanoic acids	3.9992	9.14E-04
5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone	catechols	3.7579	0.001
Arachidic acid	long-chain fatty acids	3.5643	0.002
3-(3,5-dimethoxyphenyl)propanoic acid	phenylpropanoic acids	3.4177	0.002
3-Methoxybenzenepropanoic acid	phenylpropanoic acids	-3.3913	0.002
Lathosterol	cholesterols and derivatives	-3.3125	0.003
PC(22:5(7Z,10Z,13Z,16Z,19Z)/15:0)	phosphatidylcholines.	-3.0900	0.004
PE(14:1(9Z)/20:0)	phosphatidylethanolamines	3.0858	0.005
MG(20:4(5Z,8Z,11Z,14Z)/0:0/0:0)	monoacylglycerols	2.8386	0.005
Arachidonic acid	long-chain fatty acids	2.7361	0.006
PE(20:2(11Z,14Z)/16:1(9Z))	phosphatidylethanolamines	-2.6431	0.007
PE(18:3(6Z,9Z,12Z)/18:0)	phosphatidylethanolamines	2.5371	0.009
PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/16:1(9Z))	phosphatidylcholines	-2.5295	0.009
SM(d18:1/20:0)	phosphosphingolipids	-2.5247	0.010
PC(22:5(4Z,7Z,10Z,13Z,16Z)/P-18:0)	glycerophosphocholines	2.4524	0.010
PC(20:2(11Z,14Z)/15:0)	phosphatidylcholines	2.3434	0.012
PC(18:0/14:0)	phosphatidylcholines	2.2732	0.013
2-Phenylpropionate	phenylpropanoic acids	2.1895	0.014
Austalide B	xanthenes	2.1605	0.015
PC(15:0/16:1(9Z))	phosphatidylcholines	2.1461	0.016
Cinnamic acid	cinnamic acid	-2.0840	0.016
All-trans-13,14-dihydroretinol	retinoids	2.0625	0.017
Daidzein	isoflavanols	-2.0071	0.019
4-Hydroxyretinoic acid	retinoids	-1.9769	0.020
8,9-Epoxyeicosatrienoic acid	long-chain fatty acids	-1.9727	0.021
Oleic acid	long-chain fatty acids	1.9725	0.022
PE(20:0/22:5(4Z,7Z,10Z,13Z,16Z))	phosphatidylethanolamines	1.9618	0.022
Cholesterol	cholesterols and derivatives	1.9132	0.023
PE(P-16:0/22:5(7Z,10Z,13Z,16Z,19Z))	phosphatidylethanolamines	1.8872	0.024
PC(18:3(9Z,12Z,15Z)/20:3(8Z,11Z,14Z))	phosphatidylcholines	1.7852	0.027
4-Carboxyphenylglycine	alpha amino acids	1.7760	0.027
MG(18:1(9Z)/0:0/0:0)	monoacylglycerols	1.6828	0.032

Pregnenolone	gluco/mineralocorticoids, progestogins and derivatives	1.6643	0.032
20alpha-Dihydroprogesterone	gluco/mineralocorticoids, progestogins and derivatives	-1.5982	0.035
3,7-Dihydroxy-12-oxocholanoic acid	bile acids, alcohols and derivatives	1.5959	0.035
1-Hydroxy-2,12,15-heneicosatrien-4-one	long-chain fatty alcohols	-1.5859	0.035
alpha-Tocopherol	tocopherols	1.5823	0.035
LysoPC(O-18:0/0:0)	lysophospholipids	-1.5572	0.037
Goshuyic acid	long-chain fatty acids	-1.4876	0.039
Vaccenic acid	long-chain fatty acids	1.4607	0.041
10Z-Nonadecenoic acid	10z-nonadecenoic acid	-1.4373	0.042
PC(18:4(6Z,9Z,12Z,15Z)/18:2(9Z,12Z))	phosphatidylcholines	-1.4336	0.043
PC(16:0/18:1(9Z))	phosphatidylcholines	-1.4330	0.043
L-glycyl-L-hydroxyproline	dipeptides	1.4164	0.044
LysoPC(20:0/0:0)	lysophospholipids	1.3859	0.047
PC(20:2(11Z,14Z)/14:1(9Z))	phosphatidylcholines	1.3656	0.048
LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	lysophospholipids	-1.3603	0.049

^degree of change in the relative abundance of plasma metabolites when comparing a standard to a low protein diet in summer (negative and positive numbers show down and upregulation, respectively). Significant difference at $P<0.05$.

Table 12. Differentially abundant plasma metabolites identified in lactating sows fed a standard vs. low protein diet during winter.

Compound name	Sub Class	log2fold^	*P* value
Sinapic acid	hydroxycinnamic acids	-6.3413	6.90E-06
Vinyl caffeate	coumaric acids and derivatives	-4.5996	7.45E-05
2,4'-Dimethoxyphloretate	phenylpropanoic acids	4.1080	8.73E-05
3,4-Dimethoxycinnamic acid	coumaric acids and derivatives	3.8212	6.64E-04
3-(3,5-dimethoxyphenyl)propanoic acid	phenylpropanoic acids	-3.4602	0.001
5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone	catechols	-3.4505	0.002
MG(20:4(5Z,8Z,11Z,14Z)/0:0/0:0)	monoacylglycerols	-3.0510	0.003
Indole-3-carboxaldehyde	indoles (aromatic heterocycles)	3.0267	0.004
LysoPC(O-18:0/0:0)	lysophospholipids	2.9936	0.005
3-Methoxybenzenepropanoic acid	phenylpropanoic acids	2.7548	0.008
PE(20:2(11Z,14Z)/16:1(9Z))	phosphatidylethanolamines	2.5781	0.009
3,4,5-Trimethoxycinnamic acid	coumaric acids and derivatives	-2.5597	0.009
Arachidonic acid	long-chain fatty acids	2.4651	0.010
(9Z,11E,13E,15Z)-4-Oxo-9,11,13,15-octadecatetraenoic acid	long-chain fatty acids	2.4307	0.011
PS(14:0/18:2(9Z,12Z))	phosphatidylserines	2.3936	0.013
PC(22:1(13Z)/18:2(9Z,12Z))	phosphatidylcholines	2.3677	0.015
Heptadecanoic acid	long-chain fatty acids	-2.3407	0.016
3,7-Dihydroxy-12-oxocholanoic acid	bile acids, alcohols and derivatives	2.2558	0.017
Chenodeoxycholic acid glycine conjugate	bile acids, alcohols and derivatives	2.1748	0.020
LysoPC(P-18:0/0:0)	lysophospholipids	2.1303	0.023
9-Octadecenal	fatty aldehydes	-2.0503	0.027
Valerenic acid	sesquiterpenoids	1.9705	0.030
Corticosterone	21-hydroxysteroids	-1.9415	0.031
3b,7a-Dihydroxy-5b-cholanoic acid	bile acids, alcohols and derivatives	1.8690	0.035
Austalide B	xanthenes	1.7937	0.042
5-Hydroxy-L-tryptophan	serotonins	1.7698	0.043
PC(20:4(5Z,8Z,11Z,14Z)/P-16:0)	phosphatidylcholines.	-1.7417	0.045

^degree of change in the relative abundance of plasma metabolites when comparing a standard to a low protein diet in winter (negative and positive numbers show down and upregulation, respectively). Significant difference at $P<0.05$.

Table 13. Metabolic pathways altered between standard and low protein diets in lactating sows during summer.

Metabolic Pathway	Total compounds	Hits[1]	-log10(p)	*P* value
Biosynthesis of unsaturated fatty acids	36	3	2.4445	0.0035
Glycerophospholipid metabolism	36	3	2.4445	0.0035
Arachidonic acid metabolism	44	3	2.195	0.0063
Retinol metabolism	17	2	2.014	0.0096
Linoleic acid metabolism	5	1	1.3492	0.0447
Steroid biosynthesis	41	2	1.2879	0.0515

[1]Number of metabolites identify (from the total compounds) as part of the metabolic pathway

Table 14. Metabolic pathways altered between standard and low protein diets in lactating sows during winter.

Metabolic Pathway	Total compounds	Hits[1]	-log10(p)	*P* value
Glycerophospholipid metabolism	36	3	3.2152	0.0006
Arachidonic acid metabolism	44	2	1.6973	0.0200
Linoleic acid metabolism	5	1	1.5889	0.0257
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	15	1	1.1216	0.0755
Ether lipid metabolism	20	1	1.0016	0.0996

[1]Number of metabolites identify (from the total compounds) as part of the metabolic pathway

5. Application of Research

Reduced ADFI as well as increased RR and body temperatures were found to be consistent/reliable indicators of HS in lactating sows, aligning with what is already well described in the literature (Bjerg et al., 2020; de Oliveira et al., 2024).

The present results demonstrated that adding an umami flavour in feed (750 ppm) was insufficient to improve the performance of lactating sows under HS. Considering the appetite enhancing and cooling effects of umami flavour and essential oils, respectively (Orani et al., 1985; Guzmán-Pino et al., 2019; Roura et al., 2016), the testing of a broader range of concentrations to determine their acceptance threshold and best dose-response effect in sows under HS, may merit further study (particularly essential oils, which consumption was close to nil). In contrast to the previous additives, lowering the CP level in the diet (by 3% or more from standard concentrations) improved the ADFI and reduced body temperatures of animals under hot environmental conditions. Although some of the benefits of the LP diet were not identified in all experiments, such as higher ADFI and lower RR (potentially due to differences in animals, housing conditions/environmental temperatures, and experimental designs), a reduction in body temperatures was consistently observed.

This indicates that lowering dietary CP levels may reduce, as initially hypothesized, hindgut fermentation and internal heat production (Le Bellego et al., 2001; Kerr et al., 2003). In particular, the effects of LP diets on hindgut fermentation may need to be confirmed through additional HS studies focused on hindgut metabolic products. Piglets born from sows fed LP diet (group that was used in both summer and winter trials, n=41) were shown to have lower birth weights (Figure 25, A) suggesting that the commercial diet tested (CP level of 13.5%) could have been potentially deficient in some functional AA essential for fetal growth (e.g., Gln, Tau, Arg, Gly and Pro). Additional studies in this area are needed considering that differences in piglets' birth weights were not observed when comparing diets within the larger summer and winter cohorts (n=400 and n=56, respectively, Table 7).

Long-chain fatty acids, glycerophospholipids, gluco/mineralocorticoids and alpha AA/AA derivates, were some of the most abundant types of metabolites shown to be altered by HS. Thus, our results suggest that changes in plasma levels of compounds such as Trp, pregnenolone or arachidonic acid could be used as biomarkers of HS. These metabolites and related precursors/derivates were consistently affected across experiments and tissue analyses. In particular, blood increments of arachidonic acid and pregnanolone and lower concentrations of Trp under hot weather conditions might be used as biomarkers to identify heat sensitive sows before visible HS symptoms appear. Consistent with the plasma metabolome results, a negative correlation between the abundance of proteins related to lipid and AA metabolism in the liver and feed intake reduction during HS was identified. Thus, decreases in the activity of lipid and AA enzymes in the liver have the potential to be used as indicators of heat sensitivity in lactating sows as well. The overall lower concentration of blood lipids observed in sows exposed to HS is compatible with the reduced P2 backfat change identified in the summer vs. the winter trial and the lower adipose tissue mobilization described in pigs under HS in previous studies (Pearce et al., 2013; Sanz Fernandez et al., 2015; Zhao et al., 2018).

When comparing diets, long-chain fatty acids, glycerophospholipids, AA, and bile acids were among the most abundant subclass of blood metabolites altered (the last group mainly under thermoneutral conditions). A lower abundance of AA, but higher concentrations of lipids, were identified in the plasma of SP sows compared to the LP group in summer. In particular, reduced levels of arachidonic acid, arachidic acid and other precursors of inflammatory molecules, were observed in LP vs. SP sows. Moreover, the low levels of cholesterol and bile acids identified in the LP compared to the SP treatment, seems to indicate that the first group was able to better cope with environmental hyperthermia as high blood levels of these metabolites have been described as biomarkers of HS in pigs (Fang et al., 2020). Likewise, the higher blood abundance of aromatic AA in LP vs SP sows (particularly in Exp 2) illustrates potential reduced stress responses in the LP group under high temperatures (Badakhshan et al., 2021). Whereas the reduced plasma level of AA may be indicative of an altered intestinal absorption and/or increased body muscle breakdown for energy generation to combat HS, the high abundance of lipids may describe a degree of alteration in liver function related to lipid metabolism under HS (Pearce et al., 2014; Morales et al., 2016; Wen et al., 2019). Additional studies

focused on gut, muscle and liver function in lactating sows fed low dietary CP levels under high temperatures may help corroborate the previous statements.

Interestingly, the microbiome analysis showed an increment in phylogenetic diversity and in the abundance of several bacteria with SCFA production capacity under high environmental temperatures, aligning with the enhanced microbial TCA cycle described in HS sows (i.e., indicator of increased microbial activity) and with previous HS studies in broiler chickens (Shi et al., 2019; Liu et al., 2020). Moreover, 5 of these bacteria, belonging to Anaerovoracaceae, Muribaculaceae, *Treponema*, *Cryptobacteroides*, Bacteroidales were found to be positively correlated with body temperatures (i.e., ET and EaT), suggesting that changes in their levels could be used as indicators of HS in sows. Reduced nutrient absorption in the small intestine and increased hindgut fermentation of proteins and carbohydrates may explain these microbiota changes (Michael and Thomas, 2007). However, additional microbiome and gut health research is required to confirm this hypothesis and the reliability of the proposed biomarkers. The microbiota of sows fed the LP treatment showed an upregulation of several pathways related to vitamin B_{12} metabolism irrespective of the environmental temperatures. Vitamin B_{12} plays a pivotal role in red blood cells formation, ATP production, detoxification of ROS and DNA synthesis, suggesting that the use of LP diets may benefit sows beyond reducing hindgut fermentation and body temperatures (Smołucha et al., 2024) by increasing the activity of beneficial bacteria under HS.

The use of LP diets and some of the highlighted blood biomarkers as early predictors of HS could enable the prevention, early detection and management of HS episodes in sows, helping producers improve the wellbeing of their animals during summer months. In addition, the ingredients required in LP formulations are generally readily available and can be included in commercial diets at reasonable costs, making the proposed strategy easy for producers to adopt. Given the ease of implementation and low investment required, these findings are likely to have a positive impact on the Australian pig industry. However, lowering the CP content in excess may have negative impacts on piglet birth weight. Additional studies aimed at identifying some of the biomarkers in more accessible body fluids, such as milk and saliva, could help facilitate their routinary use in farms. Moreover, the application of osmoprotectants, such as the AA Bet and Pro, in conjunction with LP formulations is a subject that warrants investigation as the combination of these dietary strategies could provide additional performance and welfare benefits to sows under hot weather conditions (Ratiyanto et al., 2009; Phang et al., 2010, 2019).

Finally, it is important to mention that moderate to strong correlations were found across studies between surface (i.e., eye and ear) and internal temperatures (i.e., rectal and vaginal). This suggests that the first group could be used by trained personnel to non-invasively identify lactating sows at risk of HS in commercial farms. However, it must be taken into consideration that surface temperatures were on average 1-1.5°C lower than internal recordings and showed higher variability within and between animals, making them less reliable for the identification of HS as compared to other measurements. Moreover, temperature recordings and temperature-humidity indexes allow for a slower identification of

heat sensitive animals under hot weather conditions when compared to metabolic biomarkers, leaving a small window for interventions before the onset of HS (Liang et al., 2024). The recording of sows' temperatures for the early identification of HS in farms has important limitations to be considered. The increments in ADFI and the reduction of body temperatures (particularly RT) and RR observed across weeks (period 1 to 4 in Exp 1 and 2) showed sows have an innate capacity to adapt to high environmental temperatures (Gomez-Prado et al., 2022). These results are in agreement with a previous report on chronic HS that illustrates signs of acclimation in pigs following 1 week of exposure to high temperatures (Vasquez et al., 2022). The adaptive capacity of sows should be considered when planning HS amelioration strategies to maximize their effectiveness and prevent performance losses.

6. Conclusion

Using an LP diet improved the ADFI and reduced internal body temperatures and RR. However, LP diets reduced the piglet's birth weight and did not improve the piglets' ADG and the sows' reproductive performance. The blood abundance of pro-inflammatory molecules, such as arachidonic acid and its derivates/precursors, and previously described HS biomarkers, such as cholesterol, were reduced in LP compared to SP sows indicating reduced metabolic stress due to environmental hyperthermia in the first group. Similarly, the LP diet enhanced the gut microbiota vitamin B_{12} synthesis pathways and aromatic AA blood levels when compared to the SP treatment, indicating that the first group of sows had an improved metabolic capacity to cope with HS. Further work is required to determine if some of the plasma metabolites identified under HS can be observed in more accessible body fluids to facilitate their use in farms as indices of HT in sows.

7. Limitations/Risks

Risk 1: Confounding effect of higher levels of available methionine (Met) in LP diets

Although diets were formulated to be as close as possible in energy, crude fibre and essential AA content across experiments, the LP diets had a higher calculated abundance of available/standardised ileal digestible Met. The higher availability of Met in LP diets could have influenced some of the results observed as this AA has been identified to promote intestinal integrity and antioxidant functions in pigs under HS (Morales et al., 2022). However, these effects have been reported at concentrations of 20% (or higher percentages) above requirements, a threshold/level that was not reached in any of the experiments conducted in this project.

Risk 2: Temperature and diet interaction inconsistencies

The relatively mild summer temperatures experienced during the commercial study (Experiment 3) may have undermined some of the benefits of the LP diet. In addition, temperature differences across experiments may explain some of the inconsistencies in performance and physiological parameters observed in LP sows between experiments. In particular, the benefits of LP diets on feed intake and reproductive performance under hot weather conditions may need further testing.

Risk 3: Potential functional (non-essential) AA deficiencies and increased costs of LP diets

Depending on the level of CP to be used in commercial diets, supplementation of functional (non-essential) AA to prevent low birth weights may be needed. This may limit the degree of CP reduction and effectiveness of LP diets in gestation or increase their cost due to the requirement of additional supplements.

Risk 4: Sampling limitations/difficulties for the measurement of biomarkers

The need for blood samples to detect HS biomarkers can make their adoption/use in farms difficult. Additional studies aimed at identifying some of the highlighted biomarkers in milk and/or saliva may facilitate their routinary use in the pig industry.

8. Recommendations

As a result of the outcomes in this study, the following recommendations have been made:

1. Reducing the CP level (3% or more) in lactation diets will improve sow feed intake and reduce RR and internal body temperatures in sows during summer/hot weather conditions. However, if LP diets are to be used during gestation, depending on the level of protein restriction, supplementation of functional AA, such as Gln, Tau, Arg, Gly and Pro, could be an option to prevent potential negative impacts of AA deficiencies on litter growth/birth weight. However, due to the high cost of these additives, NEAA supplementation should be avoided when possible.

2. Low protein diets can be used to improve the metabolic capacity of lactating sows to cope with environmental hyperthermia by increasing the gut microbiota vitamin B12 synthesis and reducing the blood levels of pro-inflammatory molecules, such as arachidonic acid.

3. Blood levels of Trp, arachidonic acid and pregnenolone have the potential to be used as indices of heat tolerance in lactating sows to identify individuals at higher risk of HS. Further research should be conducted to determine if changes in the level of these and other long-chain fatty acids, glycerophospholipids, gluco/mineralocorticoids and alpha AA identified in this study can be detected in less invasive body fluids.

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