UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CÂMPUS DE JABOTICABAL

ADAPTIVE RESPONSES OF PIGS EXPOSED TO ENVIRONMENTAL CHALLENGES

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Mestre em zootecnia
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Jaboticabal, December 16, 2022

AUTHOR'S CURRICULUM DATA

ALÍCIA ZEM FRAGA - was born on the 20th of March 1993 in Muriaé, Minas Gerais – Brazil. Graduated in Animal Science (from 2011 to 2016) from the University Federal of Viçosa (UFV), Minas Gerais – Brazil where she had her first contact with nutrition and health of pigs (internship carried out from the second to tenth graduation period). Her Master Degree in Animal Science (from 2016 to 2018) was at Veterinary and Agrarian Science Faculty – UNESP Universidade Estadual Paulista “Júlio de Mesquita Filho” – Jaboticabal, São Paulo, Brazil with emphasis on protein and energy metabolism in pigs exposed to heat stress. Her supervisor was Dr. Luciano Haushild and co-advisor was Dr. Paulo HRF Campos and she spent three months at L’Institut National de Recherche pour l’Agriculture, l’Alimentation et l’Environnement, INRAE (Saint Gilles, France) under Dra. Nathalie Le Floc’h supervision. In the same year after obtaining the title of Master Degree, Alícia started Doctorate cotutela in Animal Science (dual program PhD) at Unesp, Brazil and Agrocampus, France where she shared her doctoral research in both institutions (3 years at UNESP and 16 months at INRAE, Saint Gilles). In her PhD, Alícia studied adaptive responses of pigs exposed to different environmental challenges conditions under supervison of Dr. Luciano Hauschild and Dra. Nathalie Le Floc’h. During her Master Degree and PhD, Alícia received scholarship from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil). On the 16 of December 2022, Alícia submitted the current thesis work for evaluation by the jury committee.

… “And the world will be as one!”

Imagine – John Lennon

DEDICATION

The hands described in the Acknowledgments section,

I dedicate.

ACKNOWLEDGEMENTS

Before typing in the computer, I wrote every word of this thesis on paper. In schematic format, single sentences or paragraphs that have been written and rewritten. Faced with the technological innovations that we are daily confronted, some people might think it's a time delay or some specific limitation on my part. It might even be. But I prefer to believe in the power of hands - without linking them to the use of technology. After all, there were several hands that guided me. Hands that boosted, soothed, strengthened, and celebrated. Hands that taught – and these hands will never be replaced by any technology. Among all, I highlight the hands of God and the hands of my parents. There is no distance that cannot be reached by the love of a daughter, so I feel my father's presence (in memoriam) every day. And I'm sure his hands and heart are with mine. My mother, who has strong and wise hands. I have a mother who abdicated of her dreams so that my brothers and I could build ours. A woman who built my solid roots and gave me wings. And many were the “flights” - But all my “landings” were always to meet her. For all your love and dedication, I thank you mom. I also thank you my family and Joca for his unconditional love (some angels have wings, others have paws).

Through Science I discovered myself. Faced with my ignorance, insecurity, and fear it was in the academic/scientific environment that awoke my curious and restless side. To science and its new discoveries and beginnings, my admiration. To public universities (especially to Brazilian universities) and their transforming and questioning environment, my respect. I bet and defend the power of universities; especially the solitary, thinking and inclusive side of Brazilian public universities. I am proud to be part of the university environment – which is much more than academic. It was in this environment that I thought of many old thoughts and habits. This environment constructed and (de)constructed me, making me think and question about my role as a citizen. I remember the first day that I entered in a public university (Viçosa, 2011) and I was with my mother. Today it makes sense that my first time at university was with her: Education is an act of love. Therefore, to the Professors/Researchers/Collaborators who are part of this environment, my gratitude. I thank the wise hands of Prof Dr Luciano

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[1] *Merci beaucoup*: Dra Nathalie Le Floc'h et ta belle famille (puisque 2017), Françoise Thomas (puisque 2017), Raphaël COMTE (puisque 2017), Aira SERVIENTO, Mariana MESCOUTO, Francis EUGENIO, Amandine CAUCHI, Anastasia AGOUROS, Angélique CORSET, Camille JUIGNÉ, Chloé VAN BAELEN, Clement GARÇON, Elise DUBOIS, Emma GOULEZ, Jean-Charles ANGER, Julie TANG, Lorry BÉCOT, Maëva DURAND, Mathilde LANTHONY, Valeriia KHINTSYSKA, Dr Elodie MERLOT, Laurence LE-NORMAND, Marie-Hélène LOHAT, Josselin DELAMARRE, Patrice ROGER, Hervé DEMAY, Simon DUTEIL, Mathieu MONVOISIN, Myriam BURBAN, Laurance THEBAULT, Sophie DARÉ, Colette MUSTIÈRE, Fabiano BELÉM, Tales SILVA, et Mariana ROZA.

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LIST OF ABBREVIATIONS

Item	Term
AA	Amino acids
ADFI	Average daily feed intake
ADG	Average daily gain
Arg	Arginine
BCAA	Branched chain amino acids
BW	Body weight
Cys	Cysteine
Cys2	Cystine
DAO	Diamine oxidase
dROM	Diacron-reactive oxygen metabolites
g	Grams
Gln	Glutamine
IL	Interleukins
Ile	Isoleucine
TNF	Tumor necrosis factor
Kg	Kilograms
Leu	Leucine
LPS	*Escherichia coli* lipopolysaccharide
RFI	Residual feed intake
SOD	Superoxide dismutase
Val	Valine
ºC	Degrees Celsius

CERTIFICATE OF THE ETHICS COMMITTEE IN THE USE OF ANIMALS[2]

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MINISTERE DE L'ENSEIGNEMENT SUPERIEUR, DE LA RECHERCHE ET DE L'INNOVATION

Paris, le 25 juin 2020

Objet : Notification de décision relative à l'autorisation de projet utilisant des animaux à des fins scientifiques

Direction générale de la recherche et de l'innovation

Service de la performance, du financement et de la contractualisation avec les organismes de recherche

Département des pratiques de recherche réglementées

Cellule Animaux utilisés à des Fins Scientifiques - AFiS -

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75231 Paris Cedex 05

En application des dispositions du code rural et de la pêche maritime, notamment ses articles R.214-87 à R.214-126, le projet :

- référencé sous le numéro APAFIS#23735-2020012214413172 v2
- ayant pour titre : Apport d'acides aminés fonctionnels sur la capacité des porcs en croissance à répondre à un challenge inflammatoire basé sur la dégradation des conditions d'hygiène du logement
- déposé par l'établissement utilisateur : INRA 1421 UEPR Saint Gilles, numéro d'agrément D3527532, dont la responsable est Madame Hélène LUCAS,
- et dont la responsabilité de la mise en œuvre générale du projet et de sa conformité à l'autorisation est assurée par : Madame Nathalie LE FLOCH,

est autorisé.

L'autorisation de projet est accordée, sous réserve de la validité de l'agrément de l'établissement utilisateur, pour une durée de 3 ans à compter de la présente notification.

Le projet précité a été évalué sur le plan éthique par le comité d'éthique en expérimentation animale n°007 et a reçu un avis favorable.

Ce projet fera l'objet, à l'issue de sa réalisation, d'une appréciation rétrospective. Il vous appartiendra de prendre contact directement avec le comité d'éthique.

Pour la ministre et par délégation
le chef du département
des pratiques de recherche réglementées

Laurent PINON

Madame Hélène LUCAS
INRA 1421 UEPR Saint Gilles

[2] The certificate refers to the third study (Chapter 5) of this thesis work in which animal experimentation was performed.

ADAPTIVE RESPONSES OF PIGS EXPOSED TO ENVIRONMENTAL CHALLENGES

ABSTRACT – High ambient temperatures and poor hygienic housing conditions alter the welfare, health, and performance of pigs. This thesis was conducted to better understand the adaptive responses of pigs exposed to environmental challenges (high ambient temperature and poor hygiene conditions). We investigated the effects of genetic selection and nutritional strategy on adaptive responses. In a first study, our results show that genetic selection for lean body mass deposition modulates the feeding behaviour of pigs exposed to high ambient temperatures. In a second study, we showed that pigs selected for high feed efficiency had a greater capacity to use amino acids (AA) for immune purposes than less efficient pigs, which may explain their better ability to cope with poor hygiene conditions. Finally, a third study showed that supplementing the diet with a mixture of AA and grape polyphenols can contribute to the weaning transition and better prepare pigs to cope with an inflammatory challenge caused by poor hygiene conditions. This nutritional strategy is therefore interesting to accompany demedicalisation in pig farming. Our studies have provided new knowledge on the effects of genetic selection and nutrition on adaptive responses to two types of environmental challenges. This knowledge can help to modify farming practices adapted to modern genotypes more susceptible to stress and disease and to address the challenge of reducing antibiotic use in intensive farming conditions.

Keywords: amino acids, genetic selection, health, inflammation

RÉPONSES ADAPTATIVES DES PORCS EXPOSÉS AUX CHALLENGES ENVIRONNEMENTAUX

RESUME – Des températures ambiantes élevées et les mauvaises conditions d'hygiène de logement altèrent le bien-être, la santé et les performances des porcs. Ce travail de thèse a été réalisé pour mieux comprendre les réponses adaptatives des porcs exposés à des challenges environnementaux (température ambiante élevée et mauvaise conditions d'hygiène). Nous nous sommes intéressés aux effets de la sélection génétique et d'une stratégie nutritionnelle sur les réponses d'adaptation. Dans une première étude, nos résultats montrent que la sélection génétique pour le dépôt de masse maigre module le comportement alimentaire des porcs exposés à des températures ambiantes élevées. Dans une seconde étude, les porcs sélectionnés sur une efficacité alimentaire élevée avaient une plus grande capacité à utiliser les acides aminés (AA) à des fins immunitaires que les porcs moins efficaces, ce qui peut expliquer leur meilleure capacité à faire face à de mauvaises conditions d'hygiène. Le troisième étude a montré que la supplémentation avec un mélange d'AA et de polyphénols de raisin peut contribuer à la transition du sevrage et mieux préparer les porcs à faire face à un challenge causé par de mauvaises conditions d'hygiène. Cette stratégie nutritionnelle est donc intéressante pour accompagner la démédicalisation en élevage porcin. Nos études ont apporté des connaissances nouvelles sur les effets de la sélection génétique et la nutrition sur les réponses adaptatives à deux types de challenges environnementaux. Ces connaissances peuvent aider à modifier les pratiques d'élevage adaptées aux génotypes modernes plus susceptibles au stress et aux maladies et permettre de relever le défi de réduction de l'utilisation des antibiotiques dans les conditions d'élevage intensives.

Mots clés: acides aminés, inflammation, santé, sélection génétique

PREFACE

The work performed during the course of this thesis resulted in the following scientific contributions:

Journal Publications

1. **Alícia Zem Fraga**, Luciano Hauschild, Paulo Henrique Reis Furtado Campos, Marcio Valk, Débora Zava Bello, Marcos Kipper, Ines Andretta. Genetic selection modulates feeding behavior of group-housed pigs exposed to daily cyclic high ambient temperatures. PLoS One, v. 17, p. e0258904, 2022.
2. **Alícia Zem Fraga**, Isabelle Louveau, Paulo Henrique Reis Furtado Campos, Luciano Hauschild, Nathalie Le Floc'h. Selection for feed efficiency elicits different postprandial plasma metabolite profiles in response to poor hygiene of housing conditions in growing pigs. PLoS One, v. 16, p. e0246216, 2021.
3. **Alícia Zem Fraga**, Paulo Henrique Reis Furtado Campos, Luciano Hauschild, Tristan Chalvon-Demersay, Martin Beaumont, Nathalie Le Floc'h. A blend of functional amino acids and grape polyphenols improves the pig capacity to cope with an inflammatory challenge caused by poor hygiene of housing conditions. BMC Veterinary Research, v. 19, p. 1-11, 2023.

Abstracts published in conference proceedings

1. Paulo Henrique Reis Furtado Campos, **Alícia Zem Fraga**. Improving pigs resilience and adaptation to sanitary challenges via nutrition: a focus on energy and amino acids. Rio de Janeiro. 26th International Pig Veterinary Society Congress, IPVS 2022.
2. **Alícia Zem Fraga**, Paulo Henrique Reis Furtado Campos, Luciano Hauschild, Tristan Chalvon-Demersay, Nathalie Le Floc'h. A mix of amino acids and polyphenols reduced inflammation caused by poor hygiene of housing and subsequent metabolic consequences. Rio de Janeiro. 26th International Pig Veterinary Society Congress, IPVS 2022.
3. **Alícia Zem Fraga**, Paulo Henrique Reis Furtado Campos, Luciano Hauschild, Tristan Chalvon-Demersay, Nathalie Le Floc'h. A blend of amino acids

and polyphenols improves the pig capacity to cope with weaning and poor hygiene challenge. 15th International Symposium on Digestive Physiology of Pigs, DPP 2022, Rotterdam.

4. **Alícia Zem Fraga**, Paulo Henrique Reis Furtado Campos, Luciano Hauschild, Isabelle Louveau, Nathalie Le Floc'h. Effects of housing hygiene conditions on amino acid postprandial profiles in pigs selected for low and high residual feed intake. 6th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP 2019), Belo Horizonte.

5. Nathalie Le Floc'h, Paulo Henrique Reis Furtado Campos, **Alícia Zem Fraga**, Isabelle Louveau. Modulatory effects of housing conditions on energy related metabolites and insulin in pigs divergently selected for residual feed intake. 6th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP 2019), Belo Horizonte.

6. **Alícia Zem Fraga**, Paulo Henrique Reis Furtado Campos, Raphael Perini Caetano, Welex Cândido da Silva, Alini Mari Veira, Luan Sousa dos Santos, Luciano Hauschild. Efficiency of energy deposition in growing-pigs fed high-fat / low-protein diets under daily cyclic high ambient temperature conditions. 9th Workshop on Modelling Nutrient Digestion and Utilization in Farm Animals (Modnut 2019), Ubatuba.

7. **Alícia Zem Fraga**, Raphael Perini Caetano, Thayssa de Oliveira Littiere, Vinicius Eduardo Moreira, Paulo Henrique Reis Furtado Campos, Luciano Hauschild. Efeito do sistema de alimentação sequencial no comportamento alimentar de suínos em condição de estresse cíclico por calor. PorkExpo 2018, Foz do Iguaçu.

8. **Alícia Zem Fraga**, Brandon Rincon, Joseane Penteado Rosa, Paulo Henrique Reis Furtado Campos, Luciano Hauschild. Desempenho e composição corporal de suínos em crescimento e terminação alimentados com diferentes estratégias nutricionais em estresse cíclico por calor. Congresso Latino-Americano de Nutrição Animal, Campinas, 2018.

Training and courses carried out during this thesis are presented in Annex I. Scientific publications and indicators are presented in Annex II.

CHAPTER 1 – GENERAL INTRODUCTION

1.1 Introduction

Pigs in commercial farms are frequently confronted to many challenges including social (early weaning, housing density, and animal mixing) and environmental factors (degree of sanitation in farms, ambient temperature, etc; Pastorelli et al., 2012). Furthermore, the continual exposition of the pigs to biotic (i.e., bacteria, fungi, and virus) and abiotic (i.e., gas and dust) agents as it occurs when biosecurity rules and environmental conditions are not respected, may result in immune system stimulation (Le Floc'h et al., 2021). This situation may be intensified in the context of global warming, since increase in high ambient temperature and heat wave frequency have been associated with the dissemination of pathogens and vectors predisposing animals to infections (Kimaro and Chibinga, 2013). Modern genotypes selected for improved lean tissue accretion may not be able to allocate enough resources to other demanding processes than growth when facing environmental and health challenges (Rauw et al., 1998). Besides, improved lean pigs produce more metabolic heat and may have a reduced capacity to cope with high ambient temperatures (Brown-Brandl et al., 2004). Taken together, these aspects may induce metabolic responses and inflammatory disorders, which decrease the well-being, health status and growth performance of highly performing pigs (Nakov et al., 2019).

In this context, antimicrobial molecules were being widely used to attenuate the impact of continuous immune system stimulation caused by environmental and sanitary-related issues. However, the antimicrobial resistance has increased the demand to find non-antibiotics strategies (Chalvon-Demersay et al., 2021). In Brazil, antimicrobial use in pig for prophylactic (treatment to defending or protecting from disease or infection before the disease happens) and metaphylactic (treatment after the diagnosis of infection and/or clinical disease) purposes has been not prohibited yet (Dutra et al., 2021). However, some antimicrobials used as growth promoters were prohibited in Brazil in November 2016 (colistin) and in January 2020 (lincomycin, tiamulin, and tylosin; MAPA, 2020). This situation is different from some countries in Europe. In 1986,

Sweden was the first country in the world to legally ban antimicrobial growth promoters use in animals (including treatment via feed or drinking water; Waluszewski et al., 2021), and an EU-wide ban on the use of antimicrobials as growth promoters began in January 2006 (European Union, 2005). Even for metaphylactic purposes, veterinarians in France have considerably limited the prescription of antimicrobials in the last 15 years (Ducrot et al., 2022). Due to this event, alternatives have been researched and proposed. Because of their health-promoting effects (controlling of inflammation, increase intestinal development and functions, modulate gut microbiota, etc; Liu et al., 2018), nutritional strategies including feed additives may help to attenuate the negative impacts of demedicalization on pigs exposed to environmental and sanitary challenges.

Pig's responses to stressful situations including behavioral, physiological, and metabolic defense mechanisms in an attempt to restore homeostasis[3] and body integrity (Campos et al., 2017). These responses are integrated into complex relationships whose objective is to annulate or minimize the effects of a stressor (Figure 1). Overall, this thesis was performed to improving our understanding of the adaptive responses of pigs exposed to environmental challenges conditions and how genetic selection and/or nutritional strategies may influence it. For this purpose, environmental challenges (variation of temperature throughout the day and poor hygiene of housing conditions) and management practices commonly carried out in pig farms (in particular, weaning) were considered in the thesis as stressors. In turn, the adaptive responses of pigs exposed to these challenges were evaluated through changes in behavioral, health status, and/or metabolism. It should be noted that assessing the impact of a challenge over time allows a better understanding of an animal's robustness[4] and provide useful information of individuals based on their thermoregulatory responses, productive potential, and well-being.

[3] In 1926, the term homeostasis was first mentioned by Walter Bradford Cannon in an attempt to extend and codify the principle of “la fixité du milieu intérieur” or a constant interior body environment (Davies, 2016). This principle had been postulated in 1865 by Claude Bernard (french medical and physiologist; considered the founder of experimental physiology). Currently defined, homeostasis is a dynamic regulatory process by which biological systems maintain a constant internal state (Billman, 2020).

[4] For the purposes of practical pig breeding, robustness is defined as the ability to combine high production potential with resilience to stressors, allowing the expression of production potential in a wide variety of environmental conditions (Knap, 2005). Indeed, is a complex and dynamic characteristic composed of several components, including response rates and recovery from environmental disturbances (Friggens et al., 2017).

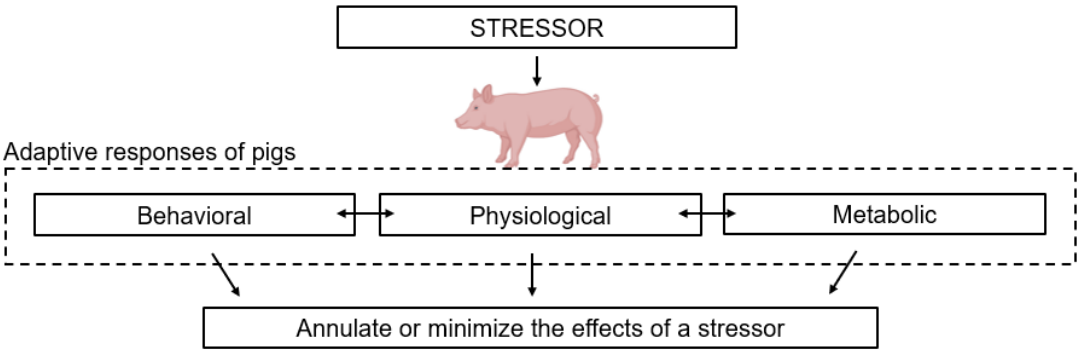

Figure 1. Pig's responses including behavioral, physiological, and metabolic defense mechanisms whose objective is to annulate or minimize the effects of a stressor.

The thesis is composed of 7 chapters. A brief literature review followed by the main objectives and hypotheses are presented in the Chapter 2. In the Chapters 3, 4 and 5, the results of the studies carried out during the thesis work are presented in the format of scientific manuscripts. Regarding the Chapter 3, it was evaluated the effect of genetic selection for lean mass deposition on the feeding behavior of group-housed pigs exposed to daily cyclic high ambient temperatures. In the Chapter 4, it was compared plasma metabolite profiles in response to poor hygiene of housing conditions in growing pigs divergently selected for residual feed intake (RFI). The Chapter 5 was performed to evaluate whether a nutritional strategy (blend of amino acids and grape extract polyphenols, AAP) provided during the post-weaning period may improve the pig's capacity to cope with the weaning transition and poor hygiene conditions in the beginning of the growing period. In the Chapter 6, experimental limitations and final considerations are presented. Finally, the perspectives and their implications for further research are summarized and discussed in the Chapter 7.

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CHAPTER 2 – LITERATURE REVIEW

2.1 Environmental challenges in pig production

Current predictions indicate that global ambient temperature will increase from 1.5 to 2.0°C in 2050 as well as frequency, duration, and intensity of heat stress events (data compared to the year 1900; IPCC, 2021). Regarding swine industry in tropical and temperate regions, high ambient temperatures may affect pig metabolism, health, and welfare resulting in reduced growth performance and economic losses (Poullet et al., 2022). For instance, annual economic loss due to heat stress for US swine industry was estimated to be around $300 million (St-Pierre et al., 2003). When compared with other livestock species, pigs are particularly sensitive to heat stress. Pigs have few functional sweat glands and thick layer of subcutaneous adipose tissue which make them more depend on the respiratory route for heat dissipation (Ross et al., 2015). Furthermore, the long-term focus on pig breeding programs worldwide has been for high productivity (Guy et al., 2012). However, the increase in genetic selection for production traits (growth performance, lean mass deposition and reproductive capacity) has resulted in pigs with lower capacity to cope with heat stress. For instance, growing-finishing pigs select to lean tissue accretion had lower average daily feed intake and daily gain (ADFI and ADG, respectively) in daily cyclic high ambient temperature exposure when compared with non-select pigs (Fraga et al., 2019). This lower capacity to cope with heat stress may be explained by the increased metabolic heat production as result of greater lean tissue deposition (Brown-Brandl et al., 2004). In the scenario of increased pig production in tropical and subtropical regions associated with lower capacity of modern genotypes to cope with high ambient temperatures, increased attention has been paid to the quantification of the effects of high temperatures on pig growth performance (Campos, 2014). In a recent study, Santos et al. (2021) reported that synthetic-sired pigs (SYN) had faster growth rate than Hampshire-sired pigs (HAM) at thermoneutral conditions (22ºC). Despite both lines been affected by high ambient temperature exposition (33ºC), HAM pigs had less impaired growth performance than SYN pigs (between 22ºC and 33ºC conditions, the ADFI decreased by 37% and 45% for HAM and SYN pigs, respectively), suggesting

more prominent effects of heat stress for pigs with faster growth potential (Santos et al., 2021).

Global warming is also associated with the development and dissemination of disease vectors, parasites, and pathogens in the environment. This situation is especially important in tropical climate areas, in which high ambient temperatures and sanitary challenges may occur concomitantly and may induce animal responses to both challenges (Campos et al., 2017). Increase in environmental pathogenic pressure can be aggravated when biosecurity rules, animal density, and degree of hygiene of the facilities are not respected. Sanitary challenges, including poor hygiene of housing conditions, result in immune system activation which in turn, may negatively impact health status and growth performance of pigs (Le Floc'h et al., 2014a; Campos et al., 2017). For instance, decrease in ADFI (5%) and ADG (10%) from 0 to 40 days post-weaning were reported for pigs kept in poor sanitary conditions when compared with non-challenged pigs (Le Floc'h et al., 2009). Accordingly, lower average body weight (BW) after 6 weeks exposed to poor hygiene conditions was observed for pigs housed in poor than in good hygiene conditions (7kg/animal of difference between hygiene conditions; Chatelet et al., 2018). One of the reasons of this lower performance is because immune challenge exposure may affect nutrient repartitioning away from productive processes toward immune-related functions. During an immune response, amino acids (AA) are diverted from growth functions to the synthesis of acute phase proteins, immune cell proliferation and other compounds related with body defenses (Le Floc'h et al., 2018). Greater incidence of soft feces and diarrhea (Pastorelli et al., 2012a) and lower villus height (Cho et al., 2021) were observed in pigs raised in poor than those raised in good hygiene conditions indicating intestinal inflammation and disrupted integrity in sanitary challenge conditions.

Studies evaluating the effects of high ambient temperature and sanitary challenge (reported hereafter as environmental challenges) on physiological and metabolic responses are utmost toward a better understanding of pig adaptive response on farm conditions. In the first study of this thesis work (Chapter 3), growing-finishing pigs were exposed to daily cyclic high ambient temperature, a condition in which pigs are usually raised in tropical climate areas. Furthermore, poor hygiene of housing condition as a methodological approach to study the

effects caused by the activation of pig's immune system was used in the second and third study (Chapter 4 and 5) of the current thesis.

2.2 Adaptive responses of pigs exposed to heat stress and sanitary challenge conditions: A brief overview

Adaptation can be defined as an organism's response to its surrounding environment (Engle, 2011). Since environmental challenges can substantially stimulate the adaptive responses of animals, the concept of adaptive capacity has recently been defined as the ability of a species or population to cope with climate changes (Beever et al., 2016). When exposed to a stressor (considered in this thesis work as environmental challenge that stimulate physiological, metabolic, or behavioral adjustments; Chu et al., 2021), adaptive responses are triggered in an attempt to support vital body functions of animals within the normal range without compromising their productive, reproductive, and growth activities. However, depending on the magnitude and degree of exposure to the stressor, adaptive capacity may be overwhelmed and the animal may fail to maintain homeostasis and growth activities in their normal ranges. Since exposure to environmental challenge negatively affect well-being, health, and growth performance of the animals (Rojas-Downing et al., 2017), research that considers the associated effects of environmental challenge and adaptation on livestock have important implications for the development of animal production systems (Escarcha et al., 2018). Furthermore, a better understanding of the impact of these challenges on adaptive responses of animals can provide useful information to support the development of strategies that minimize the effects of stress-induced responses.

As pigs are homeothermic animals, they have the ability to maintain their body temperature relatively constant, within a narrow range, despite fluctuations in ambient temperature (Gourdine et al., 2021). When exposed to high ambient temperature, in order to decrease heat production and/or increase body heat dissipation, thermoregulatory responses are actived (Campos et al., 2017). Pigs dissipate heat through behavioral adjustments, and sensible and latent heat losses. Sensible mechanism of heat loss (which includes heat exchanges by conduction, convection and/or radiation; Figure 2) are dependent of gradient of temperature between animal and ambient. In a condition where the ambient

temperature is close to skin temperature, animals decrease sensible heat loss and increase latent heat loss mechanisms. Latent losses include evaporation of sweat from the body surface and water evaporation from the respiratory tract (Campos, 2014) and are dependent on water vapor pressure gradient (Mayorga et al., 2019), which may partially explain why the associate effects of high temperature and high relative humidity increase the effect of heat stress. For pigs, the major component to increase evaporative heat loss is through increasing the respiratory rate. In agreement, greater respiratory rate was observed for pigs housed at high ambient temperature (32.2°C and 30.0°C; night and day, respectively) than pigs under thermoneutral conditions (21ºC; Rudolph et al., 2022). Decreased feed intake and consequently, the caloric increment associated with nutrient digestion and metabolism and physical activities, constitutes an adaptive mechanism of pigs under high ambient temperature (Renaudeau et al., 2012). Accordingly, Santos et al. (2018) reported that the time spend feeding, feed intake rate, and feed intake per meal were 15% lower for growing pigs exposed to heat stress[5] conditions (30ºC) when compared with those raised in thermoneutral conditions (22ºC). Furthermore, Renaudeau et al. (2011) estimated that each additional degree in ambient temperature between 20 to 30°C in 50kg BW pigs results in a decrease of 32 and 18 g/day in ADFI and ADG, respectively. Apart from growth performance impacts, high ambient temperature exposure may also induce negative consequences on pig health. When compared with pair-feeding pigs under thermoneutral conditions (22°C), heat-stressed pigs (33°C) had greater serum concentrations of diamine oxidase (DAO[6]), in association with lower concentrations of superoxide dismutase (SOD; Xia et al., 2022). These findings suggest that heat stress affects the intestinal barrier function and antioxidant defense system independent of reduced feed intake. Accordingly, lower villus height: crypt depth ratio on jejunum was observed in heat-stressed pigs (35ºC) than pair-feeding pigs raised in thermoneutral conditions (20ºC; Pearce et al., 2013).

[5] Animals are heat-stressed when the environmental temperature is higher than the upper critical temperature of the thermoneutral zone (Rauw et al., 2017).
[6] Diamine oxidase is a digestive integrity biomarker. In humans, an increase in plasma DAO concentrations reflect impairment of intestinal morphology and lower nutrient absorption (Cai et al., 2019).

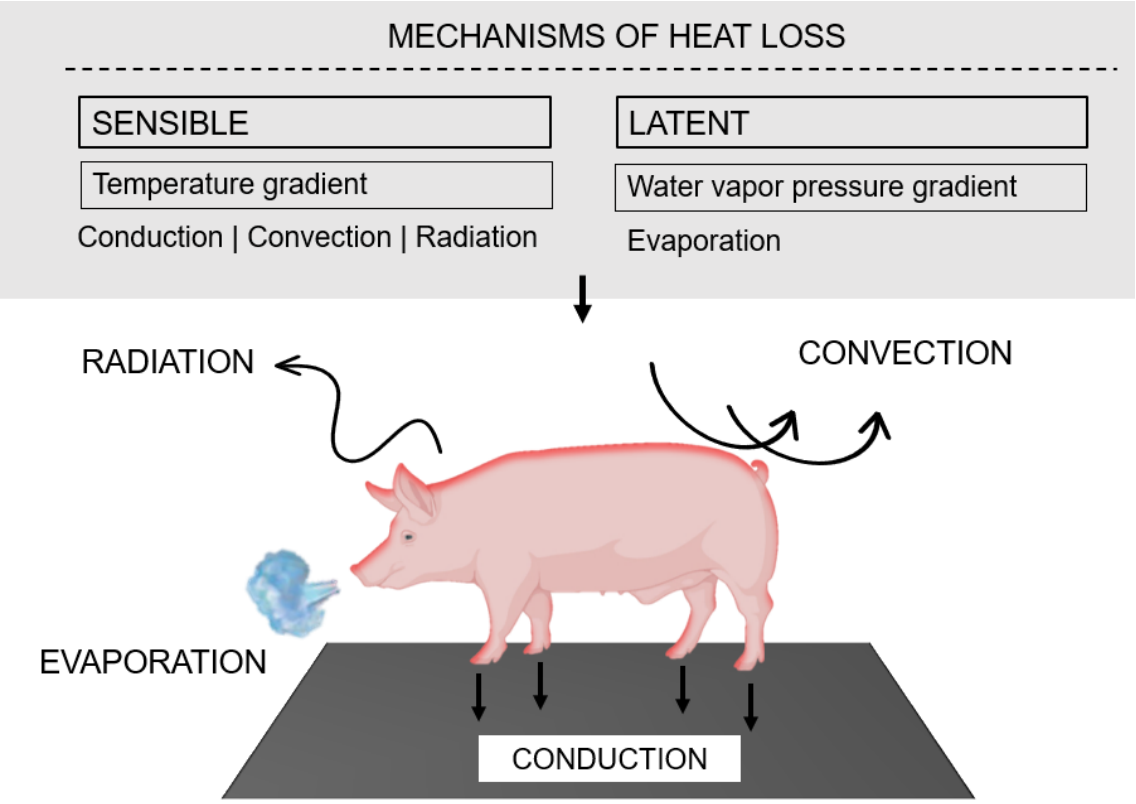

Figure 2. Mechanisms of heat loss in response to high ambient temperature.

Heat stress is not the only factor that can result in adaptive responses. Physiological and metabolic disturbances in pigs are also observed during an immune system stimulation (Campos et al., 2017). For instance, greater concentrations of interleukins 1 and 6 and tumor necrosis factor (IL-1, IL-6, and TNF, respectively) were observed in *Actinobacillus pleuropneumoniae*-infected pigs when compared with non-challenged pigs (Wyns et al., 2015). Pro-inflammatory cytokines (in particular, IL-1, IL-6 and TFN) are produced mainly by dendritic cells and macrophages during the early stages of inflammation (innate immunity[7]) and are related with synthesis of acute phase proteins, fever, and other metabolic and neurological adjustments (Nordgreen et al., 2020). Accordingly, greater plasma concentrations of haptoglobin, an acute phase protein, were observed in pigs housed in poor than in good hygiene conditions, which was associated with greater inflammatory status (Chatelet et al., 2018). Phagocytic cells that participate in both innate and adaptive immunity[7] produce reactive oxygen species (Robinson, 2008; Lim et al., 2017) and therefore, a

[7] Innate immunity constitutes a rapid and nonspecific immune response without involving an immunological memory. Chemical-mechanical barriers represents the first line of defense against pathogens, including skin, stomach pH, enzyme secretion, and mucus (Riera Romo et al., 2016). As an immediate response after challenge (innate immunity), is observed an increase in plasma concentrations of cytokines in particular IL-1, IL-6, and TNF (Le Floc'h et al., 2004). Depending on the magnitude and nature of the challenge, are observed specific responses (acquired or adaptive immunity) that include the synthesis of T and B lymphocytes (Riera Romo et al., 2016). In addition to enhancing the production of defense cells, this type of response establishes long-term memory mechanisms (Marshall et al., 2018).

prolonged immune response can also induce imbalance of the antioxidant system leading to oxidative stress. Accordingly, greater oxidative stress characterized by grater plasma concentrations of diacron-reactive oxygen metabolites (dROM[8]) were observed in pigs housed in poor than those housed in good hygiene conditions (Sierzant et al., 2019). Reduction of voluntary feed intake is also a response of animals housed in sanitary challenging conditions. Pastorelli et al. (2012b) described a reduction from 8 to 23% in feed intake of pigs exposed to different sanitary challenges when compared with non-challenged animals. Since sick animals reduce their feed intake, metabolic changes including mobilization of body reserves to support the synthesis of immune cells and other compounds related with immune response are observed. Glucocorticoids form a key component of the immune response (Whirledge and Cidlowski, 2010) and are involved in metabolic adjustments to support immune functions, such as: 1) Mobilization of energy stores through gluconeogenesis and glycogenolysis, 2) Enhanced protein catabolism to provide AA which can be utilized as an additional substrate contributing to the syntheses of immune components, 3) Increased lipolysis, and 4) Induce insulin resistance (Tsigos et al., 2020). Greater circulating levels of cortisol and higher mRNA expression of corticotrophin-releasing factor in jejunum (Guo et al., 2022) in addition to greater plasma concentrations of insulin and glucose (Campos et al., 2019) were reported in immune-challenged pigs. Furthermore, lower protein retention was observed in pigs challenged by *Escherichia coli* lipopolysaccharide (LPS) when compared with non-challenge pigs (McGilvray et al., 2019).

2.3 Modulatory effects of genetic selection and nutrition on pig adaptive responses exposed to environmental challenges

The genetic selection of pigs has focused mainly on productive traits. However, increase production levels may result in high physiological demands that may affect the capacity of pigs to cope with challenges (Prunier et al., 2010). Pigs showed a reduced curvilinear feed intake with increase in ambient temperature where a more pronounced effect was observed in recent studies, suggesting that modern genotypes could be more sensitive to heat stress than moderate growth lines (Renaudeau et al., 2011). Pigs selected for greater lean

[8] dROM: Measure of oxidative stress. The dROM test measures the concentration of hydroperoxides generated from the peroxidation of lipids, proteins, or nucleic acid (Kilk et al., 2014).

tissue accretion may have a reduced capacity to cope with heat stress because they produce more metabolic heat (Brown-Brandl et al., 2004). Therefore, adaptive thermoregulatory responses including changes of feeding behavior pattern may be more important for pigs with greater lean growth potential (Campos et al., 2017). Investigating plasticity of feeding behavior (i.e. how animals modify their feeding behavior; Poullet et al., 2022) of pigs when exposed to high ambient temperature can provide critical information for improving feeding strategies, growth performance, and animal well-being (Gomes et al., 2021). Indeed, moderate correlations were found between behavior and performance traits. For duroc barrows, feed intake rate (defined as the feed intake for each minute spent feeding) was positively correlated with average ADFI and ADG (0.29 and 0.38, respectively; Rauw et al., 2006). Besides leaner genotypes, selection for most efficient animals has gained attention (Boddicker et al., 2011). Feed cost is the most expressive economically index of livestock production (60-70% of total production costs; Rauw et al., 2020) and therefore, breeding strategies has focused on optimizing animal feed intake. Residual feed intake (RFI) is a measure of feed efficiency. For a similar production level, high-RFI pigs eat more than predicted and therefore are less efficient than low-RFI pigs (HRFI and LRFI respectively; Gilbert et al., 2017). It could be expected that most efficient pigs had lower capacity to cope with challenges than less efficient pigs because selected pigs for high production efficiency are more susceptible to have immune issues (Rauw et al., 1998). However, when LRFI and HRFI pigs were housed in poor hygiene of housing conditions, LRFI pigs had less impaired growth performance and health than HRFI pigs (Chatelet et al., 2018) which suggests a better ability of LRFI line to cope with poor hygiene conditions. Therefore, in the second study of the current thesis (Chapter 4), we hypothesized that the difference in coping ability between pigs from LRFI and HRFI lines may involve changes in their metabolism. Our objective was to evaluate whether metabolic modifications (postprandial plasma concentrations of insulin, energy-related metabolites, and AA) induced by poor hygiene of housing conditions differed between pigs from LRFI and HRFI lines.

The diet composition may also modulate the adaptive response of pigs exposed to challenge conditions. During weaning transition, pigs are exposed to multiple stressors (including changes of the diet, separation from their mothers

and mixing with unfamiliar littermates) that predisposed the animals to reduced feed intake, impaired intestinal functions, and nutrient absorption; all contributing to post-weaning diarrhea and poor growth (Campbell et al., 2013). In order to minimize the effects of weaning-induced stress especially in view of the ban of in-feed antibiotics in livestock production, nutritional strategies including feed additives have been widely studied (Zheng et al., 2021). Nutrients (along with feed additives, extracts, and microorganisms) can modulate the immune response, gut development, and antioxidant capacity of pigs. Therefore, a better understanding of their modulatory effects may support nutritionists in formulating more adapted diets and contribute to the development of nutritional strategies to improved animal performance and health (Rodrigues et al., 2022). Therefore, in the Chapter 5 of the thesis it was evaluated the dietary supplementation with a blend of some AA and grape extract polyphenols as a nutritional strategy for pigs exposed to challenge conditions (weaning transition and a subsequent challenge caused by poor hygiene of housing conditions). More details are discussed in the following section.

2.4 Potential use of amino acids and grape extract polyphenols for immune-challenge pigs

Besides being building blocks of proteins, AA are known to be nutrients that contribute to health maintenance. Changes in AA plasma concentrations have been observed in inflammatory-challenged pigs (Campos et al., 2019; Le Floc'h et al., 2014b) and could be associate with some major factors, such as: 1) Synthesis of immune components to support innate and/or adaptive responses, 2) Redistribution from growth and production functions towards tissues involved in inflammatory and immune responses, 3) Decreased anabolism and/or increased catabolism of skeletal muscle to increase the availability of nutrients as consequence of reduced feed intake. The plasma kinetics of AA evaluation (that is, the appearance and disappearance profile in the plasma) allows a better understanding of the metabolic alterations during an immune system activation (Campos et al., 2019). Changes in postprandial plasma profile of AA in response to an immune challenge were potentially explored in this thesis work (Chapter 4). Functional AA was defined as those AA that exert functions other than being precursors of proteins (Wu, 2013). Their potential use to preserve and improve

intestinal health, oxidative homeostasis, microbiota balance and immune functions were recently reviewed by Chalvon-Dermasay et al. (2021). For instance, dietary supplementation with arginine (Arg; Wu et al., 2010), branched-chain amino acids (BCAA; Zhang et al., 2013), cysteine (Cys, Song et al., 2016), and/or glutamine (Gln; Wang et al., 2015) separately or in a blend (Prates et al., 2021) was associated with improvement of intestinal integrity and immune status in weaned and/or inflammatory-challenge pigs. Accordingly, supplemented diet with a blend of AA (L-tryptophan, Arg, Cys and BCAA) supported pigs weaning transition and alleviated post-weaning diarrhea (Wessels et al., 2021). A summary of studies that evaluated supplementation of Arg, Cys (and cystine; Cys2), Gln (and glutamate; Glu), and BCAA for pigs under challenge conditions are presented in Table 1.

Besides AA, plants extract containing proanthocyanidins, the most common and consumed dietary polyphenols, are also characterized by diverse bioproperties such as antioxidant activity and ability to modulate the intestinal microbiota (Andersen-Civil et al., 2021). As a rich source of proanthocyanidins, grape extract polyphenols have been increasingly studied as a potential health-promoting dietary component. Previous studies have demonstrated a reduced post-weaning diarrhea incidence in piglets fed with a diet supplemented with grape polyphenols by improvement of mucosal morphology (Han et al., 2016) and antioxidant capacity (Hao et al., 2015), in addition to minimizing the growth depressed associated with the weaning (Han et al., 2016). Beaumont et al. (2021) recently demonstrated that feed supplementation with a blend of functional AA and grape extract polyphenols contributed to preserving the intestinal health and growth performance of piglets during the post-weaning period. More specifically, it was demonstrated that this supplementation resulted in greater production of protective bacterial metabolites by increasing the abundance of *Lactobacillus* in the jejunum and reduced the abundance of *Proteobacteria* in the caecum (Beaumont et al., 2021). The effects of a blend of functional AA (Gln, Arg, Cys2 and BCAA) and grape extract polyphenols dietary supplementation on pig capacity to cope with weaning and a subsequent sanitary challenge were potentially evaluated in the third study of this thesis work (Chapiter 5). In this study, the immunomodulatory[9] effects of the blend on the animal's robustness facing to weaned-induced stress were discussed.

[9] An immunomodulatory nutrient can be defined as a nutrient that, when fed at dietary levels higher than the established requirement, alters specific and non-specific mechanisms of the immune response (Wils-Plotz and Klasing, 2017).

Table 1. Summary studies that evaluated supplementation of arginine, cystine (and cysteine), glutamine (and glutamate), and branched-chain amino acids for pigs under challenge conditions.

Amino acid	Main results	Challenge model	Reference
Arginine (Arg)	0.8 and 1.6% L-Arg: ↑ concentrations SOD and GSH-Px and ↓ IL-6 and TNF-α	Induced oxidative stress	Zheng et al., 2013
	0.5 and 1% L-Arg: ↑ villus height and ↓ crypt depth, IL-6 and TNF-α	LPS	Liu et al., 2008
	0.5 and 1% L-Arg: ↑ villus height/crypt depth (ratio) and ↓ mast cells	LPS	Zhu et al., 2013
	1% L-Arg: ↑ growth performance, concentrations GSH-Px and ↓ crypt depth	Mycotoxin	Yin et al., 2014
Cystine (Cys2) and cysteine (Cys)	L-Cys administration: ↓ crypt depth, MPO activity, IL-6, IL-12, IL-1IL-10 and TNF-α	Colitis	Kim et al., 2009
	↑ metabolic demand of Cys is mainly related with the synthesis of GSH	LPS	Rakhshandeh et al., 2019
	NAC (500 mg/kg): ↑ ADG, claudin-1, occludin, villus height and ↓ crypt depth	LPS	Hou et al., 2012
	NAC-supplemented liquid diet (50 mg/kg BW): ↑ redox status and ↓ crypt depth	PEDV	Wang et al., 2017
Glutamine (Gln) and glutamate (Glu)	0.2% L-Gln: ↓TNF-α	Weaning and transport	Duttlinger et al., 2019
	1% (L-Gln plus L-Glu): ↑ growth performance (ADG, ADFI and feed conversion) and ↓ diarrhea	Weaning	Teixeira et al., 2014
	0.5, 1, 2, or 4%: ↑ villus height, GSH and ↓ diarrhea	Weaning	Rezaei et al., 2013
	2% L-Glu: ↑ concentrations GSH-Px and ↓ crypt depth	Mycotoxin	Duan et al., 2014
Branched chain amino acid (BCAA)	1% leucine (L-Leu): ↑ mucin production, villus heiht, phosphorylated mTOR level, goblet cells and ↓ mean cumulative score of diarrhea	Rotavirus	Mao et al., 2015
	1% isoleucine (L-Ile): ↑ growth performance and ↓ diarrhea	Rotavirus	Mao et al., 2018

LPS: Escherichia coli lipopolysaccharide, PEDV: Porcine epidemic diarrhea virus, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, IL: Interleukin, TNF: tumor necrosis factor, GSH: glutathione (reduced form), MPO: myeloperoxidase, NAC: N-acetylcysteine, ADG: average daily gain, ADFI: average daily feed intake, BW: body weight, mTOR: mechanistic target of rapamycin signaling.

2.5 Conclusions of the literature review

Pig responses to stressful situations include behavioral, physiological, and metabolic defense mechanisms in an attempt to re-establish homeostasis and survival in a specific environment. Depending on the magnitude and degree of exposure to the stressor, adaptive responses may not be sufficient and under these conditions, are observed undesirable effects on welfare, health, and growth performance of animals. Because of more susceptibility of modern genotypes to environmental challenges, global warming issues (including development of diseases), sanitary-related problems, and stressful management practices (in particular, weaning), the negative effects of challenge exposure may be especially important in the current intensive production systems. Therefore, the current livestock systems will need to adapt to this and other challenges (limited resources, increased production, etc.) in order to support the growing demands for food without compromising the ability of the future generations to also meet their requirements (Andretta et al., 2021).

The literature review evidenced the adaptive responses of pigs to environmental challenge conditions (high ambient temperature and sanitary challenges) and the potential effects of genetic selection and nutrition on these responses. Understanding the adaptive responses of pigs in the context of challenge contribute to a better comprehension of the interactions between animal-environment. Furthermore, the thematic of “adaptive responses of animals exposed to environmental challenges” raises important questions: How to apply the concept of sustainability of production systems in a condition where animals are more and more exposed to environmental challenges? How to support the growing demand for animal products while respecting the welfare of farm animals? What is the role of scientific research in this context? And finally, how far can human intervention go?

2.6 General objective and hypothesis

The main objectives of this thesis were to bring new knowledge on the adaptive responses of pigs exposed to environmental challenge conditions (daily cyclic high ambient temperatures and poor hygiene of housing conditions) and to determine if and how genetic selection and/or nutritional strategies (in particular, amino acids) may influence these responses.

Three studies were designed to evaluate the following hypothesis (Figure 3):

- First study (Publication nº1): Genetic selection for lean tissue accretion modulates feeding behavior of growing-finishing pigs exposed to daily cyclic high ambient temperatures.
- Second study (Publication nº2): Differences in the ability to cope with poor hygiene conditions between less and more efficient line pigs can be explained by changes in postprandial plasma profiles of AA and metabolites.
- Third study (Submitted): Beneficial effects of AA (glutamine, arginine, cystine, valine, isoleucine, and leucine) and grape extract polyphenols supplementation persist after the post-weaning period and confer a greater ability of pigs to cope with a subsequent inflammatory challenge caused by poor hygiene conditions.

2.7 Schematic representation of the thesis

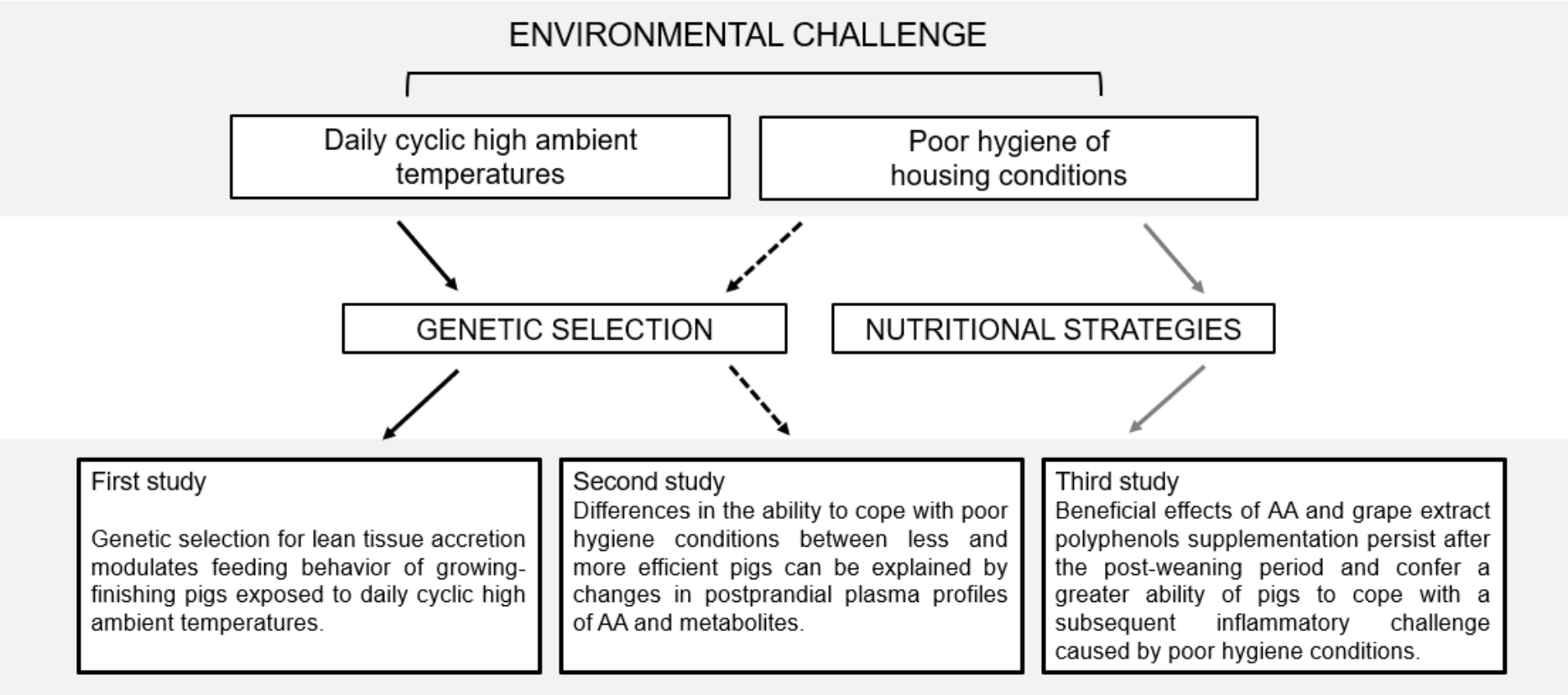

Figure 3. Three studies were designed to evaluate the main objective of this thesis: Understanding of the adaptive responses of pigs exposed to environmental challenge conditions (daily cyclic high ambient temperatures and poor hygiene of housing conditions) and how genetic selection and/or nutritional strategies (in particular, amino acids) may influence these responses.

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CHAPTER 3 – GENETIC SELECTION MODULATES FEEDING BEHAVIOR OF GROUP-HOUSED PIGS EXPOSED TO DAILY CYCLIC HIGH AMBIENT TEMPERATURES[10]

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Abstract

This study was conducted to evaluate the effect of genetic selection (Lines A and B; Line A pigs have a greater proportion of Pietrain genes than those from Line B and therefore, selected for improved lean tissue accretion) on the feeding behavior of group-housed pigs exposed to daily cyclic high ambient temperatures. Feeding behavior of 78 barrows housed together in a single room was recorded in real time by five automatic feeders. The feeders registered each visit of each pig (day, hour, min, and second) and the amount of feed requested. Daily cyclic high ambient temperature was induced exposing pigs at 22°C from 18.00 to 10.00 h and 30°C from 10.01 to 17.59 h. From this temperature variation, day-period was divided into: 22°C(06-10h), from 6.00 to 10.00 h; 30°C(10-18h), from 10.01 to 17.59 h; and 22°C(18-06h), from 18.00 to 5.59 h. Meal criteria was estimated based on the probability of animals starting a new feeding event within the next minute since the last visit (Pstart). After defining the meal criteria, the number of meals (n), feed intake rate (g/min), feed intake (g/meal), feeder occupancy (min/meal), and interval between meals (min) of each animal were calculated. Greatest probability of starting to feed was observed at 22°C(06-10h), followed by 30°C(10-18h) and then 22°C(18-06h). Regardless of time period, pigs from line A had greater feed intake rate and lower feed intake, feed occupancy per meal and probability of starting a meal when compared with line B pigs. Only line A pigs had greater feed intake and feeder occupancy per meal at 22°C(18-06h) than remainder of the day. This indicates that pig feeding pattern is strongly related to the circadian rhythm. However, the genetic selection for improved lean tissue accretion may modulate pigs feeding behavior under daily cyclic high ambient temperatures.

Introduction

Pigs show a diurnal feeding pattern characterized by two peaks of feeding activity over 24 h-day [1]. However, the feeding behavior of pigs may be affected by several factors, including genetic selection [2] and environmental conditions [3]. Mainly through changes in meal size, lower feed intake for pigs under cyclic high ambient temperature has been observed when compared to thermoneutral conditions [4]. Despite such scientific finding, most of the information of heat stress on feeding behavior of pigs were obtained from experimental protocols with animals housed individually (or with few animals per pen), and/or exposed to constant high temperatures during the day [5]. Such studies do not apply to commercial conditions, especially in tropical areas, in which group-housed pigs are usually reared in semi-open buildings and then exposed to daily temperature variations. In addition, animals selected for improved lean tissue accretion produce more metabolic heat and may have a reduced capacity to cope with heat stress [6].

A better understanding of feeding behavior may contribute to the genetic selection of individuals based on their thermoregulatory responses, productive traits, and well-being. Furthermore, the detection of changes in feeding behavior may be an interesting and useful tool for identifying heat stress in the early stages of exposure and may help in decision-making to prevent major losses. Equipment such as electronic feeders provides a promising approach to evaluate pigs feeding behavior responses since it allows recording automatically information on feed intake of individual pigs in a group-housed condition [7]. In a previous study, we have demonstrated that pigs selected for

improved lean tissue accretion (i.e., with a greater proportion of Pietrain genes) had lower growth performance when housed under daily cyclic high ambient temperature conditions [8]. We hypothesized that in such conditions, pigs prioritize feed intake in the coolest hours of the day and that their feeding behavior is modulated by genotype. Therefore, in the current study we further evaluated the effects of genetic selection on the feeding behavior of group-housed growing-finishing pigs exposed to daily cyclic high ambient temperatures.

Material and methods

All experimental procedures were reviewed and approved according to the current ethical standards of the Ethical Committee for Care and Use of Experimental Animals of the School of Agricultural and Veterinarian Sciences of São Paulo State University (protocol N° 18077/16).

Data origin

Pigs feeding database was obtained from our previous study [8]. In this study, the feeding behavior of 78 barrows (22 ± 2.5 kg of body weight, BW) from two commercial genetic lines (39 pigs of each genetic line, reported hereafter as Line A and Line B; Agroceres PIC, Rio Claro, Brazil) was recorded in real-time by five automatic feeders (AIPF; University of Lleida, Lleida, Spain). Both lines consisted of Large White × Landrace × Duroc × Pietrain multiple crosses, with Line A pigs having a greater proportion of Pietrain genes than those from Line B, as previously described [9]. Pigs were housed together in a single room (1.19 m2/animal; without environmental enrichments) with a full concrete floor equipped with an evaporative pad cooling system (Big Dutchman, Araraquara,

SP, Brazil) and electric heaters, automatically controlled to maintain the ambient temperature at 22°C from 18.00 to 10.00 h and 30°C from 10.01 to 17.59 h. These temperatures aimed to simulate daily cyclic ambient temperature variations that pigs are usually exposed to in tropical climate areas.

Pigs remained in the experiment for 102 days, which consisted of an 18-day adaptation period and a subsequent 84-day experimental period, divided into growing phase 1 (30 to 45 kg BW; days 0 to 20); growing phase 2 (45 to 75 kg BW; days 21 to 48); and finishing phase (75 to 105 kg BW; days 49 to 83). Pigs had free access to water delivered via 10 beat ball drinkers distributed all over the pen and were fed *ad libitum* with diets formulated to meet their nutritional requirements of the animals with the highest potential for lean deposition (Line A) according to NRC [10] recommendations. The photoperiod was fixed from 6.00 to 18.00 h of artificial light controlled by a timer switch. At the beginning of the adaptation period, one transponder (plastic button tag containing passive transponders of radio frequency identification; Allflex, Joinville, SC, Brazil) was inserted in the right ear of each pig using tagger pliers, and then animals were introduced to the electronic feeders. The use of exclusive identification codes per pig allowed recording individual feed intake over the trial with all animals housed in the same pen in a single group.

The functioning of these feeders was previously described [11]. The electronic feeders (AIPF) identify each pig when its head is introduced into the feeder and deliver feed in response to each animal request. The serving size was 15 g at the beginning and 25 g at the end of the experiment. A time lag (18 sec) was imposed to ensure that pigs consumed each serving before requesting a new

one. Pigs tended to leave the feeder hopper empty or to leave small amounts of feed after each visit. All pigs in the pen could access any of the feeders.

Overview of data compilation

Feed intake was calculated using the feeding information of each pig (AIPF Software). The feeders were equipped with a monitoring tool that continuously registered each visit of each pig with start and stop times (day, hour, minute, and second) and the amount of feed requested. Feeding information collected on days on which animals were handled (weighed or scanned, six days) or on days that some eventuality happened (equipment malfunctions, momentaneous data record error, etc., which correspond to 12 days) were removed from the data. The total information from the initial data set consisted of 129,167 records, which were then analyzed using R Statistical Software (version 2.14.0; R Foundation, Austria).

The presence of outliers was evaluated through graphical analyses and by daily records of anomalies during the experimental period. Therefore, the first procedure was data filtering. Data of animals whose interval between visits was greater than 720 min (3.78% of observations from the initial database) were arbitrarily excluded from the database and assumed to be physiologically noncompatible data [12]. After a graphical analysis of the new database, a second filter criterion was applied. Data of feeder occupancy greater than 30 min (0.48% of observations) and zero feed intake (0.35% of observations) were also excluded. Records without feed intake may be associated with animal-feeder interactions, in which quick entry and exit of animals at the feeder are recognized by the software. Therefore, data filtering, although arbitrary and

variable according to the database, is a biologically relevant step since it allows a more realistic view and avoids possible incorrect conclusions garnered from the analysis of feeding behavior. After the filtering steps, no other discrepancies were observed in the database (more details can be found in S1 Dataset).

Meal criteria

A pig visit to the feeder is not necessarily a meal. As aforementioned, a visit record can be caused by the quick entry and exit of the animal, which may even be considered a nonfeeding event. Therefore, expressing feeding behavior data in terms of meals is considered a more appropriate unit [13]. A meal is defined as a cluster of feeding visits interrupted by short pauses [14]. Then, a criterion clustering visits into a single meal must be applied. This point is important for studies of animal behavior since the absence of the definition of a meal criterion (or even the mathematical method to be used) may result in the loss of biologically significant information. For this purpose, the probability of animals starting a new feeding event within the next minute since the last visit (Pstart) was calculated for each factor of our study (i.e., periods of the day and genetic lines). Thus, the meal criteria were estimated as the first point that minimizes Pstart curve as was previously described [15]. To reduce the effect of random variation in the Pstart function, a simple moving average (SMA) over 5-min intervals was used.

Calculations and Statistical Analysis

After defining the meal criteria, a new database was created using the meal criterion to group visits that have composed the same meal. The new database

consisted of 39,680 records. This database was used to calculate the number of meals (n), feed intake rate (defined as the feed intake for each minute spent feeding, g/min), feed intake (g/meal), feeder occupancy (min/meal), and the interval between meals (min) of each animal. As pig feeding behavior changes throughout the 24-h day [1], for a more adequate approach and considering temperature variation during the day, the day was divided into three periods: 22°C(06-10h), from 6.00 to 10.00 h; 30°C(10-18h), from 10.01 to 17.59 h; and 22°C(18-06h), from 18.00 to 5.59 h. Because the periods of the day had different durations, feeding behavior variables were expressed per hour.

The Cramer-von Mises test was used to verify the normality of studentized residuals. The data were analyzed using the linear mixed effects model framework. The implementation was via the MIXED procedure of SAS (version 9.3, SAS Inst. Inc., Cary, NC) and were presented hereafter as least squares means. The individual pig was considered the experimental unit and the initial BW (weight of the animal on the first day of the experimental period) was used as covariable between genetic lines when significant in the model. The model included the fixed effects of the period of the day (TP), genetic line (GL), experimental phase (P), and their interactions (TP×GL, TP×P, GL×P, and TP×GL×P) as follow:

$$Yijmk = \mu + \alpha i + \beta j + \gamma m + (\alpha\beta)ij + (\alpha\gamma)im + (\beta\gamma)jm + (\alpha\beta\gamma)ijm + eijmk$$

where Yijmk is the observed variable (observation for the *k*th animal from the (i,j,m)th cell), μ is the overall mean, αi is the effect of the *i*th level of the TP factor, βj is the effect of the *j*th level of the GL factor, γm is the effect of the *m*th level of the P factor, and their interactions (that is, *i*th level of TP with the *j*th

level of GL, (αβ)ij; *i*th level of TP with the *m*th level of P, (αγ)im; *j*th level of GL with the *m*th level of the P, (βγ)jm; and *i*th level of TP with the *j*th level of GL with the *m*th level of the P, (αβγ)ijm). The e_{ijmk} was considered the random error of *k*th observation from (i,j,m)th. The repeated measurement option was used with a compound symmetry covariance structure to account for the animal effect over sampling time. Differences were considered significant if $p < 0.05$. When there was an effect of an interaction between period and genetic line ($p < 0.05$), adjusted means of genetic lines were compared in each period using the Tukey-Kramer test.

Prandial correlations

The prandial correlations were calculated for each animal individually through Pearson's correlation between the variables “meal size, min” and “interval between meals, min”. The correlation between “meal size” and “interval before meal” (pre-prandial) indicates satiety mechanism; whereas the correlation between “meal size” and “interval after meal” (post-prandial) indicates hunger mechanism [16]. The pigs were classified in four groups according to the significant correlation coefficients ($p < 0.05$) that showed: pre-prandial, post-prandial, pre- and post-prandial regulations, or no prandial correlations when both were not significant.

Results

General observations

Room ambient temperature averaged 22.3 ± 0.4°C from 18.00 to 10.00 h and 30.2 ± 0.5°C from 10.01 to 17.59 h. The daily relative humidity averaged 70.35

± 3.20%. These values were in accordance with our predefined objectives. Performance data were reported in our previous study [8], and individual pig behavior profiles are available in S2 Fig. Briefly, irrespective of the growth phase, pigs from genetic line A had lower average daily feed intake (ADFI), average daily gain (ADG), and final BW than pigs from line B ($p < 0.01$). At the end of the experiment, the difference in BW between lines was 11 kg on average (112.15 and 129.58 kg for line A and line B pigs, respectively; $p < 0.01$).

Pstart and meal criteria

Among the three periods, Pstart difference was observed up to approximately 100 min, a period in which the greatest probability of starting to feed was observed at 22°C$_{(06\text{-}10h)}$, followed by 30°C$_{(10\text{-}18h)}$ and 22°C$_{(18\text{-}06h)}$ (Fig 1). The meal criteria values were 48 min at 22°C$_{(06\text{-}10h)}$, 47 min at 30°C$_{(10\text{-}18h)}$ and 49 min at 22°C$_{(18\text{-}06h)}$; and for line A and line B were 52 min and 46 min, respectively. Therefore, as we had different periods (n = 3) and genetic lines (n = 2), the meal criteria applied in our database was the average of these five results (48 min). After defining the meal criteria, differences between the genetic lines (with a lower probability of line A to start a meal compared with line B) were observed mainly up to 450 min (Fig 2).

Fig 1. Probability of growing-finishing pigs exposed to daily cyclic high ambient temperature starting a new feeding event within the next minute since the last visit (Pstart) at 22°C$_{(06\text{-}10h)}$, 30°C$_{(10\text{-}18h)}$, and 22°C$_{(18\text{-}06h)}$.

Fig 2. Probability of growing-finishing pigs exposed to daily cyclic high ambient temperature starting a meal according to the interval between

meals (min) considering two genetic lines. Genetic Line A pigs had greater proportion of Pietrain genes than those from genetic Line B pigs.

Feeding behavior responses

Exploratory data analysis

Feeding behavior responses of growing-finishing pigs are presented in Table 1. Residuals of all dependent variables were normally distributed, and initial BW as covariate was significant ($p < 0.01$) for all feeding behavior variables. All responses were influenced by period, genetic line, and experimental phase ($p < 0.01$).

Regardless of line and experimental phase, feed intake rate was greater at $22°C_{(06\text{-}10h)}$, followed by $30°C_{(10\text{-}18h)}$, and then $22°C_{(18\text{-}06h)}$ ($p < 0.05$; Table 1). A GL×P interaction was observed for feed intake rate ($p < 0.01$; Table 1). Regardless of time period, line A pigs had greater feed intake rate than line B pigs in growing phase 2 (26.53 vs 24.98 g/min; $p < 0.01$) and total period (26.73 vs 25.95 g/min; $p = 0.01$).

Interactions between TP×GL, TP×P, and GL×P were significant for feed intake and feeder occupancy per meal (Table 1). At $22°C_{(06\text{-}10h)}$ and $30°C_{(10\text{-}18h)}$, pigs from line A had lower feed intake and feeder occupancy per meal than pigs from line B ($p < 0.05$; TP×GL). In contrast, at $22°C_{(18\text{-}06h)}$, line A pigs had greater feed intake and feeder occupancy per meal than line B pigs ($p < 0.05$). Despite of no significant differences among periods in growing phase 1, in the following phases, feed intake and feeder occupancy were decreased at $22°C_{(06\text{-}10h)}$ compared with $30°C_{(10\text{-}18h)}$ and $22°C_{(18\text{-}06h)}$ ($p < 0.05$; TP×P). Besides, except in

growing phase 1, in which feed intake and feeder occupancy per meal did not differ between lines, both were lower for line A than line B pigs in the subsequent phases ($p < 0.05$; GL×P).

A TP×GL interaction was observed for the interval between meals ($p = 0.02$; Table 1). At $22°C_{(06\text{-}10h)}$ and $30°C_{(10\text{-}18h)}$, pigs from line A had greater interval between meals than pigs from line B ($p < 0.05$). At $22°C_{(18\text{-}06h)}$, no difference between lines was found ($p = 0.34$). For both lines, regardless of period, the increase in BW between stages of growth was associated with an increased feed intake rate, feed intake per meal, and interval between meals ($p < 0.05$).

Table 1. Feeding behavior according to periods of the day and genetic lines of growing-finishing pigs exposed to daily cyclic high ambient temperature during 84-days of the experimental period.[1,2]

Periods	$22°C_{(06\text{-}10h)}$		$30°C_{(10\text{-}18h)}$		$22°C_{(18\text{-}06h)}$		Statistical analysis (*p*-value)[4]			
Genetic Lines[3]	A	B	A	B	A	B				
Number of animals	39	39	39	39	39	39	TP×GL	TP×P	GL×P	TP×GL×P
Feed intake rate, g/min	27.71 (0.5)	26.86 (0.8)	27.11 (1.2)	26.00 (2)	25.38 (0.5)	24.93 (2)	0.40	0.13	<0.01	0.98
Feed intake, g/meal	182.25^{c} (8)	256.46^{b} (12)	241.67^{b} (15)	299.91^{a} (9)	306.33^{a} (3)	267.74^{b} (4)	<0.01	<0.01	<0.01	0.17
Feeder occupancy, min/meal	6.96^{e} (3)	9.78^{cd} (3)	9.29^{d} (4)	11.69^{ab} (0.5)	12.43^{a} (0.4)	10.81^{bc} (2)	<0.01	0.01	<0.01	0.61
Interval between meals, min	162.19^{bc} (7)	128.60^{d} (4)	180.21^{a} (12)	150.14^{c} (5)	184.06^{a} (9)	173.22^{ab} (4)	0.02	0.56	0.08	0.39
Number of meals[5], n/pig	0.29 (0.1)	0.49 (0.1)	0.28 (0.4)	0.38 (0.3)	0.21 (0.2)	0.22 (0.2)	<0.01	<0.01	0.09	0.01

[1]Data after application of meal criteria defined as 48 min and expressed per hour of the day since the periods had different duration ($22°C_{(06\text{-}10h)}$: from 6.00 to 10.00 h, $30°C_{(10\text{-}18h)}$: from 10.01 to 17.59 h; $22°C_{(18\text{-}06h)}$: from 18.00 h to 5.59 h).

[2]Least squares means and standard error (SE).

[3]Genetic line A pigs had a greater proportion of Pietrain genes than those from genetic line B pigs.

[4]Probability of periods of the day (n = 3; TP), genetic line (n = 2; GL), experimental phase (n = 3; P), and interactions between TP×GL, TP×P, GL×P, and TP×GL×P. There was an effect of TP, GL, and P ($p < 0.01$) for all variables studied. For variables whose TP×GL interaction was significant, average values were analyzed by Tukey' test. Initial BW as a covariate was significant for all variables; $p < 0.01$.

[5]The TP×GL×P interaction for the number of meals is presented in Table 2.

[a,b,c] Values within a row with different superscripts differed between genetic lines in each period of the day ($p < 0.05$).

Interaction between TP×GL×P was significant for number of meals (Table 1). Overall, at 22°C$_{(06\text{-}10h)}$ and 30°C$_{(10\text{-}18\ h)}$, pigs from line A had lower number of meals than pigs from line B ($p < 0.05$; Table 2). Besides, an increase in the number of meals was observed only in the early stages (from growing phase 1 to growing phase 2), at 22°C$_{(06\text{-}10h)}$ for pigs from line A, and at 22°C$_{(06\text{-}10h)}$ and 30°C$_{(10\text{-}18h)}$ for pigs from line B ($p < 0.05$; Table 2). Only line B pigs became more diurnal throughout the trial, with 77, 79, and 83% of the meals consumed during the light period (at 22°C$_{(06\text{-}10h)}$ and 30°C$_{(10\text{-}18h)}$) in growing phases 1 and 2 and the finishing phase, respectively.

Table 2. Number of meals (n/pig) according to periods of the day, genetic lines, and experimental phases of growing-finishing pigs exposed to daily cyclic high ambient temperature.[1,2]

Periods	$22°C_{(06\text{-}10h)}$		*p*-value[4]	$30°C_{(10\text{-}18h)}$		*p*-value[5]	$22°C_{(18\text{-}06h)}$		*p*-value[6]
Genetic Lines[3]	A	B	A vs B	A	B	A vs B	A	B	A vs B
Number of animals	39	39		39	39		39	39	
Growing phase 1 (21 days)									
Number of meals, n/pig	0.21* (0.02)	0.34# (0.03)	<0.01	0.23 (0.02)	0.29# (0.02)	0.85	0.15 (0.01)	0.19 (0.05)	0.98
Growing phase 2 (28 days)									
Number of meals, n/pig	0.32* (0.01)	0.57# (0.01)	<0.01	0.31 (0.03)	0.42# (0.05)	<0.01	0.25 (0.02)	0.26 (0.01)	0.99
Finishing phase (35 days)									
Number of meals, n/pig	0.35 (0.01)	0.55 (0.02)	<0.01	0.30 (0.03)	0.44 (0.01)	<0.01	0.24 (0.01)	0.21 (0.05)	0.99
Total period (84 days)									
Number of meals, n/pig	0.29 (0.02)	0.49 (0.02)	<0.01	0.28 (0.01)	0.38 (0.02)	<0.01	0.21 (0.01)	0.22 (0.01)	0.99

[1]Data after application of meal criteria defined as 48 min and expressed per hour of the day since the periods had different duration ($22°C_{(06\text{-}10h)}$: from 6.00 to 10.00 h, $30°C_{(10\text{-}18h)}$: from 10.01 to 17.59 h; $22°C_{(18\text{-}06h)}$: from 18.00 to 5.59 h). Additional information regarding the pig feeding 272 behavior profile for each experimental phase is available in S3 Fig.

[2]Least squares means and standard error (SE).

[3]Genetic line A pigs had a greater proportion of Pietrain genes than those from genetic line B pigs.

[4,5,6]Statistical analysis between genetic lines in each experimental phase for $22°C_{(06\text{-}10h)}$[3], $30°C_{(10\text{-}18h)}$[4], and $22°C_{(18\text{-}06h)}$[5].

[*,#]Means differed between experimental phases for pigs from line A* and line B[#] ($p < 0.05$).

Descriptive statistics

Descriptive statistics for the total experimental period (from 0 to 84 days) are presented in Figure 3. For feed intake rate, regardless of the experimental phase, the interval values were 27.24 and 20.47 g/min for pigs from lines A and B, respectively (Fig 3). Despite greater dispersion data for line A pigs, the feed intake rate followed a similar pattern throughout the 24h-day for both lines (Fig 4A).

Average values of feed intake and feeder occupancy per meal were lower for line A than line B pigs (Fig 3). Such results were associated with a peak in feed intake and feeder occupancy per meal at $30°C_{(10\text{-}18h)}$ for line B pigs (at 11.00 h; Fig 4B and 4C) and a 14% difference in diurnal feed consumption between lines. In contrast, at $22°C_{(18\text{-}06h)}$ (specifically from 19.00 to 3.00 h), line A pigs had greater feed intake and feeder occupancy per meal than line B (Fig 4B and 4C; $p < 0.05$).

Pigs from line A had greater average value of interval between meals when compared with pigs from line B (175.49 vs 150.86 g/meal; Fig 3). In particular, greater values of interval between meals for line A pigs were observed mainly during the diurnal period (from 12.00 to 16.00h, Fig 4D). Differences between lines with a lower number of meals in line A than line B pigs were observed for all descriptive statistics (Fig 3). This effect was associated with two peaks in the number of meals at approximately 8.00 h and 17.00 h for line B pigs (Fig 4E).

Fig 3. Description statistics of feeding behavior responses for two lines[1] of growing-finishing pigs exposed to daily cyclic high ambient temperature during 84-days of the experimental period. [2,3]

[1]Genetic Line A pigs had a greater proportion of Pietrain genes than those from genetic Line B pigs.

[2]Data after application of meal criteria defined as 48 min. Such data refer to the total experimental period, regardless of the experimental phase.

[3]Interval value: difference between the minimum and maximum value.

Fig 4. Circadian variation of A) Feed intake rate, B) Feed intake per meal, C) Feeder occupancy per meal, D) Interval between meals, and E) Number of meals for two lines[1] of growing-finishing pigs exposed to daily cyclic high ambient temperature during 84-days of the experimental period. [2]

[1]Genetic Line A pigs (———) had greater proportion of Pietrain genes than those from genetic Line B pigs (- - - - -).

[2] Average data of total experimental period throughout 24 h-day, regardless of the experimental phase.

Prandial correlations

In our study, 49% of group-housed pigs (17 pigs from line A and 21 from line B) showed both pre- and post-prandial regulation. In addition, 21% of pigs (13 pigs from line A and 3 from line B) showed no prandial correlation, 24% (7 pigs from line A and 12 from line B) showed only a post-prandial correlation, and the remaining 6% of the group (2 pigs from line A and 3 from line B) showed only a pre-prandial correlation. Therefore, except for the “no prandial correlations”

group, in which the number of animals from line A was numerically greater than that from line B, little difference between lines was observed.

Discussion

Feeding data from two pig genetic lines with different proportions of Pietrain genes were analyzed to evaluate the genetic selection effect on the feeding behavior of growing-finishing pigs under daily cyclic high ambient temperature conditions. Our major and original finding is that differences in feeding behavior between lines reared under daily ambient temperature variations may partially explain differences in growth performance, which was previously reported [8]. The greatest probability of animals starting to feed was found at $22°C_{(06\text{-}10h)}$, followed by $30°C_{(10\text{-}18h)}$ and then $22°C_{(18\text{-}06\ h)}$, suggesting pigs feeding patterns are strongly related to the circadian rhythm. In addition, because pigs from line A had a lower probability of starting a meal than pigs from line B, differences between “hunger” and “satiety” mechanisms could also be attributed to the genetic line effect on feeding behavior. From these results and assuming that feeding behavior patterns can be evaluated through pre- and post-prandial correlations [16], we discuss whether pigs differently selected for lean mass and presumably with different metabolic heat production and thermoregulatory responses, could present different feeding behavior patterns when exposed to daily cyclic high ambient temperature conditions.

In the current study, the greater probability of pigs starting to feed at $22°C_{(06\text{-}10h)}$ compared with $30°C_{(10\text{-}18h)}$ and $22°C_{(18\text{-}06h)}$ reflects the strong influence of circadian rhythm on feeding patterns and may have induced the concomitant access of pigs to the feeders. As a result, pigs ate faster and had lower feed

intake and feeder occupancy per meal at 22°C$_{(06\text{-}10h)}$ than the remaining of the day. Indeed, greater feed intake rate was highly associated with a shorter feeder occupation time [2], indicating that pigs with faster feed intake rate spend less time feeding. Accordingly, diurnal feeding activities were reported in group-housed pigs exposed to thermoneutral conditions [1], constant high ambient temperature [17], and cyclic ambient conditions [4]. From these results and considering that catabolic and anabolic status follows a circadian rhythm [18], it can be assumed that the periods of activity and feeding inactivity of pigs change over the 24-h day.

When exposed to thermoneutral [1] and constant high ambient temperature conditions [17], group-housed pigs showed a biphasic feeding pattern, characterized by two peaks of feeding activity over 24-h day (a first peak at the beginning and another at the end of the day). Such feeding behavior (two peaks) is defined mainly by decreased melatonin levels in the morning and cortisol in the afternoon [18]. Despite such scientific evidence, similar results in the current study were observed only for line B pigs, whose number of meals also presented two peaks (at 8.00 h and 17.00 h) and there was a peak in feed intake per meal at 11.00 h. In agreement, two peaks in the number of visits (one at 9.00 h and the other at 15.00 h) and a greater feed intake per visit value (at approximately 18.00 h) was previously reported for pigs under thermoneutral conditions [2]. Furthermore, pigs from line A had a lower number of meals, feed intake, and feeder occupancy per meal compared with line B during the diurnal period (22°C$_{(06\text{-}10h)}$ and 30°C$_{(10\text{-}18h)}$). Greater diurnal feed intake per meal (mainly between 15.00 h and 18.00 h; [1]) and lowest number of visits to the feeder from 20.00 to 4.00 h [19] were reported for group-housed pigs raised at

thermoneutrality. From these results, it can be suggested that only pigs from line B had feeding behavior patterns similar to nonchallenged pigs, evidencing a better capacity of these animals to cope with our experimental conditions. Because the interval between meals is commonly opposite of meal frequency [18], the greater interval between meals was observed for line A pigs at 22°C$_{(06\text{-}10h)}$ and 30°C$_{(10\text{-}18h)}$.

Regarding 22°C$_{(18\text{-}06h)}$, pigs from line A had substantially greater feed intake and feeder occupancy per meal compared with pigs from line B. It should be noted that line A pigs had a higher proportion of Pietrain genes, which can explain their greater potential for lean depositions than line B pigs [8]. Because the energetic cost of proteogenesis is greater than that of lipogenesis [20], increased lean tissue accretion has been related with increased metabolic heat production [21] with consequent greater susceptibility to heat stress [20]. Therefore, the greater heat production of modern lean genotypes in association to their greater protein deposition and growth rate may explain the increased feeding activity of line A pigs during the nocturnal period. In fact, it seems that pigs from line A may have tried to compensate for the lower feeding activity in the diurnal period changing part of their feeding behavior to the night. Adaptive mechanisms such as changes in feeding behavior patterns and decrease of feed intake can help pigs to reduce metabolic heat production and/or increase heat dissipation when exposed to high ambient conditions [17, 22]. From these scientific findings, it can be suggested that line A pigs are more dependent on thermoregulatory responses to maintain homeothermy. The magnitude of these results is in accordance with the pig's flexibility to modify their feeding patterns when exposed to limiting conditions [23, 24].

In the current study, the greater feed intake rate of line A pigs in growing phase 2 and total period may be understood as an attempt of these animals to meet their nutritional requirements; however, their behavioral activities may have been limited during the trial. Indeed, feed intake rate is an intrinsic characteristic of the individual and positively correlated with feed intake [25], protein retention [26], and feeding motivation [27]. Therefore, results of greater feed intake rate, associated with lower feed intake, feeder occupancy per meal, and probability of line A pigs starting a meal compared with line B pigs, suggest that pigs from line A were presumably more affected by our experimental conditions. In addition, although no difference between lines was reported in growing phase 1 for feed intake and feeder occupancy per meal, in the subsequent phases, both were lower for line A than line B pigs. Such metabolic and behavioral differences between lines may partially explain the contrasting performance results, in which lower values of ADFI, ADG, and final BW were observed for pigs from line A than line B [8]. Besides, feed intake of pigs became more diurnal with increasing BW and/or age as previously reported [28,1]. Despite such scientific findings, an increase in diurnal feed intake and the number of meals throughout the trial were observed only for line B pigs (in which, 77, 79, and 83% of the meals were performed during light period in growing phases 1 and 2 and finishing phase, respectively). These findings offer compelling evidence that genetic selection for lean growth may result in changes in pigs feeding behavior activity when exposed to daily cyclic high ambient temperatures.

When comparing lines, the number of line A pigs with “no prandial correlations” was greater than that of line B (13 vs 3 pigs). This result demonstrates that

some individuals from line A started to feed randomly without necessarily having a stimulus, and such a response may be a consequence of their lower ability to cope with daily cyclic high ambient temperatures conditions. As a result, a greater dispersion of feeding behavior responses (mainly for feed intake rate, feed intake, and feeder occupancy per meal) was observed for line A than line B pigs. The impact of between-animal variation was highlighted in a previous study [5], which reported that characteristics intrinsic to animals such as breed, BW, and physiological status could be an additional source of variation among studies. These findings are in close agreement with the large variability of results that exist between studies considering the high ambient temperature effects.

Regardless of the period and genetic line, the increase in BW throughout the experimental phases was mainly associated with a greater feed intake rate and feed intake per meal, which resulted in an increase in total feed intake. Similarly, an increase in feeding rate, meal size, and total feed intake was observed with an increase in BW [4,29]. Since the feeding rate increased by approximately 0.47 g/min per kg increase in BW, such result may be a consequence of increase in body size and physical capacity of feed intake [25].

In conclusion, despite our initial hypothesis that pigs could prioritize their feed intake in the coolest hours of the day (i.e., at 22°C$_{(06\text{-}10h)}$ and 22°C$_{(18\text{-}06h)}$), our results demonstrate that there was a considerable and dynamic genetic selection effect able to modulate the pigs feeding behavior responses when exposed to daily temperature variations. The greatest probability of animals starting to feed found at 22°C$_{(06\text{-}10h)}$, followed by 30°C$_{(10\text{-}18h)}$, and then 22°C$_{(18\text{-}06}$

h), suggest that pigs feeding pattern is strongly related to the circadian rhythm. Furthermore, the greater feeding activities of line A at 22°C(18-06h) than the remaining of the day supports the pigs' capacity to modify their feeding activity under daily cyclic high ambient temperatures. Since our results showed that genetic selection affects feeding behavior under daily cyclic high ambient temperature conditions, research on genetic selection for thermotolerant animals is encouraged.

Declaration of interest

None.

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Supporting information

S1 Dataset. **Data filtering diagram**. (PDF)

S2 Fig. **Individual pigs behavior profile during the total experimental period (days 0 to 83) throughout 24 h-day**. (PDF).

S3 Fig. **Pigs feeding behavior profile in the growing phase 1 (days 0 to 20), growing phase 2 (days 21 to 48), finishing phase (days 49 to 83), and total experiment period (days 0 to 83). Average data throughout 24 h-day for each genetic line are presented separately**. (PDF)

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Fig 1.

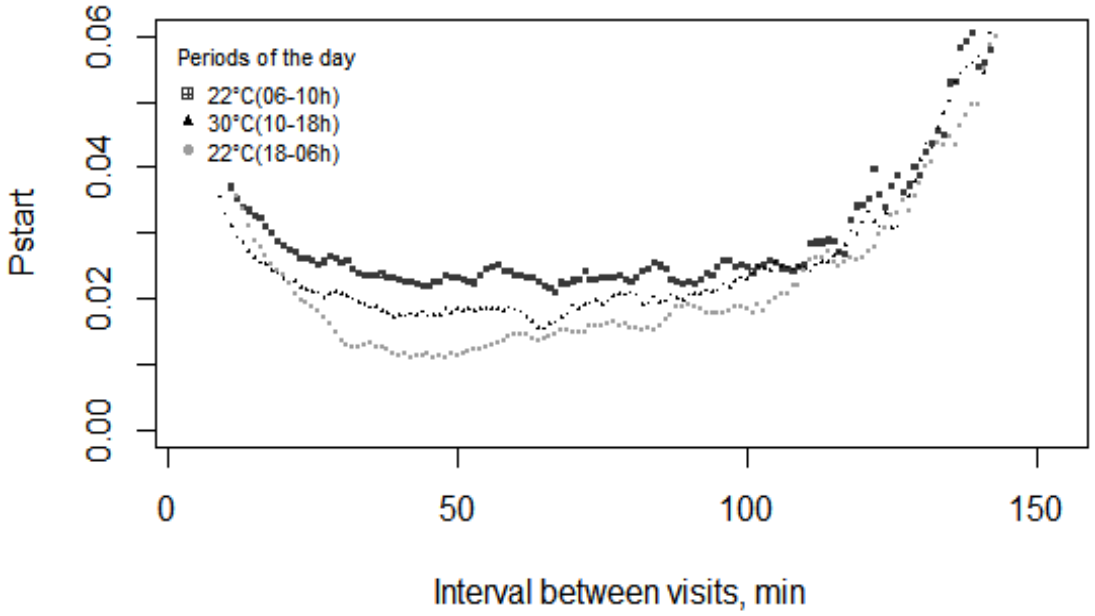

Periods of the day
22°C(06-10h)
30°C(10-18h)
22°C(18-06h)
Pstart
0.00
0.02
0.04
0.06
0
50
100
150
Interval between visits, min

Fig 2.

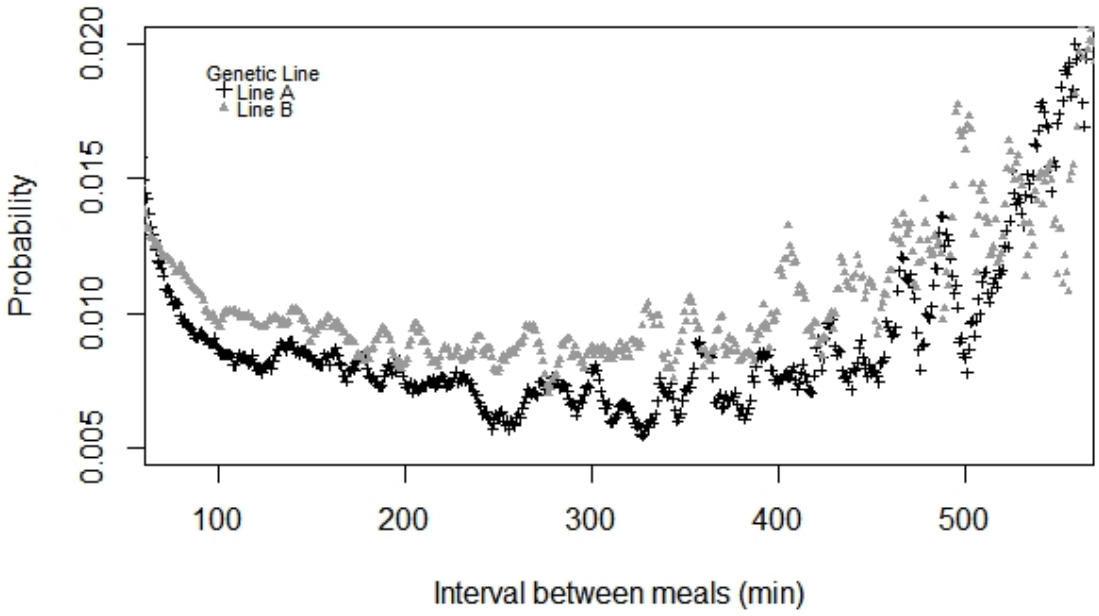

Genetic Line
Line A
Line B
Probability
0.005
0.010
0.015
0.020
100
200
300
400
500
Interval between meals (min)

Fig 3.

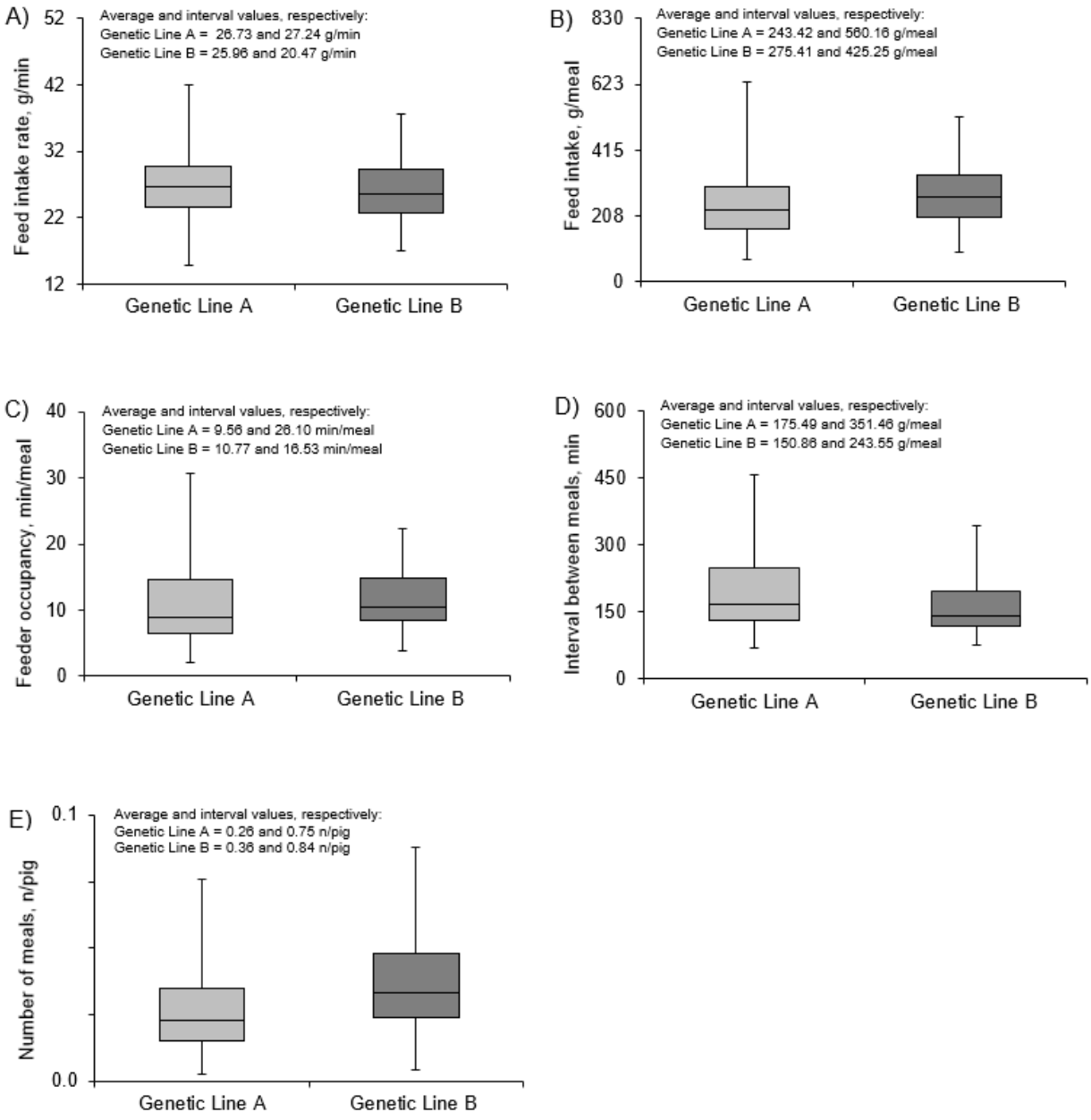
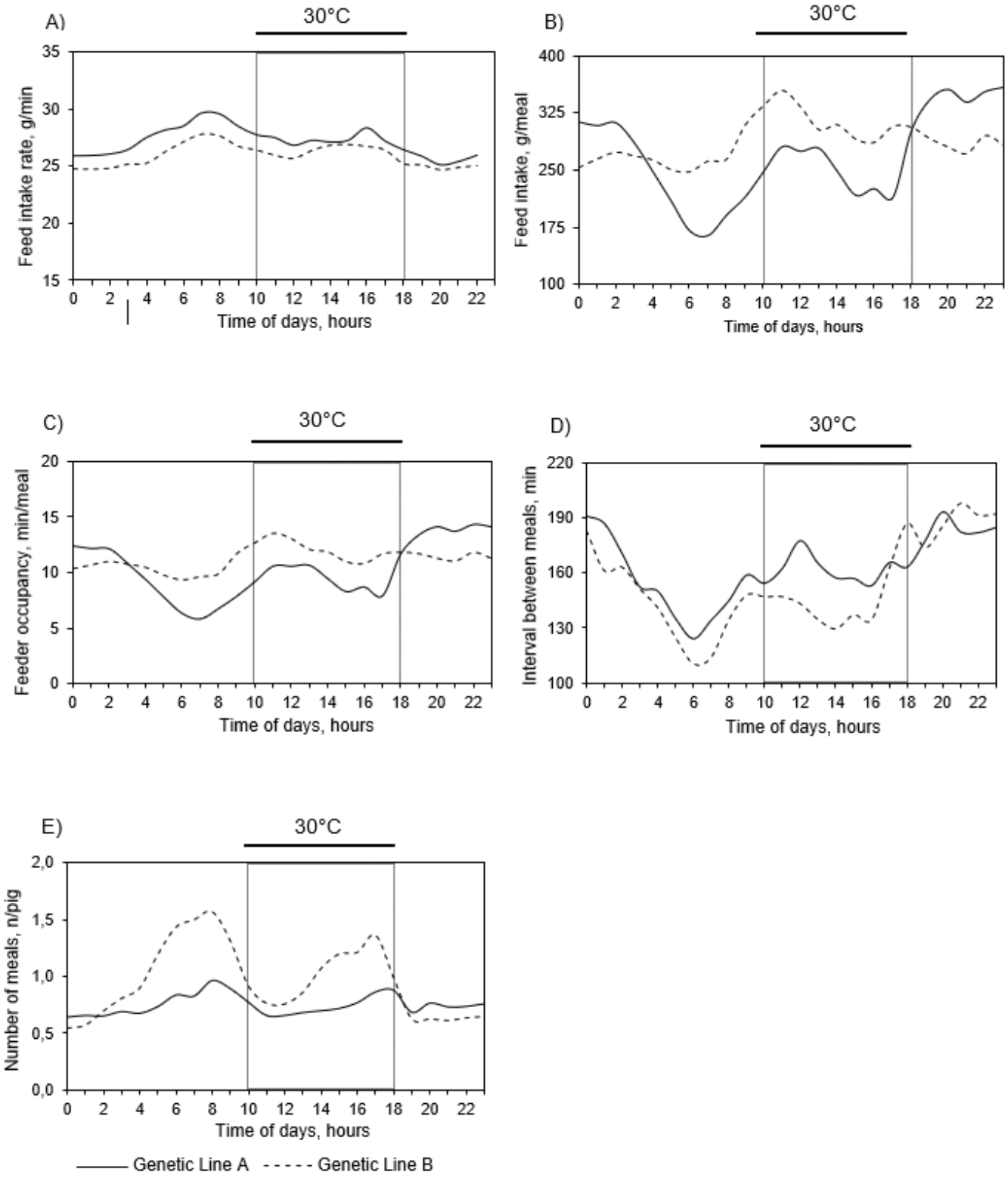

A)
Average and interval values, respectively:
Genetic Line A = 26.73 and 27.24 g/min
Genetic Line B = 25.96 and 20.47 g/min
Feed intake rate, g/min
52
42
32
22
12
Genetic Line A
Genetic Line B
B)
Average and interval values, respectively:
Genetic Line A = 243.42 and 560.16 g/meal
Genetic Line B = 275.41 and 425.25 g/meal
Feed intake, g/meal
830
623
415
208
0
Genetic Line A
Genetic Line B
C)
Average and interval values, respectively:
Genetic Line A = 9.56 and 26.10 min/meal
Genetic Line B = 10.77 and 16.53 min/meal
Feeder occupancy, min/meal
40
30
20
10
0
Genetic Line A
Genetic Line B
D)
Average and interval values, respectively:
Genetic Line A = 175.49 and 351.46 g/meal
Genetic Line B = 150.86 and 243.55 g/meal
Interval between meals, min
600
450
300
150
0
Genetic Line A
Genetic Line B
E)
Average and interval values, respectively:
Genetic Line A = 0.26 and 0.75 n/pig
Genetic Line B = 0.36 and 0.84 n/pig
Number of meals, n/pig
0.1
0.0
Genetic Line A
Genetic Line B

Fig 4.


A)
30°C
Feed intake rate, g/min
Time of days, hours
B)
30°C
Feed intake, g/meal
Time of days, hours
C)
30°C
Feeder occupancy, min/meal
Time of days, hours
D)
30°C
Interval between meals, min
Time of days, hours
E)
30°C
Number of meals, n/pig
Time of days, hours
Genetic Line A
Genetic Line B

S1 Dataset. Data filtering diagram.

Criteria used for data filtering (see "*Overview of data compilation*" topic).

Days of data record:

18 days of adaptation + 84 days of experimental period

Excluded[1]: 30 days of data record

- Adaptation period[2]: 18 days
- Animal scanner[3]: 3 days
- Animal weighing[3]: 4 days
- Equipment malfunctions[4]: 2 days

[1] Such days were excluded from the database because they were considered without interest for this study (atypical conditions).
[2] Animals adaptation period to automatic feeders.
[3] For more details see our previously publication Fraga et al., 2019 (doi: 10.1093/jas/skz123).
[4] Such data was likely generated by a technical system failure. However, this was considered a one-off limitation, given that a fairly limited number of experiment days were removed from the dataset.

Initial database: 72 days

Assessed for eligibility (n = 129,167 records)

First data filtering: Interval between visits greater than 720 min (4,883 records were excluded = 3.78% of observations from the initial database).

From the total of deleted records (3.78%):

- 58.0% were from Genetic Line A pigs
- 42.0% were from Genetic Line B pigs

Data "interval between visits" summary		
	Before data filtering	After data filtering
Minimum	0.02	0.02
First quartile	3.17	1.13
Median	10.14	7.05
Mean	53.70	50.52
Third quartile	310.18	54.15
Maximum	992.30	719.45

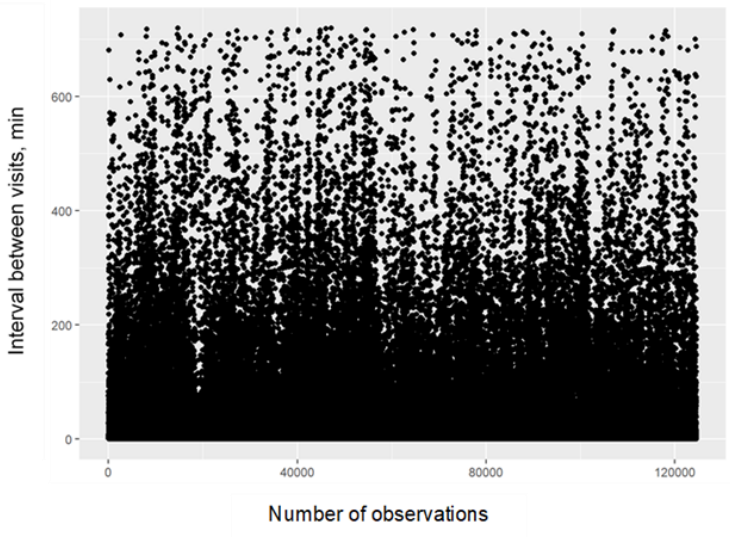


Figure 1. Interval between visits distribution after data filtering.

Second data filtering: Feeder occupancy greater than 30 min (597 records were excluded = 0.48% of observations from the database generate after the first data filtering).

From the total of deleted records (0.48%):

- 51.8% were from Genetic Line A pigs
- 48.2% were from Genetic Line B pigs

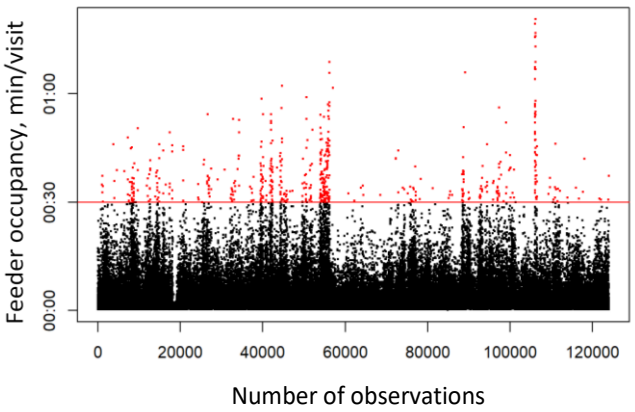


Figure 2. Feeder occupancy distribution before data filtering.

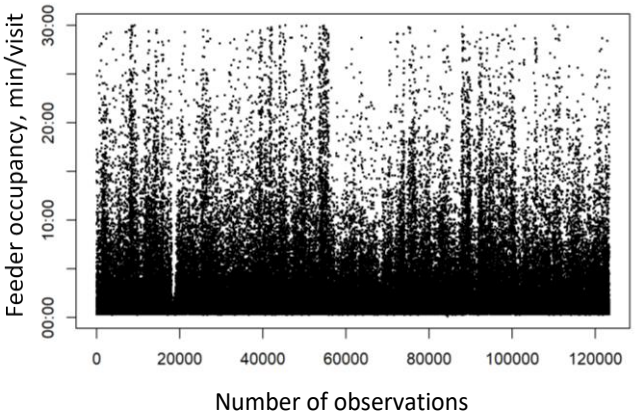


Figure 3. Feeder occupancy distribution after data filtering.

Third data filtering: Data records with zero feed intake (433 records were excluded = 0.35% of observations from the database generate after the second data filtering).

From the total of deleted records (0.48%):

- 61.2% were from Genetic Line A pigs
- 38.8% were from Genetic Line B pigs

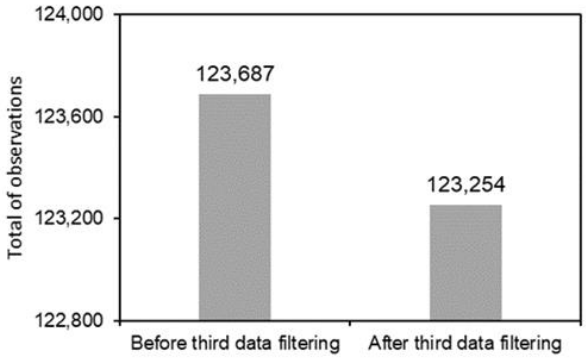


Figure 4. Total of observation before and after third data filtering.

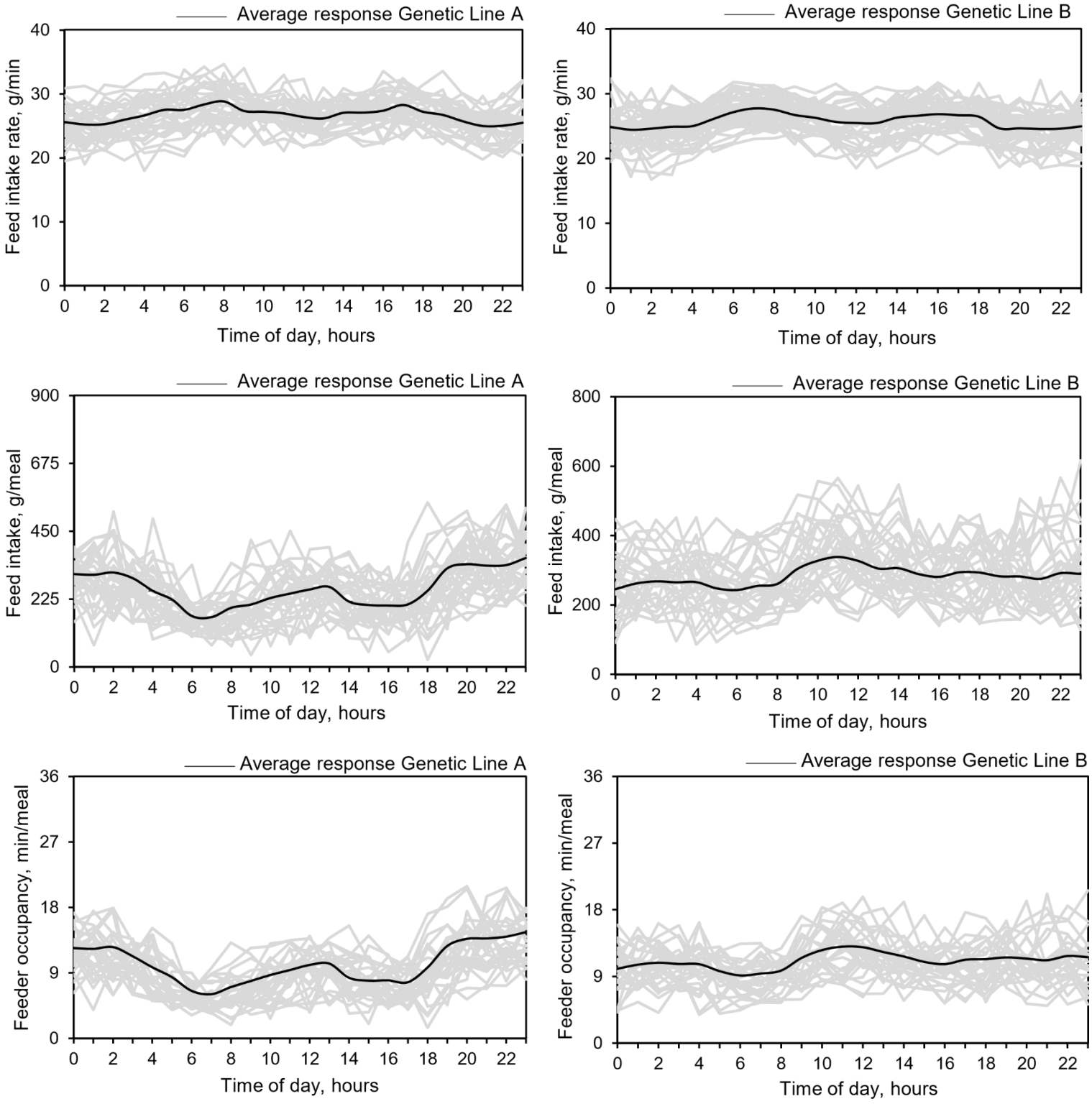
Final database

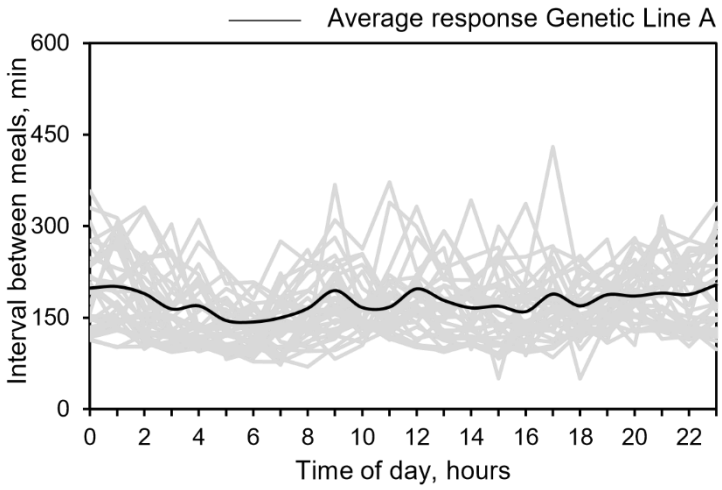
n = 123,254 records

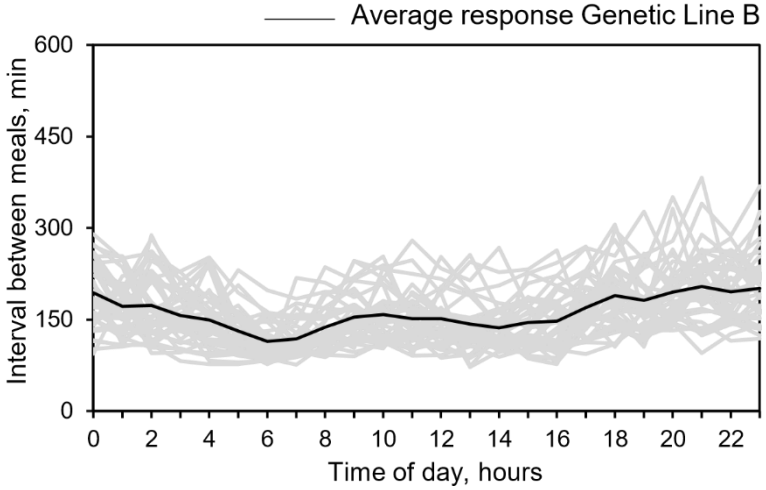
Database used to calculate meal criteria

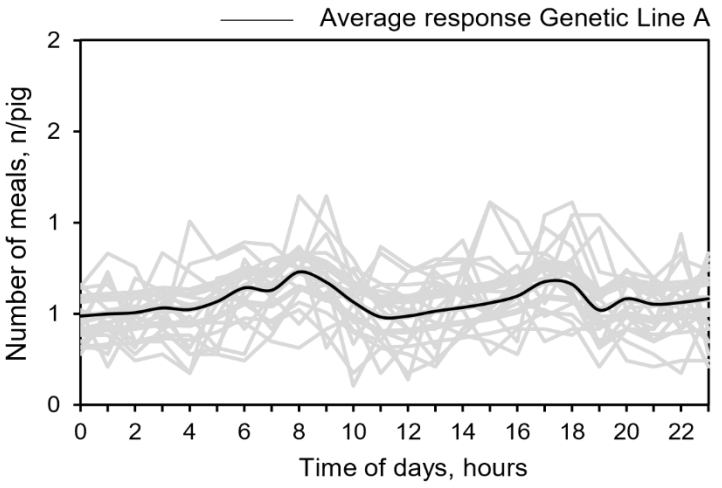
S2 Fig.

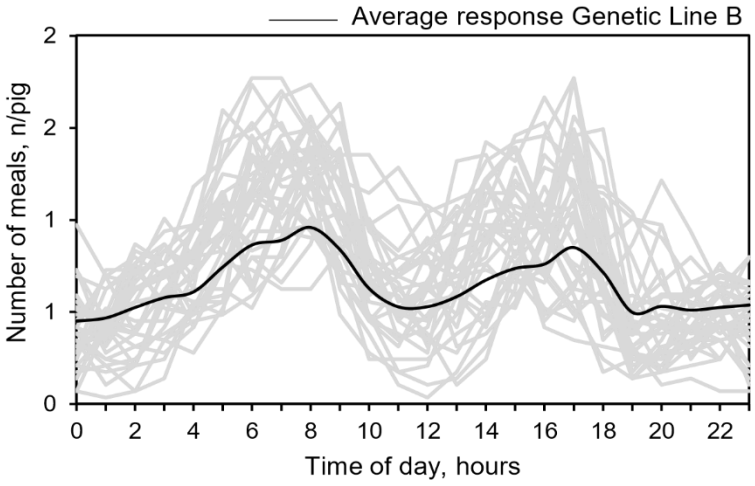
Individual pigs behavior profile during the total experimental period (days 0 2 to 83) throughout 24 h-day.




Average response Genetic Line A
Interval between meals, min
600
450
300
150
0
0 2 4 6 8 10 12 14 16 18 20 22
Time of day, hours

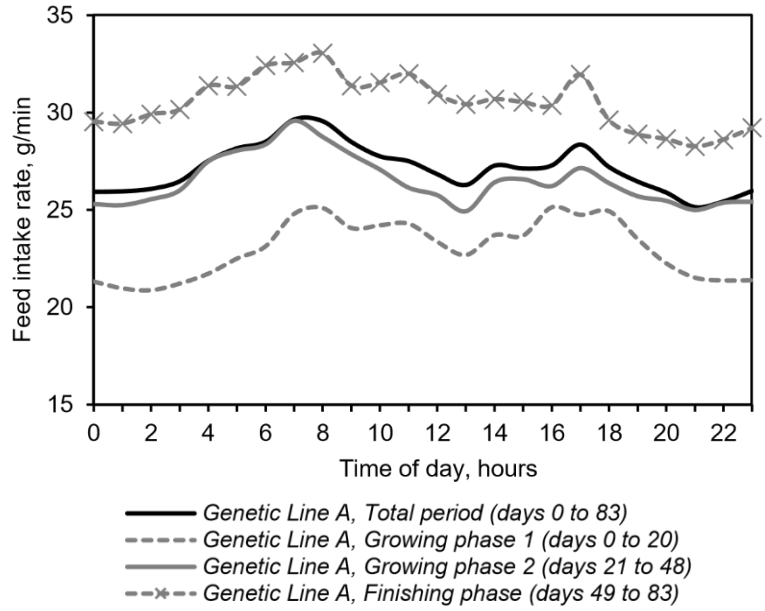

Average response Genetic Line B
Interval between meals, min
600
450
300
150
0
0 2 4 6 8 10 12 14 16 18 20 22
Time of day, hours

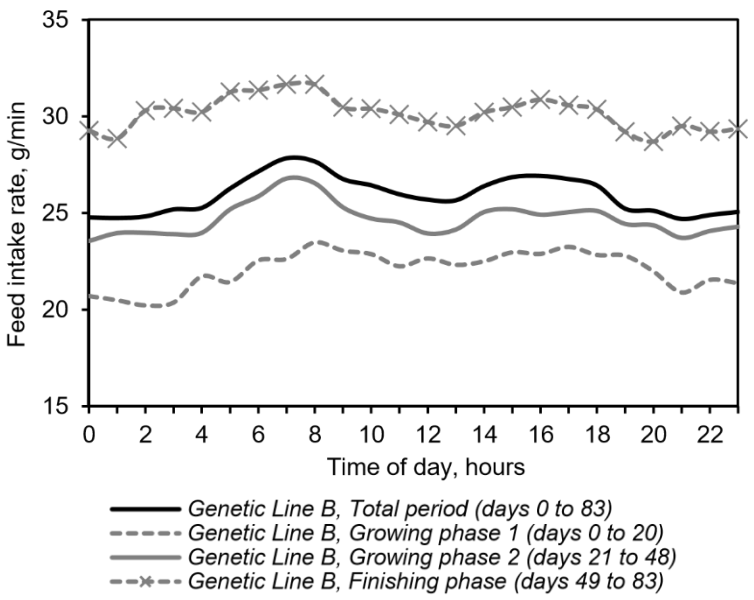

Average response Genetic Line A
Number of meals, n/pig
2
2
1
1
0
0 2 4 6 8 10 12 14 16 18 20 22
Time of days, hours

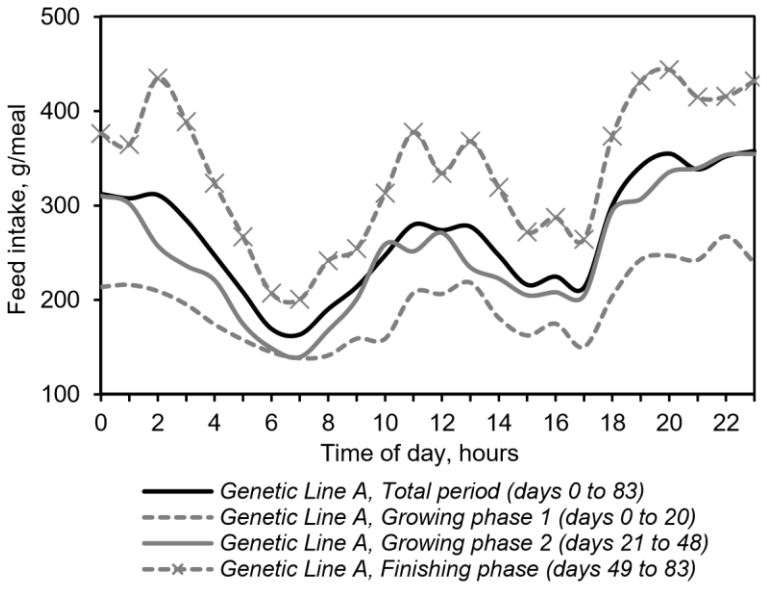

Average response Genetic Line B
Number of meals, n/pig
2
2
1
1
0
0 2 4 6 8 10 12 14 16 18 20 22
Time of day, hours

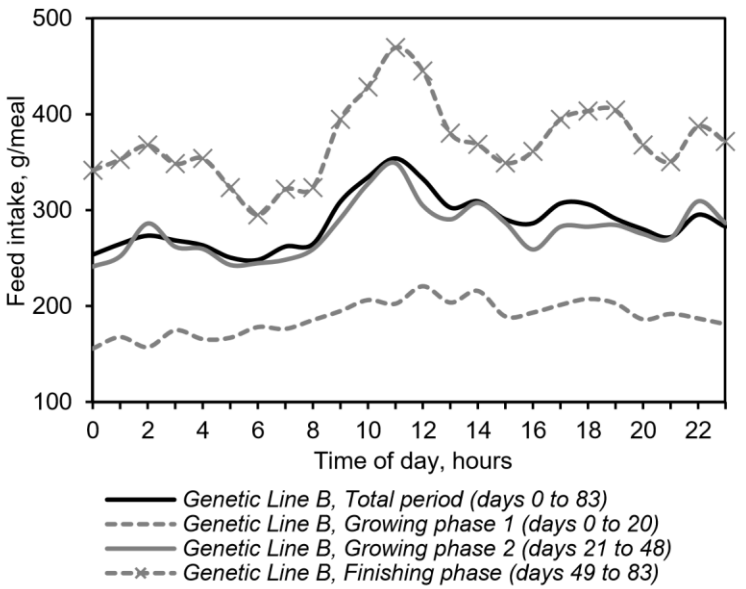
S3 Fig.

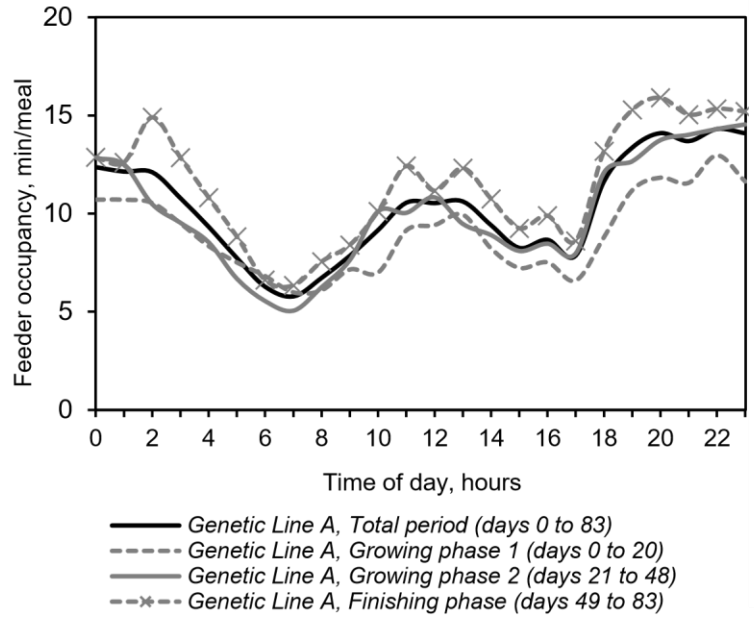
Pigs feeding behavior profile in the growing phase 1 (days 0 to 20), growing phase 2 (days 21 to 48), finishing phase (days 49 to 83), and total experiment period 3 (days 0 to 83). Average data throughout 24 h-day for each genetic line are presented 4 separately.

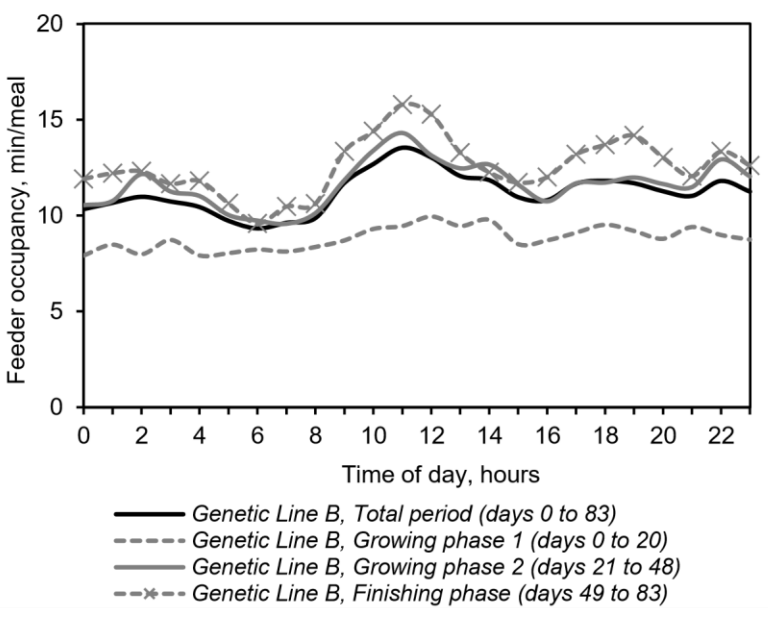


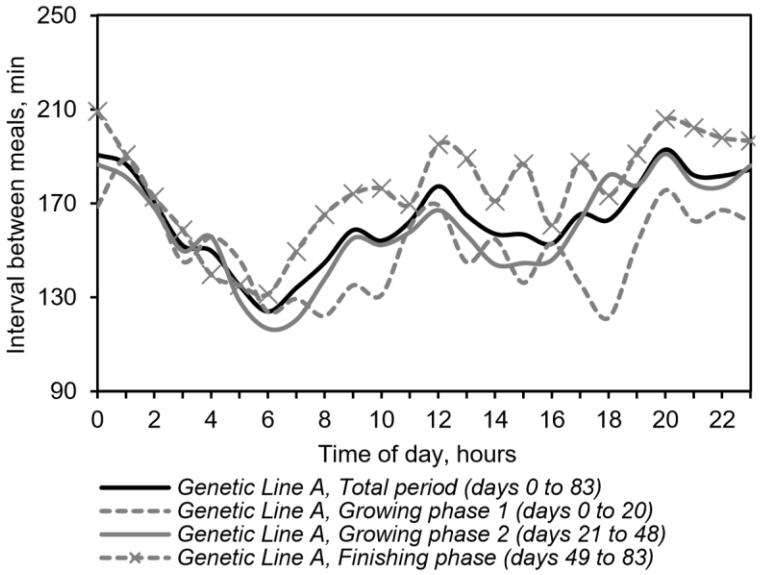


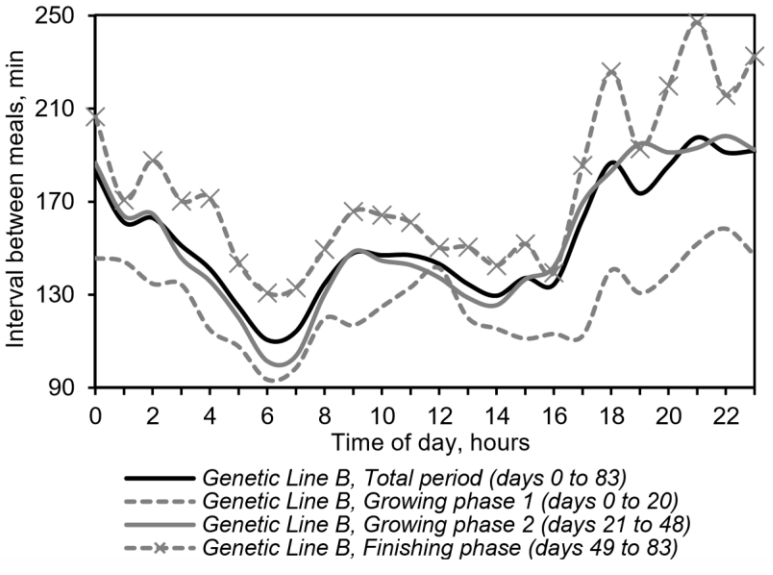


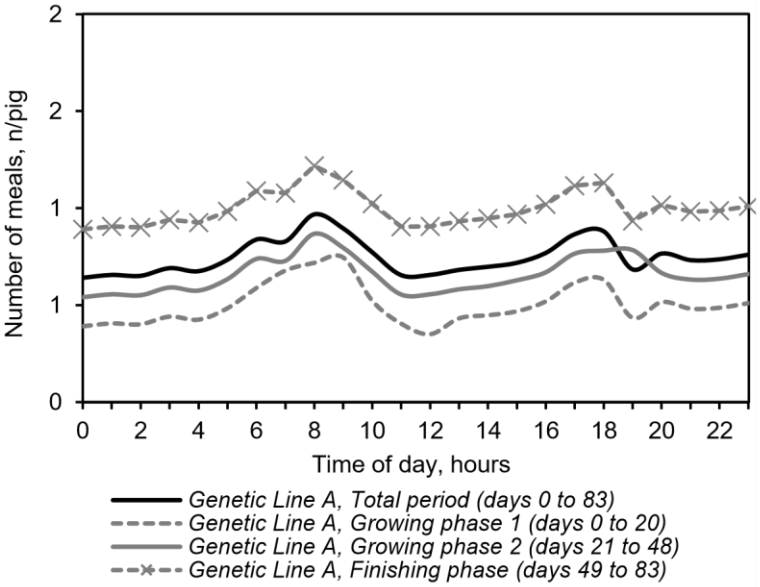


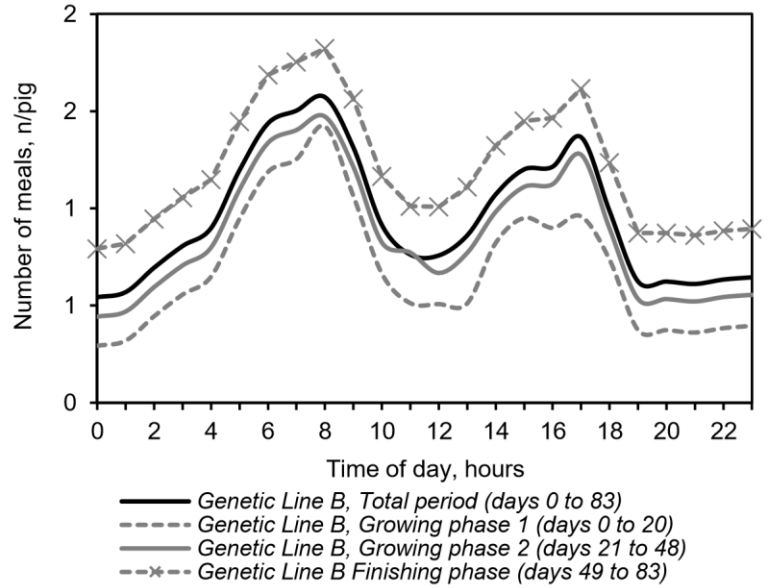

Feeder occupancy, min/meal
Time of day, hours
Genetic Line A, Total period (days 0 to 83)
Genetic Line A, Growing phase 1 (days 0 to 20)
Genetic Line A, Growing phase 2 (days 21 to 48)
Genetic Line A, Finishing phase (days 49 to 83)


Feeder occupancy, min/meal
Time of day, hours
Genetic Line B, Total period (days 0 to 83)
Genetic Line B, Growing phase 1 (days 0 to 20)
Genetic Line B, Growing phase 2 (days 21 to 48)
Genetic Line B, Finishing phase (days 49 to 83)


Interval between meals, min
Time of day, hours
Genetic Line A, Total period (days 0 to 83)
Genetic Line A, Growing phase 1 (days 0 to 20)
Genetic Line A, Growing phase 2 (days 21 to 48)
Genetic Line A, Finishing phase (days 49 to 83)


Interval between meals, min
Time of day, hours
Genetic Line B, Total period (days 0 to 83)
Genetic Line B, Growing phase 1 (days 0 to 20)
Genetic Line B, Growing phase 2 (days 21 to 48)
Genetic Line B, Finishing phase (days 49 to 83)


Number of meals, n/pig
Time of day, hours
Genetic Line A, Total period (days 0 to 83)
Genetic Line A, Growing phase 1 (days 0 to 20)
Genetic Line A, Growing phase 2 (days 21 to 48)
Genetic Line A, Finishing phase (days 49 to 83)


Number of meals, n/pig
Time of day, hours
Genetic Line B, Total period (days 0 to 83)
Genetic Line B, Growing phase 1 (days 0 to 20)
Genetic Line B, Growing phase 2 (days 21 to 48)
Genetic Line B Finishing phase (days 49 to 83)

CHAPTER 4 – SELECTION FOR FEED EFFICIENCY ELICITS DIFFERENT POSTPRANDIAL PLASMA METABOLITE PROFILES IN RESPONSE TO POOR HYGIENE OF HOUSING CONDITIONS IN GROWING PIGS[11]

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[11] The chapter is presented according to PlosOne formatting.

Abstract

This study was conducted to compare postprandial plasma concentrations of insulin, energy-related metabolites, and amino acids measured after a 6-week challenge consisting of exposure to good or poor hygiene of housing conditions of 24 growing pigs divergently selected for low-RFI (LRFI) and high-RFI (HRFI). Blood indicators of immune responses were assessed from samples collected before 0 (W0), and 3 (W3), and 6 weeks (W6) after pigs transfer to their respective hygiene of housing conditions. Plasma haptoglobin concentrations and blood neutrophil granulocyte numbers were greater in poor than in good hygiene of housing conditions at W3. Plasma concentrations of total immunoglobulin G were greater ($p = 0.04$) in poor than in good hygiene of housing conditions at W6. At W6, pigs were fitted with an intravenous catheter for serial blood samplings. Low-RFI pigs had greater insulin ($p < 0.001$) and lower triglyceride ($p = 0.04$) average plasma concentrations than HRFI pigs in both conditions. In poor hygiene of housing conditions, the peaks of insulin and glucose were observed earlier and that of insulin was greater in LRFI than in HRFI pigs. Irrespective of genetic line, average plasma concentrations of histidine, isoleucine, leucine, methionine, threonine, valine, and alanine were greater in poor compared with good hygiene of housing conditions. Only HRFI pigs had greater lysine, asparagine, proline, and tyrosine plasma concentrations in poor than in good hygiene of housing conditions. Conversely, arginine, tryptophan, proline, and tyrosine plasma concentrations were lower only for LRFI pigs housed in poor hygiene conditions. Our results suggest that, contrary to HRFI, LRFI pigs increase or maintain their utilization of tryptophan, arginine, and lysine when housed in poor hygiene conditions. This indicates that this

difference may contribute to the better capacity of LRFI to cope with poor hygiene of housing conditions.

Introduction

Selection of pigs for residual feed intake (RFI) has been used to improve feed efficiency. Briefly, RFI is the difference between the observed feed consumption of an animal and that predicted by the estimated requirements for maintenance and growth of a reference population. High-RFI (HRFI) pigs eat more than predicted and therefore are less efficient than low-RFI (LRFI) pigs [1]. Difference in feed efficiency between RFI lines is explained by changes in physical activity [2], heat production [3], and in the partition of nutrients between maintenance and growth [5]. Difference in nutrient partitioning is suspected to alter pig ability to allocate nutrients for stress and immune responses when facing environmental challenges [6]. In commercial farms, pigs are often exposed to stressful situations such as weaning, mixing, high stocking density, transport, and poor hygiene of housing conditions resulting in immune system activation. In turn, immune activation, including inflammation, results in changes in nutrient metabolism, which therefore reduces nutrient availability for growth [7].

The better ability of LRFI compared with HRFI growing pigs to cope with an immune challenge caused by poor hygiene of housing conditions was previously reported [8]. More specifically, we showed that after being housed six weeks in poor hygiene conditions, growth performance and health, assed by lung lesion scoring and blood indicators, were more affected in HRFI pigs than in LRFI pigs. We hypothesized that this difference in coping ability between RFI pigs may involve changes in their metabolism after a 6-week exposure to contrasted hygiene of housing conditions. To evaluate this metabolic response,

we analysed, on a subset of pigs from the study by Chatelet et al. 2018 [8], the patterns of plasma postprandial concentrations of insulin, energy-related metabolites and amino acids (AA), as it has been shown previously that they reflect changes in the use of nutrients for anabolic and catabolic processes. The same methodology was recently reported to describe the metabolic status of growing pigs in response to an inflammatory challenge and high ambient temperature [9] or to compare castrated and entire male pigs [10]. Therefore, the present study was carried out to compare, in LRFI and HRFI pigs, the effects of poor hygiene of housing conditions on pre- and postprandial plasma concentrations of insulin, energy-related metabolites, and free amino acids.

Material and methods

The experiment was conducted at INRAE UE3P (Saint-Gilles, France) in accordance with the ethical standards of the European Community (Directive 2010/63/EU) and was approved by the local ethical committee (Comité rennais d'éthique en matière d'expérimentation animale or CREEA N° 07). The experiment approval number is APAFIS#494-2015082717314985.

Animals, Diets, and Experimental Design

The experiment was conducted on a subset of 24 Large-White pigs from a larger study (n = 160 pigs) previously described [8]. Animals with representative body weight (BW) of each experimental group (see below) were selected at 12 weeks of age. Selected pigs were fed, housed, and submitted to the same experimental procedures as the whole set of pigs, before being involved in the serial blood sampling 6 weeks later.

Pigs originated from the 8th generation of a selection program for divergent RFI conducted at INRAE. Briefly, the lines were established using the RFI selection criterion between 35 and 95 kg BW, calculated as: RFI = ADFI − (1.24 × ADG) − (31.9 × BFT), where ADFI was the average daily feed intake (g/day), ADG the average daily gain (g/day) and BFT was the backfat thickness (mm) at 95 kg [11]. The study was performed as a 2 × 2 factorial design including four experimental groups: HRFI and LRFI pigs housed in good hygiene conditions (good-HRFI, good-LRFI); and HRFI and LRFI pigs housed in poor hygiene conditions (poor-HRFI, poor-LRFI). Briefly, poor hygiene of housing conditions consisted of no cleaning nor sanitation of the room after the previous occupation by non-experimental pigs [8]. In contrast, good hygiene of housing conditions included room cleaning, disinfection, and adoption of strict biosecurity precautions throughout the experimental period. The staff wore clean boots, clothes and gloves before entering the room.

According to their allocation, pigs were placed in one of the two experimental rooms (good or poor hygiene of housing conditions) for 6 weeks. In each room, pigs were housed in individual concrete floor pens (85 × 265 cm) equipped with a feed dispenser and a nipple drinker. Pigs had free access to water and were fed ad libitum a standard diet formulated to meet the nutritional requirement of growing pigs (Table 1). After 6 weeks, the 24 selected pigs (n = 6 per experimental group) were fitted with an intravenous ear catheter following a minimally invasive procedure. Briefly, after an overnight fast, pigs were premedicated with 15 mg/kg of ketamine injected intramuscularly (Imalgène 1000, Merial, Lyon, France) and were then anesthetized by inhalation of sevoflurane (Sevoflurane, Baxter, Maurepas, France) using a facemask. An

intravenous catheter was inserted through a small incision on the flap of the ear. The external part of the catheter was fixed on the ear skin and a connector was added for blood samplings the day after catheter insertion. No drug was used to avoid interference with the health status of pigs.

Table 1. Ingredients and chemical composition of the diet.

Items	Standard diet
Ingredient composition as-fed basis, %	
Wheat	32.17
Corn	15.00
Barley	24.95
Wheat bran	5.00
Rapeseed meal	7.00
Soybean meal	11.47
Vegetable oil	1.00
Calcium carbonate	1.51
Dicalcium phosphate	0.10
Salt	0.45
Liquid lysine (50.0% of L-Lysine)	0.53
DL-Methionine	0.04
L-Threonine	0.10
L-Tryptophan	0.07
Vitamin and mineral premix[1]	0.50
Phytase and organic acids	0.11
Chemical composition	
ME, MJ/kg	12.64
NE, MJ/kg	9.48

CP, %	15.50

[1] Mineral vitamin supplement (per kg of diet): Vit. A (1.000.000 UI); Vit. D3 (200.000 UI); Vit. E (4.000 UI); Vit. K3 (400 mg); Vit. B1 (400 mg); Vit. B2 (800 mg); Vit. B6 (200 mg); Niacin (3.000 mg); Pantothenic acid (2.000 mg); Folic acid (200 mg); Biotin (40 mg); Vit. B12 (4.4 mg); Copper (2.000 mg); Iodine (40 mg); Manganese (8.000 mg); Selenium (30 mg); Zinc (20.000 mg); Ferrous sulphate (11.200 mg); Ferrous carbonate (4.800 mg); and Choline chloride (100.000 mg).

Blood Sample Collection

Blood samples were collected at fasted state by jugular vein puncture at week zero (W0; before pig transfer to the respective hygiene conditions) and week three (W3), and from the intravenous ear catheter at week six (W6). These samples were used to measure blood indicators of immune responses (immunoglobulin G (IgG), haptoglobin, and number of blood neutrophil granulocytes). Serial blood samplings were performed at W6 the day after catheter insertion. After being fasted overnight, each pig was offered 300 g of the standard diet at 08-h, irrespective of the hygiene conditions and the genetic line. This meal size was determined to ensure that all pigs eat their meal in less than 10 min [10] and corresponded to 15% of the ADFI. Blood samples (6 ml) were collected from the catheter before the meal delivery (fasted state; t0), and then at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, and 240 min after the meal delivery. Blood samples were collected on ethylenediaminetetraacetic acid (EDTA) tubes for insulin, glucose, free fatty acid (FFA), triglyceride, and urea measurements; and on heparinized tubes for AA analyses. Samples were immediately placed on ice, except for EDTA tubes used for measuring the number of blood neutrophil granulocytes, and then centrifuged (1800 × g) for 10

min at 4°C. Plasma was collected and stored at -80°C for AA, and at -20°C for other plasma parameters.

Blood Cell and Plasma Variable Analyses

The number of blood neutrophil granulocytes was measured with a haematology automated cell counter calibrated for pigs (MS9; Melet Schloesing Laboratories, Osny, France). Quantitative sandwich ELISA tests were used to quantify total IgG plasma concentrations [12]. Plasma concentrations of haptoglobin (phase haptoglobin assay T801; Tridelta Development Ltd, Maynooth, Ireland), glucose (Kit Glucose RTU, ref. 61269; Biomérieux, Marcy-l'Etoile, France), and free fatty acids (Kit WAKO NEFA; Sobioda, Montbonnot-Saint-Martin, France), were analysed by an automated enzymatic method using commercial kits and a multianalyzer apparatus (Konelab 20i, ThermoFisher Scientific, Courtaboeuf, France). For plasma triglyceride and urea concentration measurements, kits were obtained from Thermo Fisher Diagnostics SAS (Asnieres-Sur-Seine, France). Plasma insulin concentrations were determined using a commercial immunoassay kit (ST AIA-PACK IRI) and the AIA-1800 device (Automated Immunoassay Analyzer; TOSOH Bioscience, Tokyo, Japan). Assay sensitivity for insulin was 0.5 µUI/ml and the intra-assay CV was below 5%. Plasma free AA concentrations were determined by an ultra-performance liquid chromatography (UPLC) apparatus (Waters Acquity Ultra Performance LC, Waters, Milford, MA, USA) after derivatization of samples using the AccQ Tag Ultra method (MassTrak AAA; Waters, Milford, MA, USA) as previously described [4].

Calculations and Statistical Analysis

The pig was the experimental unit. Average plasma concentrations of insulin, energy metabolites, and AA were calculated from pre and postprandial concentrations. Average concentrations and concentrations at each sampling time were analysed using the linear MIXED procedure (SAS Inst. Inc., Cary, NC). The model included the genetic lines (LRFI or HRFI), hygiene of housing conditions (poor or good), sampling time (time), and their interactions as fixed effects. The repeated measurements option was used with a compound symmetry covariance structure to account for animal effect over sampling time. Adjusted means were compared using the Bonferroni test. Plasma concentrations of total IgG and haptoglobin, and number of neutrophil granulocytes were compared between the two hygiene conditions with a non-parametric test (Median test) using the NPAR1WAY procedure of SAS. Probabilities less than 0.05 were considered significant.

For indispensable AA, when an interaction between genetic line and hygiene of housing conditions was significant, plasma profiles were analysed by nonlinear regression using a one-compartment model with Erlang retention times [13] in combination with a constant basal concentration. The Erlang distribution was previously described by [14]. The model used to describe the change in AA concentrations over time as an asymmetric bell-shaped curve was:

$$\text{Amino acid concentration } C\,(t) = \frac{k\left(\lambda^{n} \times (t)^{n-1}\right) \times \exp(-\lambda \times t) + C\text{basal}}{(n-1)!}$$

where *C*basal is the basal concentration, *k* is a scale parameter, *λ* and *n* are shape parameters of the Erlang distribution of residence times, and *t* is the time.

For each AA plasma concentration curve, the shape parameter *n* was tested using values ranging from one to four, and the result with the lowest residual SD was retained. The model was parameterized to obtain AA concentration at *t* = 0 (*C*0), the maximum AA concentration (*C*max), and the time when AA concentration is maximum (Tmax). To test whether the parameters of the model differed between the experimental groups, a sum of squares reduction test was used [15]. As our main objective was to compare the effects of the hygiene challenge in LRFI and HRFI pigs, this test was run to compare poor-LRFI to good-LRFI, and poor-HRFI to good-HRFI. The principle was to compare a “full model” where all parameters differ between the two experimental groups with a “reduced model”, which has common parameters. This was done for each parameter successively. An F-test was used to test if the models were statistically different and a probability less than 0.05 was considered as significant.

Results

General Observations

One good-LRFI pig was excluded from analyses because it did not fully consume the 300 g of feed during the allocated time. Performance and blood indicators of immune responses are presented in Figure 1. Pig initial and final body weights were 26.5 ± 2.80 kg and 55.4 ± 6.88 kg, respectively (Fig 1A). No significant effect of hygiene of housing conditions was reported for ADG from W0 to W6 (0.683 vs. 0.614 kg/day for good and poor hygiene of housing conditions, respectively; *p* = 0.12). No difference between pigs allotted to poor or good hygiene of housing conditions were reported for any measured

variables at W0 ($p > 0.05$). At W3, plasma haptoglobin concentrations and blood neutrophil granulocyte counts were greater in pigs housed in poor than in good hygiene conditions ($p < 0.05$; Figs 1B and 1C). At W6, total IgG plasma concentrations were greater in pigs housed in poor hygiene conditions compared with pigs housed in good hygiene conditions ($p = 0.04$; Fig 1D).

Fig 1. Body weight [(A)] and blood indicators of immune and inflammatory responses [(B) Haptoglobin, (C) Neutrophil granulocytes, and (D) Immunoglobulin G] before 0 (W0), and 3 (W3) and 6 weeks (W6) after pigs transfer to good or poor hygiene of housing conditions.

Plasma Concentrations of Insulin, Energy-related Metabolites and Urea

Average concentrations of insulin, energy-related metabolites, and urea are presented in Table 2. Plasma fasted concentrations did not differ for any measured variables ($p > 0.05$). There was a sampling time effect ($p < 0.05$) for all metabolites studied. No hygiene effect and interaction between Line × Hygiene were reported ($p > 0.05$). Regardless of hygiene conditions, LRFI pigs had greater insulin ($p < 0.001$) and lower average plasma concentrations of triglycerides ($p = 0.04$) than HRFI pigs.

Table 2. Average plasma concentrations of insulin, energy-related metabolites, and urea measured in low and high residual feed intake pigs (LRFI and HRFI) housed in good (Good) or poor (Poor) hygiene conditions at week 6.

	LRFI		HRFI			*p*-value[4]		
	Good	Poor	Good	Poor	SEM	Line	Hyg	Line×Hyg
No.[1]	5	6	6	6				
Average plasma concentrations[2]								
Insulin, mU/l	27.8	29.3	17.3	15.3	1.87	<0.001	0.89	0.37
Glucose, mg/l	1050	1044	1011	1009	22	0.11	0.86	0.93
FFA[3], µmol/l	99.7	125.3	134.8	107.3	23.8	0.72	0.97	0.28
Triglycerides, mg/l	274	262	343	321	30	0.04	0.58	0.87
Urea, mg/l	140	150	143	158	13	0.70	0.36	0.86

[1]No. = number of animals per group.

[2]Average plasma concentrations include fasted and postprandial concentrations measured for 4-h after ingestion of 300 g of feed. There was no effect of the experimental treatments on fasted concentrations for any blood variables ($p > 0.05$).

[3]FFA = free fatty acids.

[4]Probability values for the effect of genetic lines (Line), hygiene conditions (Hyg), and their interaction. There was an effect of sampling time ($p < 0.05$) for all variables studied. The interactions with time are presented in Fig 2.

The Line × Hygiene × Time interaction was significant for insulin, glucose, and triglycerides (Fig 2). In poor hygiene of housing conditions only, the maximum insulin concentration occurred earlier (45 min) and was greater in LRFI compared with HRFI pigs ($p < 0.001$; Fig 2A). For glucose, the peak value did not differ between lines ($p = 0.98$) but occurred earlier (30 min) in LRFI than in HRFI pigs when housed in poor hygiene conditions ($p < 0.001$; Fig 2B). Regarding plasma triglyceride concentrations, the minimum concentration did not differ between the four experimental groups but occurred earlier (45 min) in HRFI pigs housed in poor hygiene conditions compared with the three other groups (75 min; Fig 2D).

Fig 2. Postprandial plasma profiles of [(A)] insulin and metabolites [(B) Glucose, (C) Free fatty acids; FFA, (D) Triglycerides, and (E) Urea] measured in low and high residual feed intake pigs (LRFI and HRFI) housed in good or poor hygiene conditions at week 6.

Free Plasma AA Average Concentrations

Average concentrations of plasma free AA are presented in Table 3. Except for cysteine (Cys), there was a sampling time effect ($p < 0.05$) for all AA. Regardless of hygiene of housing conditions, LRFI pigs had greater Met, Thr, asparagine (Asn), Cys, glutamine (Gln), glutamate (Glu), and glycine (Gly) ($p < 0.05$), and lower histidine (His), valine (Val), and alanine (Ala) plasma concentrations ($p < 0.05$) than HRFI pigs. Irrespective of genetic line, average plasma concentrations of His, isoleucine (Ile), leucine (Leu), Met, Thr, Val, and Ala were greater ($p < 0.05$) and Gly were lower ($p = 0.02$) in poor than in good hygiene of housing conditions. For dispensable AA, the interaction between Line and Hygiene was significant for aspartate (Asp), proline (Pro), serine (Ser), and tyrosine (Tyr). Average concentrations of Asp were greater in poor than in good hygiene of housing conditions in HRFI pigs only ($p < 0.001$). When housed in poor hygiene conditions, LRFI pigs had lower and HRFI had greater plasma concentrations of Pro ($p < 0.05$) than in good hygiene conditions. Low-RFI pigs had greater average concentrations of Ser in poor than in good hygiene of housing conditions ($p < 0.001$) whereas average concentrations did not differ in HRFI pigs ($p = 0.72$). Poor hygiene of housing conditions resulted in lower average Tyr concentrations in LRFI and greater in HRFI pigs compared with good conditions ($p < 0.001$). For indispensable AA, the interaction between

Line and Hygiene was significant for arginine (Arg), Lys and Trp. These results are described in details in the following paragraph.

Table 3. Average concentrations of plasma free AA (nmol/ml) measured in low and high residual feed intake pigs (LRFI and HRFI) housed in good (Good) or poor (Poor) hygiene conditions at week 6.

	LRFI		**HRFI**			***p*-value[3]**		
	Good	Poor	Good	Poor	SEM	Line	Hyg	Line×Hyg
No.[1]	5	6	6	6				
Average plasma AA concentrations[2]								
Arginine	151.5^{a}	139.2^{b}	100.3^{c}	99.8^{c}	8	<0.001	0.09	0.04
Histidine	70.3	69.3	75.8	82.8	10	<0.001	0.02	0.05
Isoleucine	122.2	135.2	127.6	140.0	19	0.12	<0.001	0.08
Leucine	174.7	186.6	177.6	191.3	12	0.13	<0.001	0.10
Lysine	243.8^{a}	234.5^{ab}	216.9^{b}	246.2^{a}	15	0.16	0.02	<0.001
Methionine	40.0	45.9	30.6	36.1	11	<0.001	<0.001	0.95
Phenylalanine	83.2	82.3	84.5	85.8	8	0.84	0.14	0.58
Threonine	120.1	129.9	84.0	95.0	17	<0.001	<0.001	0.44
Tryptophan	62.5^{a}	51.7^{b}	44.2^{bc}	41.4^{c}	37	<0.001	<0.001	<0.001
Valine	250.4	267.8	260.7	279.9	15	0.04	<0.001	0.32
Alanine	448.3	496.4	527.0	564.5	3	<0.001	<0.001	0.39
Asparagine	60.3	59.1	52.3	54.1	17	<0.001	0.92	0.99
Aspartate	18.2^{a}	18.4^{a}	13.8^{c}	15.2^{b}	5	<0.001	0.16	<0.001
Cystine	40.4	41.0	36.9	38.2	10	0.04	0.20	0.66
Glutamine	592.2	590.1	522.4	517.8	7	<0.001	0.88	0.64
Glutamate	207.5	214.5	157.8	162.4	6	<0.001	0.53	0.11

Glycine	767.2	733.9	732.0	713.0	12	0.01	0.02	0.55
Proline	302.1[a]	288.6[b]	248.5[d]	274.7[c]	14	<0.001	0.06	0.02
Serine	134.1[b]	141.6[a]	125.3[b]	133.1[b]	9	<0.001	<0.001	<0.001
Tyrosine	71.0[a]	56.9[b]	48.6[c]	58.9[b]	16	<0.001	0.46	<0.001

[1]No. = number of animals per group.

[2]Average plasma concentrations include fasted and postprandial concentrations measured for 4-h after ingestion of 300 g of feed.

[3]Probability values for the effect of genetic lines (Line), hygiene conditions (Hyg), and their interaction. Except for cystine ($p = 0.18$), there was an effect of sampling time ($p < 0.05$) for all AA studied. The parameters of the model for indispensable AA whose Line×Hyg interaction was significant are presented in Table 4. There was Line x Time effect for Arg, only ($p < 0.001$).

[a,b,c] Within a row values with different superscripts differed ($p < 0.05$).

Plasma Free Indispensable AA Postprandial Profiles

Average Arg concentrations were lower in poor than in good hygiene of housing conditions in LRFI pigs only ($p = 0.04$; Table 4). This effect was associated with lower *C*0 value in LRFI pigs when housed in poor hygiene conditions ($p = 0.02$; Table 4 and Fig 3A). Lysine average concentrations ($p = 0.02$; Table 4) and *C*max ($p < 0.001$; Table 4 and Fig 3B) value were higher in poor hygiene of housing conditions in HRFI pigs only. Lower Trp concentrations were observed in poor hygiene of housing conditions in LRFI pigs only ($p < 0.001$; Table 4). For LRFI pigs, the values of *C*0, and Tmax for Trp were lower in poor than in good hygiene of housing conditions ($p < 0.001$; Table 4 and Fig 3C).

Table 4. Values of parameters describing the postprandial kinetics of plasma free arginine, lysine, and threonine in low and high residual feed

intake pigs (LRFI and HRFI) housed in good (Good) or poor (Poor) hygiene conditions at week 6.

	LRFI			HRFI		
	Good	Poor	*P*-value[3]	Good	Poor	*P*-value[3]
No.[1]	5	6		6	6	
Parameter values[2]						
Arginine						
C0, μM	88 ± 6	69 ± 5	0.02	62 ± 6	53 ± 6	0.28
Cmax, μM	193 ± 12	188 ± 7	0.62	125 ± 5	130 ± 3	0.75
Tmax, min	86 ± 4	81 ± 2	0.84	87 ± 5	96 ± 3	0.27
Lysine						
C0, μM	126 ± 10	127 ± 8	0.98	145 ± 9	132 ± 9	0.34
Cmax, μM	349 ± 2	329 ± 7	0.12	273 ± 5	315 ± 3	<0.001
Tmax, min	77 ± 3	69 ± 2	0.42	74 ± 7	86 ± 3	0.61
Tryptophan						
C0, μM	39 ± 2	27 ± 2	<0.001	25 ± 2	22 ± 2	0.79
Cmax, μM	72 ± 0	63 ± 4	0.17	52 ± 2	50 ± 7	0.88
Tmax, min	87 ± 3	68 ± 5	<0.001	91 ± 6	87 ± 4	0.29

[1]No. = number of animals per group.

[2]Values are means ± SD. C0: the concentration at t = 0, Cmax: the maximum concentration, Tmax: the time in minute at maximum concentration.

[3]Probability values for the effect of hygiene conditions.

Fig 3. Plasma profiles of free indispensable amino acids [(A) arginine; Arg, (B) lysine; Lys, and (C) tryptophan; Trp] measured in low and high residual feed intake pigs (LRFI and HRFI) housed in good or poor hygiene conditions at week 6**. The observed (Ob) and estimated (Es) values are presented.**

Discussion

This study was conducted to compare the metabolic modifications induced by poor hygiene of housing conditions in LRFI and HRFI growing pigs. Our major and original finding is that the effects of the poor hygiene of housing conditions on the postprandial profiles of insulin and some indispensable AA differ between the two RFI lines. We discuss if such differences may explain the better coping ability of LRFI pigs previously reported [8].

To compare metabolic changes induced by the experimental factors, we analysed the average postprandial plasma concentrations of insulin, energy-related metabolites, urea, and amino acids, as well as the pattern of postprandial plasma indispensable AA profiles. Briefly, after being fasted overnight, on the day of serial blood samplings, all pigs received 300 g of the same feed that they consumed in less than 10 min. Thus, differences in plasma profiles were associated to differences in both digestion and postprandial metabolism induced by the experimental factors, namely the genetic line, the hygiene of housing condition, and their interaction. In the current study, a model of poor hygiene of housing conditions was used to induce an immune system activation. The findings of greater plasma haptoglobin concentrations and blood neutrophil granulocyte counts in poor than in good hygiene of housing conditions at W3 indicate that the model successfully induced an inflammation and activated immune responses [16]. Indeed, haptoglobin a major acute phase protein in pig, and blood neutrophil granulocyte counts are both relevant indicators of inflammation in pigs [17,18]. Moreover, greater total IgG plasma concentrations in poor hygiene of housing conditions at W6 show that the

immune system was still stimulated at the time of serial blood sampling. Contrary to AA, energy-related metabolites and insulin did not differ between poor and good hygiene of housing conditions. It should be noted that in our study, insulin and energy metabolite concentrations were measured 6 weeks after the beginning of the challenge when pigs were probably recovering from inflammation as indicated by lower blood haptoglobin concentrations at W6 compared with W3. Indeed, during an inflammatory challenge, plasma glucose concentrations were restored two days after being temporarily increased in young growing pigs [4] showing the fast return to glucose homeostasis. Pigs housed in poor hygiene conditions had greater concentrations of Ala, His, Met, Thr, and branched-chain AA (BCAA) than pigs housed in good conditions. Greater AA plasma concentrations may be due to a lower AA retention as muscle protein in immune challenged pigs [19] caused by an increase in protein breakdown and/or decrease in protein synthesis. If our experiment conducted on a low number of pigs failed to report a significant but numeric reduction in ADG in response to poor hygiene, growth performance measured in the whole set of pigs was depressed after 6 weeks of housing in poor hygiene conditions and this effect was mainly caused by the activation of the immune system [8]. Conversely, lower Thr postprandial plasma concentrations were reported in pigs coinfected with *Mycoplasma hyopneumoniae* and influenza virus probably to support a great demand for immunoglobulin synthesis [12]. Such a discrepancy regarding the response of Thr concentrations in our study was unexpected since the hygiene challenge increased immunoglobulin plasma concentrations but this increase was probably too moderate to affect plasma Thr concentrations. From these results, it can be suggested that the immune

system activation impacted glucose metabolism more rapidly and for a shorter period than protein metabolism, as previously reported after a change in hormonal status in growing pigs [10]. Accordingly, in septic rats, plasma concentrations of tumor necrosis factor α measured 1.5 hours after an experimental infection showed a strong correlation with changes in protein metabolism and body weight two weeks later [20], demonstrating that prolonged effects on nitrogen metabolism may be observed while blood indicators are no more detectable or return to normal values.

Contrary to the hygiene challenge that affects metabolism and digestibility [18], the selection for RFI did not affect the digestibility of a standard low fiber feed [21]. Thus, differences in plasma profile between RFI lines are mostly explained by a difference in metabolism. Irrespective of hygiene conditions, LRFI pigs had greater average plasma concentrations of insulin and lower plasma concentrations of triglycerides than HRFI pigs. In agreement with our findings, Montagne et al. [22] observed greater plasma insulin concentrations after the ingestion of a small meal and Le Naou et al. [23] reported lower plasma triglyceride concentrations measured at fed state in LRFI compared with HRFI pigs. In pigs, plasma triglycerides result from the lipolysis of lipids stored in adipose tissue. Insulin is an anabolic hormone with a potent anti-lipolytic action on adipose tissue [24]. Therefore, lower triglyceride concentrations may be partly explained by the greater insulin concentrations in plasma. Alternatively, lower plasma triglycerides levels may also be attributed to the lower body fat content of LRFI compared with HRFI pigs [25]. Moreover, the impact of the overnight fasting associated with feed restriction on the day of serial blood samplings may be greater in HRFI pigs forcing them to mobilize their body fat

reserve. However, feed restriction of HRFI pigs did not affect their plasma triglyceride concentrations [23]. Poor hygiene of housing conditions impacted differently insulin response in LRFI and HRFI. Indeed, the postprandial peak of insulin occurred earlier and was greater in LRFI compared with HRFI pigs when housed in poor hygiene conditions. Such an effect contributes to explain why LRFI pigs maintained their growth rate in poor hygiene of housing conditions [8]. Insulin is indeed the main hormone allowing the postprandial AA utilization for protein synthesis in muscle [26].

Plasma urea concentration did not significantly differ between the two lines. Urea is produced from the deamination of AA and, at fed state, reflects the catabolism of dietary AA that are not used for body protein synthesis and deposition. Our result is in line with a previous study showing that genetic selection for RFI did not affect nitrogen metabolism when HRFI and LRFI pigs were fed the same restricted level of feed [5]. However, differences in plasma free AA average concentrations were observed between the two RFI lines. For instance, His, Val, and Ala plasma concentrations were lower in LRFI than in HRFI pigs. Lower plasma Ala concentrations at fed state were previously reported in LRFI compared with HRFI pigs [4] suggesting a lower muscular release of Ala for hepatic glucose synthesis (Cahill cycle) in LRFI pigs. This is in accordance with lower energy expenditure in LRFI than in HRFI pigs [5]. The BCAA (Ile, Leu and Val) are the major donors of the amino group for the synthesis of Ala from pyruvate in muscle [4]. In the present experiment, despite that only Val concentrations differed between the two lines, Ile and Leu plasma concentrations were numerically lower in LRFI than in HRFI pigs. Alanine synthesis in muscle and its release in the plasma in LRFI pigs may have been

reduced by the decreased availability of BCAA. In the current study, Arg and Trp average and basal plasma concentrations were lower in LRFI pigs when housed in poor compared with good hygiene conditions whereas the challenge did not affect plasma concentrations of these two AA in HRFI pigs. Besides being proteinogenic AA, both Trp and Arg are known to be involved in immune-related metabolic pathways. During immune activation, Trp is catabolized in kynurenine by the indoleamine 2,3-dioxygenase (IDO) enzyme, a metabolic pathway involved in the regulation of immune responses [27]. Arginine is an AA serving as a precursor for the synthesis of polyamines that is massively used by rapidly dividing cells like proliferating lymphocytes [28]. It is also involved in the synthesis of creatine that has anti-oxidative and anti-inflammatory functions [29]. Lower C0 values for Trp and Arg may reflect an effect of immune activation that is independent of postprandial use of AA for muscle anabolism. Besides, for Trp, the time-related variations differed between hygiene of housing conditions in LRFI pigs with earlier time at maximum plasma concentration (Tmax) in poor than those housed in good hygiene conditions. Such results might be a consequence of faster postprandial clearance of dietary Trp for both immune responses and muscle anabolism. If genetic selection for LRFI reduces the total tract digestive capacity of pigs challenged with bacterial endotoxin [30], a significant contribution of the digestive tract to Trp postprandial utilization seems unlikely since Trp is not extensively used by the gut [31]. To summarize, lower average concentrations of Trp and Arg in LRFI pigs may result from an increased utilization of these two AA for immune purposes and may contribute to support the greater ability of LRFI pigs to cope with poor hygiene of housing conditions and to maintain their growth rate in challenging conditions [8]. When

housed in poor hygiene conditions, HRFI pigs had greater average concentrations and maximum plasma concentration (Cmax) of Lys than those housed in good conditions whereas no difference between hygiene was reported in LRFI pigs. Greater plasma Lys concentrations are probably a consequence of lower body protein synthesis and reduced efficiency of nitrogen utilization for protein retention in pigs when the immune system is overstimulated [32]. Accordingly, Chatelet et al. [8] reported that ADG of the HRFI pigs was more affected by poor hygiene of housing conditions with a difference in ADG between poor- and good-HRFI pigs being twice the differences observed between poor- and good-LRFI pigs.

In conclusion, our results show that insulin, energy metabolites, and AA postprandial profiles differ between LRFI and HRFI pigs in response to poor hygiene of housing conditions. The findings that only LRFI had lower concentrations of Trp and Arg in poor hygiene of housing conditions may be associated with a greater AA utilization for supporting their immune responses. Conversely, only HRFI pigs had greater concentrations of Lys in poor hygiene of housing conditions, which could be in accordance with our previous study showing a greater impact of the challenge on average daily gain of HRFI pigs. Although our results clearly showed that selection for RFI modified the metabolic response to the hygiene challenge, it is not possible to determine whether selection has first modified the immune response or some metabolic pathways.

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Data reporting

The data were deposited in DataINRAE repository:

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580 **Fig 1.**

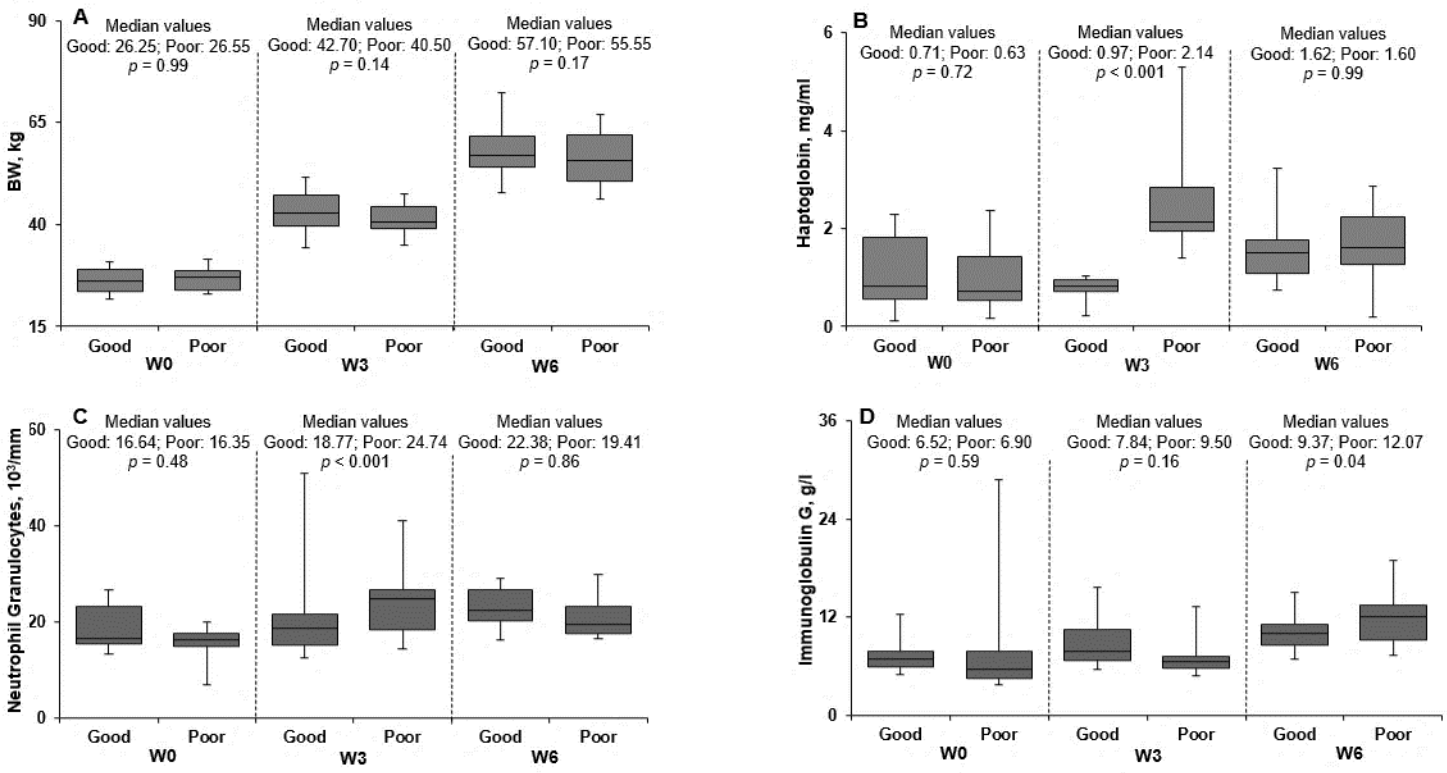

A
Median values
Good: 26.25; Poor: 26.55
p = 0.99
Median values
Good: 42.70; Poor: 40.50
p = 0.14
Median values
Good: 57.10; Poor: 55.55
p = 0.17
BW, kg
90
65
40
15
Good
Poor
W0
W3
W6
B
Median values
Good: 0.71; Poor: 0.63
p = 0.72
Median values
Good: 0.97; Poor: 2.14
p < 0.001
Median values
Good: 1.62; Poor: 1.60
p = 0.99
Haptoglobin, mg/ml
6
4
2
0
C
Median values
Good: 16.64; Poor: 16.35
p = 0.48
Median values
Good: 18.77; Poor: 24.74
p < 0.001
Median values
Good: 22.38; Poor: 19.41
p = 0.86
Neutrophil Granulocytes, 10³/mm
60
40
20
0
D
Median values
Good: 6.52; Poor: 6.90
p = 0.59
Median values
Good: 7.84; Poor: 9.50
p = 0.16
Median values
Good: 9.37; Poor: 12.07
p = 0.04
Immunoglobulin G, g/l
36
24
12
0

Fig 2.

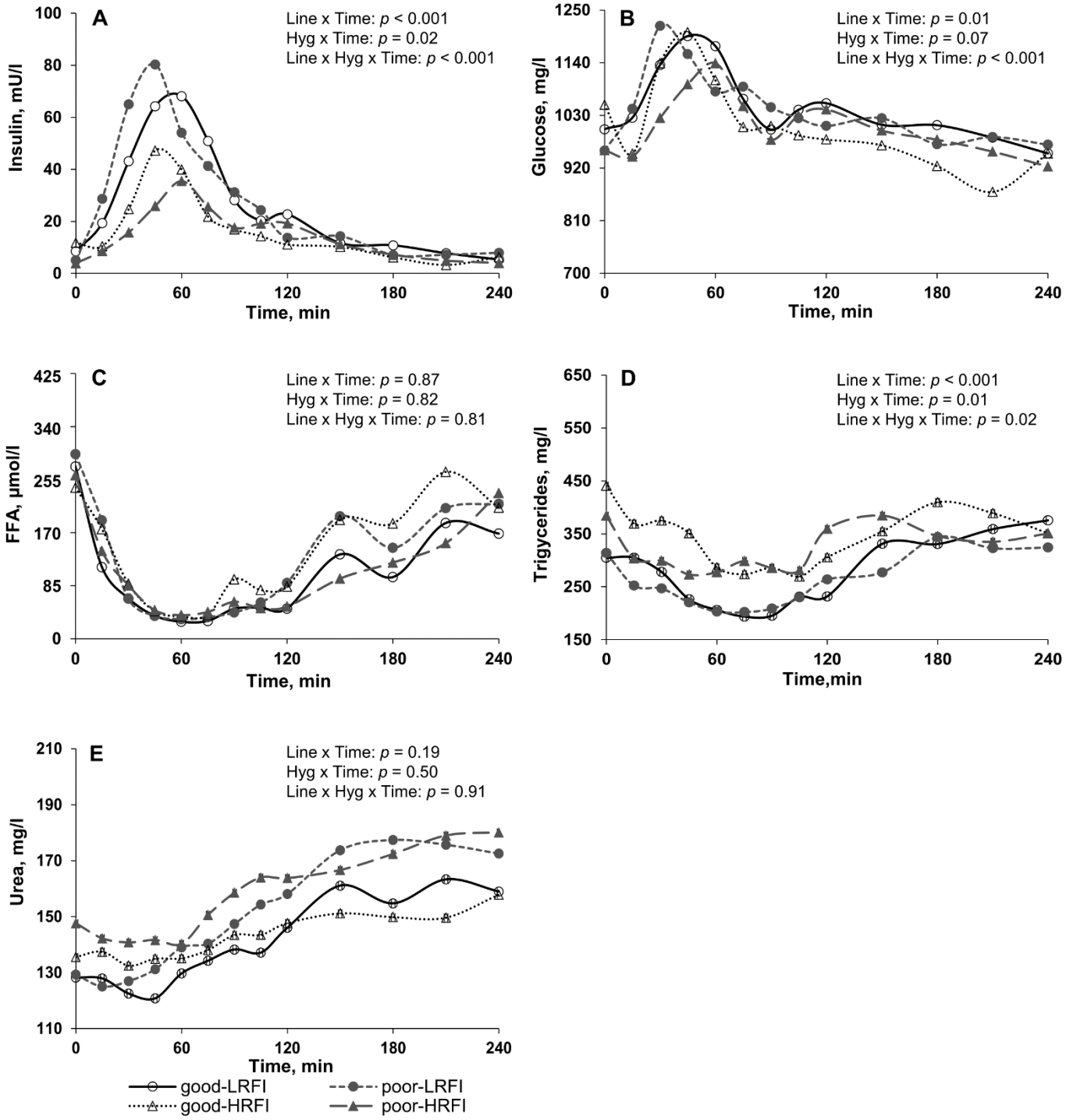
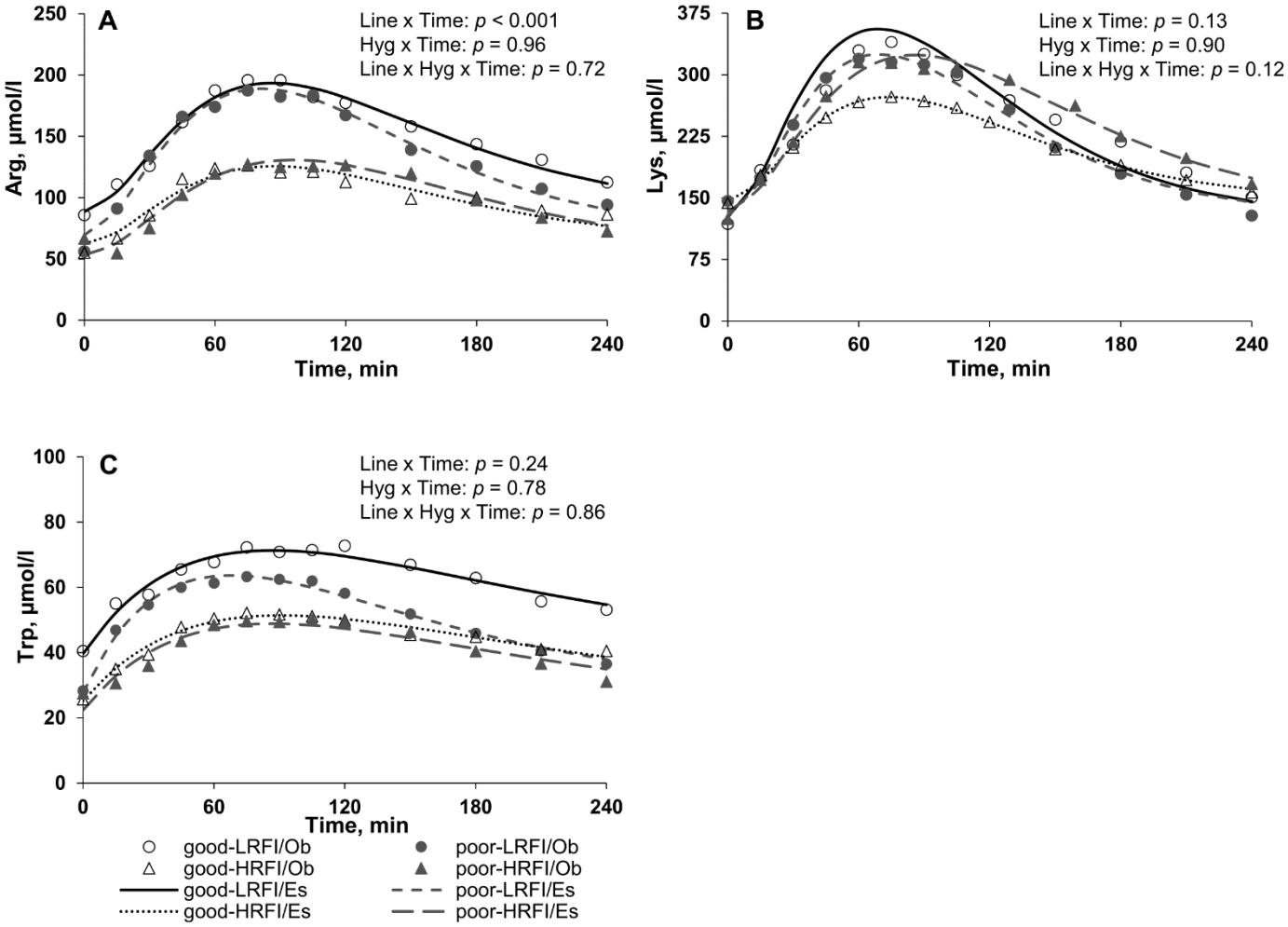

A
Line x Time: p < 0.001
Hyg x Time: p = 0.02
Line x Hyg x Time: p < 0.001
Insulin, mU/l
Time, min
B
Line x Time: p = 0.01
Hyg x Time: p = 0.07
Line x Hyg x Time: p < 0.001
Glucose, mg/l
Time, min
C
Line x Time: p = 0.87
Hyg x Time: p = 0.82
Line x Hyg x Time: p = 0.81
FFA, µmol/l
Time, min
D
Line x Time: p < 0.001
Hyg x Time: p = 0.01
Line x Hyg x Time: p = 0.02
Trigycerides, mg/l
Time,min
E
Line x Time: p = 0.19
Hyg x Time: p = 0.50
Line x Hyg x Time: p = 0.91
Urea, mg/l
Time, min
good-LRFI
poor-LRFI
good-HRFI
poor-HRFI

Fig 3.


A
Line x Time: p < 0.001
Hyg x Time: p = 0.96
Line x Hyg x Time: p = 0.72
Arg, µmol/l
Time, min
B
Line x Time: p = 0.13
Hyg x Time: p = 0.90
Line x Hyg x Time: p = 0.12
Lys, µmol/l
Time, min
C
Line x Time: p = 0.24
Hyg x Time: p = 0.78
Line x Hyg x Time: p = 0.86
Trp, µmol/l
Time, min
good-LRFI/Ob
good-HRFI/Ob
good-LRFI/Es
good-HRFI/Es
poor-LRFI/Ob
poor-HRFI/Ob
poor-LRFI/Es
poor-HRFI/Es

CHAPTER 5 – A BLEND OF FUNCTIONAL AMINO ACIDS AND GRAPE POLYPHENOLS IMPROVES THE PIG CAPACITY TO COPE WITH AN INFLAMMATORY CHALLENGE CAUSED BY POOR HYGIENE OF HOUSING CONDITIONS[12]

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[12] The chapter is presented according to BMC Veterinary Journal formatting.

Abstract

Background: Dietary supplementation with a blend of functional amino acids (AA) and grape extract polyphenols contribute to preserve intestinal health and growth performance of piglets during the post-weaning period. In the present experiment, we assessed if a supplementation with a mix of AA and grape extract polyphenols during the post-weaning period would persist to improve the pig capacity to cope with a subsequent challenge caused by poor hygiene of housing conditions. Eighty pigs weaned at 28 days of age were fed a standard diet supplemented (AAP) or not (CNT) with 0.2% of a blend of AA (glutamine, arginine, cystine, valine, isoleucine, and leucine) and grape extract polyphenols during the post-weaning period (from week 0 to 6). At week 6, pigs were transferred to a growing unit where 50% of pigs previously fed AAP and CNT diets were housed in good and the other 50% in poor hygiene conditions for 3 weeks (from week 7 to 9; challenge period). All pigs were fed a standard growing diet that was not supplemented with AAP. We measured pig growth performance, plasma indicators of inflammation, digestive integrity, and oxidative status, and scored fecal consistency. Differences were considered significant at $P \leq 0.05$.

Results: One week post-weaning, pigs fed AAP had lower plasma concentrations of haptoglobin than CNT pigs ($P = 0.03$). Six weeks post-weaning, plasma concentrations of diamine oxidase (DAO) was lower ($P = 0.03$) whereas those of vitamin E and A were greater ($P \leq 0.05$) in pigs fed AAP compared to CNT pigs. The prevalence of diarrhea was higher in CNT pigs compared to AAP pigs ($P < 0.01$). During the challenge period, only pigs previously fed CNT diet had lower growth rate in poor than good conditions ($P \leq 0.05$). They had also greater plasma concentrations of haptoglobin and oxidative stress index (OSI)

and lower plasma concentrations of vitamin E in poor than good hygiene conditions ($P \leq 0.05$).

Conclusions: Pigs fed AAP diet during post-weaning had less diarrhea and plasma concentrations of a digestive integrity marker, as well as greater plasma concentrations of antioxidant indicators during the post-weaning period. The beneficial effects of AAP supplementation persisted after the post-weaning period as evidenced by the absence of effects of the hygiene challenge on growth and health indicators in pigs previously fed APP. This clearly indicated a greater ability of pigs fed AAP to cope with the poor hygiene conditions.

Keywords: health, inflammation, weaning transition

Background

In conventional pig farming systems, pigs are usually weaned between 3 and 5 weeks of age, when animals still have an immature immune system and low digestive capacity [1]. Furthermore, during the weaning transition pigs are exposed to multiple stressors including changes of the diet, separation from their mothers and mixing with unfamiliar littermates [2,3]. The changes predisposed the animals to reduced feed intake, impaired intestinal functions, and low nutrient absorption, all contributing to post-weaning diarrhea and poor growth [3]. The post-weaning period is thus considered as one of the most critical and stressful period for the pig. Nutritional strategies have been widely studied to support immune maturation and enhance gastrointestinal morphology and functions of pigs at weaning.

Besides being building blocks of proteins, amino acids (AA) are known to be nutrients that contribute to health maintenance. Functional AA was defined as

those AA that exert functions other than being precursors of proteins [4]. Dietary supplementation with arginine (Arg), branched-chain amino acids (BCAA; leucine, isoleucine, and valine), cystine (Cys2), and/or glutamine (Gln) separately [5-8] or in a blend [9] was associated with improvement of intestinal integrity and immune status in weaned and/or inflammatory-challenged pigs. Furthermore, plants extract containing proanthocyanidins, the most common and consumed dietary polyphenols, has been increasingly studied as a potential health-promoting dietary component. Previous findings have demonstrated a reduced prevalence of post-weaning diarrhea in piglets fed with a grape extract polyphenols supplemented diet by the improvement of the immune and antioxidant capacities [10]. Recently, a study reported that diet supplementation with a blend of functional AA and grape extract polyphenols improved growth performance of piglets during the post-weaning period [11]. This beneficial effect was associated with a greater production of protective bacterial metabolites, a higher abundance of *Lactobacillus* in the jejunum and a lower abundance of *Proteobacteria* in the caecum [11]. We hypothesized that the beneficial effects of AA and grape polyphenols supplementation could persist after the post-weaning period and thereby confer a greater ability of pigs to cope with a subsequent challenge. Therefore, this study was performed to evaluate whether 0.2% of a blend of functional AA (Arg, Gln, Cys2 and BCAA) and grape extract polyphenols (AAP) supplied during the post-weaning period may improve the pig capacity to cope with the weaning transition and with an inflammatory challenge caused by poor hygiene conditions in the beginning of the growing period.

Results

Eighty pigs were weaned at 28 days of age and fed a control standard diet supplemented or not with 0.2% of a blend of functional amino acids and grape polyphenols (AAP and CNT diets, respectively) during the post-weaning period (from week 0 to 6). Six weeks after weaning (week 6), pigs were transferred in a growing unit where they were housed in good or poor hygiene conditions and fed a standard diet for 3 weeks (challenge period). The finishing period lasted 11 weeks and all animals were slaughtered at the end of the experiment (week 20; at 24 weeks of age). Two experimental groups, AAP and CNT pigs, were compared during the post-weaning period and four experimental groups were compared during the challenge period: AAP-good, AAP-poor, CNT-good and CNT-poor for pigs previously fed AAP housed in good and poor hygiene conditions or CNT housed in good and poor hygiene conditions.

General observations

Data from 80 pigs were kept for statistical analyses. One CNT-poor pig died probably due to intestinal torsion during the finishing period. Average ambient temperatures were 23.5 ± 1.5 °C (post-weaning period), 20.9 ± 1.0 °C and 21.1 ± 1.5 °C (in good and poor conditions, respectively), and 19.6 ± 1.0 °C (finishing period). Lower air concentration of carbon dioxide (625 vs 850 ppm; $P < 0.01$) and ammonia (7.5 vs 10.6 ppm; $P = 0.08$) levels were observed in good as compared to hygiene conditions. The concentration of hydrogen sulfide in the air did not differ between hygiene conditions (0.50 and 0.53 ppm in good and poor conditions, respectively; $P = 0.92$).

During the post-weaning period, the following clinical signs were recorded: thinness [AHOL_0003136; [12]] and apathy [AHOL_0003037] (three CNT pigs),

sneezing [AHOL_0003116] and cough [AHOL_0003090] (four AAP and five CNT pigs), nose wound (one AAP pig), and diarrhea [AHOL_0003105] (21 AAP and 30 CNT pigs). In CNT pigs, the number of pigs with diarrhea was greater than the number of pigs without diarrhea ($P < 0.01$; Figure 1) whereas it did not differ in AAP pigs ($P = 0.75$). During the challenge period, the following clinical signs were observed: umbilical hernia (one CNT-good pig), sneezing and cough (12 pigs: three AAP-good, four CNT-good, two AAP-poor and three CNT-poor), and diarrhea (43 pigs: one AAP-good and two CNT-good; whereas all pigs had diarrhea at least once in poor conditions). The prevalence of diarrhea did not differ between pigs previously fed AAP and CNT ($P = 0.53$), whereas it was lower in good as compared to poor hygiene conditions ($P < 0.01$). Clinical signs as thinness and apathy (one pig CNT-good), diarrhea (one pig CNT-poor), umbilical hernia [AHOL_0003267] (one pig AAP-good and one AAP-poor), and skin wound (43 pigs: 10 AAP-good, 12 CNT-poor, 11 CNT-good and 10 CNT-poor) were observed during the finishing period.

Growth performance

During the post-weaning period, there was no effect of the experimental diets (ED) on performance traits (Table 1). During the challenge period (from week 7 to 9), regardless of experimental diets fed during the post-weaning period, pigs housed in good hygiene conditions had lower feed conversion ratio (FCR, ATOL_0001580; [12]) compared to pigs housed in poor conditions (2.21 and 2.52 in good and poor conditions, respectively; $P < 0.01$). Interaction between ED and hygiene conditions (Hyg) was observed for final body weight (BW, ATOL_0000351) measured at week 9 (W9) and average daily gain (ADG,

ATOL_000989; P ≤ 0.05), and a trend for interaction was observed for average daily feed intake (ADFI, ATOL_0005508; ED×Hyg: P = 0.07, Table 2). Whereas CNT pigs had lower final BW and ADG at W9 in poor as compared to good conditions (P ≤ 0.05), there was no difference between hygiene conditions for AAP pigs (P > 0.10). A trend for lower ADFI was observed in good compared to poor conditions in AAP pigs only (1.839 and 1.945 kg/day in good and poor conditions, respectively; P = 0.07).

At the end of the finishing period, a trend for greater final BW was observed for pigs previously housed in good compared to poor conditions (128.6 and 124.5 kg in good and poor conditions, respectively; P = 0.08). Except for the finishing period where male had greater final BW as compared to females (130.6 and 122.2 kg for male and female, respectively; P < 0.01), no significant differences were observed.

Blood indicators of inflammatory and oxidative status and gut integrity

Blood variables measured during the post-weaning period are presented in Table 3. At week 0 (W0), just before pigs were assigned to received AAP or CNT diet, there was no difference between AAP and CNT pigs for any studied variable (P > 0.10). One week after weaning (W1), plasma haptoglobin concentrations [ATOL_0000935] were lower (P = 0.03) in AAP than CNT pigs. Trends for lower values of diacron-reactive oxygen metabolites (dROM, 994.95 and 1073.23 CARRU; P = 0.07) and oxidative stress index (OSI, 0.38 and 0.40; P = 0.06) were observed in AAP than CNT pigs. At the end of the post-weaning period (W6), plasma concentrations of vitamin E and vitamin A were greater in AAP compared to CNT pigs (P ≤ 0.05). Lower concentrations of diamine oxidase

(DAO; P = 0.03) and a trend for lower OSI value (0.40 and 0.43; P = 0.09) in AAP than CNT pigs were also observed at W6.

At the end of the challenge period (W9), pigs housed in good hygiene conditions had lower concentrations of white blood cells (WBC), granulocytes, and dROM compared to pigs housed in poor conditions (P ≤ 0.05; Table 4). There was an interaction between ED and Hyg for haptoglobin, vitamin E concentrations and OSI (P ≤ 0.05); however, differences between hygiene conditions were significant only for CNT pigs. When housed in good hygiene conditions, CNT pigs had greater concentrations of vitamin E (P < 0.01; Fig 2A) and lower haptoglobin and OSI (P ≤ 0.05; Fig 2B and 2C) than in poor conditions.

Fecal microbiota

The fecal microbiota was analyzed at the end of the post-weaning period (W6). There was no effect of ED on the microbiota richness and α-diversity indexes (Additional file 1). Analysis of β-diversity using the Bray–Curtis distance indicated a minor effect of ED on the microbiota structure (PERMANOVA P < 0.05 and 2% of explained variance R^2). The effect of ED was significant (P < 0.05) on the relative abundance of 3 bacterial genera representing more than 1% of the microbiota (*Streptococcus*, *Coprococcus* and a *Lachnospiraceae* genus); but these effects were not significant when considering P-values corrected for false discovery rate (Additional file 1). Overall, ED did not induce major modifications of the fecal microbiota at the end of the post-weaning period.

Discussion

The health status and growth performance of pigs were analyzed to

evaluate the effects of a dietary supplementation with a blend of functional AA (Gln, Arg, Cys2 and BCAA) and grape extract polyphenols during the post-weaning period on pig capacity to cope with weaning and a subsequent inflammatory challenge caused by poor hygiene of housing conditions. Modulatory effects on gut microbiota composition and metabolic activity, and on epithelial homeostasis in intestinal organoids were recently reported in weaned pigs fed AA and polyphenols-supplemented diet [11]. Therefore, we hypothesized that pigs fed AAP diet during the post-weaning period would be healthier and more robust at the end of post-weaning to cope with an inflammatory challenge applied in the beginning of the growing period, when they were not fed anymore this supplemented diet. Our major and original findings is that AAP diet delivered during the post-weaning period supported weaning transition and prevented the detrimental effects of poor hygiene conditions in the beginning of the growing period.

During the post-weaning period, pigs fed AAP diet had lower plasma haptoglobin concentrations, a trend to lower values of dROM and ISO, and greater concentrations of vitamins E and A compared to CNT pigs. Haptoglobin is a major acute phase protein in pigs and an increase in its plasma concentration is an indicator of inflammation and/or infection [13]. Weaning is known to increase the concentration of haptoglobin in the plasma [2, 12]. In pigs, greater plasma concentrations of haptoglobin after than before weaning were considered as inflammatory [14,2] and/or stress [14] responses related to weaning. Furthermore, dROM and ISO are indicators of oxidative stress [15]. Higher dROM and haptoglobin concentrations, and lower concentrations of vitamins E and A observed in pigs after weaning are explained by inflammation of the intestinal

mucosa and increase oxidative stress as consequence of weaning-induced stress [15-17]. Vitamins E and A are antioxidant molecules [15]. Vitamin E is mainly related to hydroperoxyl radical scavenging [18] and vitamin A acts via hydrogen atom donating [19]. Lower plasma concentrations of vitamins E and A were observed in pigs 5 days after weaning wherein vitamin E remained low until 19 days after weaning [15]. These findings were associated with a greater production of free radicals, which in turn are neutralized by antioxidant molecules (such as vitamins E and A) leading to a decrease in their concentrations [15]. Therefore, the lower haptoglobin and the trends for greater vitamins E and A concentrations in pigs fed AAP during post-weaning may be indicative of a lower inflammatory status associated with greater antioxidant potential. Furthermore, it can be hypothesized that greater plasma concentrations of both vitamins in AAP pigs may be a result of their lower utilization, probably because of the supply of polyphenols acting as exogenous antioxidants. Weaning also impairs the intestinal barrier that can result in post-weaning diarrhea [20]. More pigs were observed with than without diarrhea in CNT pigs while no difference was observed for AAP pigs, suggesting a protective effect of AAP. Diamine oxidase is a digestive integrity biomarker [21]. In humans, an increase in plasma DAO concentrations reflect impairment of intestinal morphology and lower nutrient absorption [21]. Therefore, the lower concentrations of DAO in AAP compared to CNT pigs suggest that AAP pigs had better mucosa integrity and effective barrier function, which may indicate that AAP dietary supplementation plays a protective role in gut health during the post-weaning period. Accordingly, Beaumont et al. [11] reported that a mix of AA and polyphenols upregulated the gene expression of indicators of epithelial differentiation and reduced the expression of

inflammatory indicators in pig intestinal organoids. In the same study, supplementation of weaned piglets with AA and polyphenols induced modulation of the gut microbiota by increasing the abundance of *Lactobacillaceae* in the jejunum while decreasing the abundance of the *Proteobacteria* in the caecum [11]. In contrast, in the current study, AAP supplementation had no major effect on the fecal microbiota 6 weeks after weaning. Difference between the two studies might be explained by the gut segment studied (jejunum/caecum versus fecal samples). Indeed, supplemented AA and polyphenols are probably found in greater concentrations in the middle intestine where they exert their effect on microbiota. Moreover, we analyzed the microbiota 6 weeks after weaning while the previous study analyzed samples collected 2 weeks after weaning, when the microbiota is still adapting to the new diet and environment. Since pigs from both diets started the experiment with similar BW and health indicators, the better health status observed for AAP pigs during the post-weaning period could be explained by AA and grape polyphenols biological functions. Among the AA in the blend, Cys2 and Arg are strongly associated with increase in antioxidant capacity [22] and/or regulating the inflammation in weaned pigs [23, 24]. Glutamine is a gut-trophic nutrient, whose effects are improvement of intestinal morphology and permeability, lower oxidative damage, and higher enterocyte proliferation in pigs [25, 26]. Dietary supplementation with 1% L- Gln enhanced intestinal oxidative-defense capacity of weaned pigs, demonstrated by greater jejunal concentrations of glutathione (GSH; 29%) and reduced jejunal concentrations of oxidized glutathione (GSSG; -18%) compared to non-supplemented pigs [26]. Besides AA, grape polyphenols are also related with antioxidant and anti-inflammatory effects [27]. Accordingly, pigs fed polyphenols-

supplemented diet during the post-weaning period had greater antioxidant capacity evidenced by expression of enzymatic defense system [10]. Indeed, these nutrients and nutrient-related metabolites promote health in response to stressful stimuli such as weaning [5, 11].

In the current study, we used a model of poor hygiene of housing conditions to induce a moderate inflammatory state. This model is known to reduce pig growth performance and to increase plasma haptoglobin concentration and granulocyte count in the blood [28]. Furthermore, lower growth rate and greater prevalence of diarrhea were observed in pigs housed in poor compared to good hygiene conditions [14, 29]. In addition to greater WBC and blood granulocyte count, FCR and the prevalence of diarrhea were higher in pigs housed in poor conditions compared to pigs in good conditions. Still 11 weeks after the end of the challenge, pigs previously housed in poor conditions tended to be lighter (4 kg/animal) than those previously housed in good hygiene, indicating that pigs leaving poor hygiene conditions did not completely recover from the challenge as already shown by Chatelet et al. [30]. Interestingly, during the challenge period, most of the effects of poor hygiene were reported in CNT pigs only. Growth rate, haptoglobin and vitamin E concentrations in pigs previously fed AAP diet during the post-weaning period were not affected by poor hygiene conditions. As above-mentioned, AAP pigs had lower inflammatory responses and greater antioxidant capacity in the post-weaning period. Recently, it has been shown that diet supplementation with the AA L-threonine, DL-methionine, and L-tryptophan improved antioxidant defense system in post-weaned pigs and prepared them better to cope with a subsequent Salmonella challenge [31]. These authors observed that an adaptation period with AA-

supplemented diet reduced the inflammatory responses (increase albumin and decrease haptoglobin plasma concentrations), which was accompanied by reducing colonization of the inoculated *Salmonella* [31]. Furthermore, Arg supplementation in a milk replacer diet during the pre-weaning period supported pigs' weaning transition and improved intestinal morphology (greater villus height, villous area, and relative intestine weight) in post-weaned pigs [32]. Accordingly, our results showed that the positive effects of AA and grape polyphenols supplementation extend beyond the supplemented period and may help to support health and growth performance later on.

Conclusions

Our results show an improvement of the capacity of AAP pig to cope with the weaning transition as evidenced by lower concentrations of the digestive integrity marker, greater levels of antioxidant markers at the end of the weaning period, and lower prevalence of diarrhea. The absence of negative effects of hygiene challenge in AAP pigs indicated that the beneficial effects with 0.2% AAP dietary supplementation persisted after the post-weaning period and resulted in a greater ability of pigs to cope with poor hygiene conditions. These findings support the development of nutritional strategies potentially applicable in the field to improve health of pigs during critical periods.

Methods

Experimental design

The experiment was carried out at INRAE (UE3P, Saint-Gilles, France doi.org/10.15454/1.5573932732039927E12), in compliance with the Directive

2010/63/UE on animal experimentation. The experimental protocol was approved by the regional Ethical Committee in Animal Experiment of Rennes (France) and by the French Ministry of Higher Education, Research and Innovation (authorization APAFIS#23735-2020012214413172). Eighty male and female Piétrain x Large-White x Landrace pigs born from 30 sows were selected and weaned at 28 day (8.79 kg BW; SEM: 0.12 kg). The experiment was conducted in two batches of 40 animals born at six weeks of interval. The same experimental design was replicated in both batches. For each batches, the experiment lasted 20 weeks and was divided into 3 periods: post-weaning (from week 0 to 6); challenge (from week 7 to 9); and finishing (from week 10 to 20).

During the post-weaning period, pigs were fed a control standard diet supplemented or not with 0.2% of a blend of functional AA and grape polyphenols (AAP and CNT diets, respectively). The blend contained L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine and L-glutamine (in a weight ratio 35:15:8:8:8:20) and 40 ppm of polyphenol-rich grape extract. The amino acids composing the blend were the same as the study by Beaumont et al. [11] plus glutamine known to exert trophic effect on the intestinal tract of weaned pigs [25]. The dose of grape extract polyphenols was close to the lowest dose that reduced the prevalence of diarrhea in weaned pigs in the study of Hao et al. [10]. The grape extract was obtained from seeds and skins (85 and 15%, respectively) and contains around 70% of polyphenols on dry material. Proanthocyanidins, class of polyphenols from the flavonoid family, are the predominant polyphenols in grape extract. The composition and calculated nutrient content of diets provided during the pre-starter (from week 0 to 1; post-weaning) and starter (from week 2 to 6; post-weaning) phases are presented in the Additional file 3.

At the end of W6, pigs were transferred to a growing unit where 50% of pigs fed AAP and CNT diets were housed in good and the other 50% in poor hygiene conditions for three-weeks (challenge period). At the end of challenge period (at W9), regardless of experimental diet and hygiene of housing conditions, pigs were transferred in another room within the same experimental growing unit (finishing period). Pigs were slaughtered 20 weeks after weaning.

Animals housing and feeding

During the post-weaning period, pigs were group housed in slatted floor pens (145 × 270 cm, n=5 pigs/pen) equipped with a feeder and a nipple water drinker. During the challenge period, pigs were housed in individual pens (85 × 265 cm) equipped with a feed dispenser and a nipple drinker. All pigs were fed the same standard diet formulated to meet the nutritional requirements of growing pigs (9.47 MJ of net energy/kg, starch 44.2%; fat 3.1%; crude protein 15.3% and 8.3 g of standardized ileal digestible lysine (SID Lys), SID Lys/kg). The model of good and poor hygiene condition study was described in detail in previous studies [13, 28]. To summarize, poor housing conditions consisted of no cleaning nor sanitation of the room after a previous occupation by non-experimental pigs, whereas for good hygiene conditions, the room was cleaned daily, in addition to the application of optimal aeration rate and temperature and strict biosecurity precautions. The staff put clean boots and changed clothes every time before entering the room.

During the finishing period, consecutive pens (330× 545 cm, n=15 pigs/pen) hosted alternatively pigs previously housed in good and poor hygiene conditions. Pigs were fed with the same standard finishing diet (9.69 MJ of net

energy/kg, starch 45.8%; fat 3.0%; crude protein 16.5% and 9.3 g of SID Lys/kg). Throughout the entire experimental period, the photoperiod was fixed to 12 h of artificial light (0600 to 1800 h) in temperature-controlled rooms. The ambient temperature was recorded every 30 min using 2 data loggers (LoRa® SPY, JRI, Chicago, US) located in the room's extremities and at half the height of the body of the animals. During the whole trial, feed and water were provided *ad libitum*.

Growth performance and health evaluation

Throughout the trial, each animal was individually inspected and diarrhea, coughing, lameness, and any other clinical signs were recorded two (post-weaning period) or three (challenge period) times per week. To assess the prevalence of diarrhea, a scale of three levels was used (0 = normal, 1= soft feces and 2 = diarrhea). During the challenge period, measurements of air concentration in ammonia, carbon dioxide, and hydrogen sulfide in both hygiene conditions were carried out at 30 cm above floor with a manual pump (Drager, Draeger Accuro Manual Tube Pump) at week 7, week 8 and week 9. These measurements were done between 0800 and 0900 h in two locations of the rooms.

After an overnight fasting, pigs were weighed three times during the post-weaning period: at week 0 (just before the pigs weaning), one and six weeks after the weaning; and at the end of challenge and finishing periods. Feed consumption was estimated weekly during post-weaning and challenge periods as the difference between allocated feed and refusals.

Blood and fecal sampling

The days of BW recording, blood samples were collected between 0800 and 1000 h via jugular punctures into heparinized and ethylenediaminetetraacetic acid (EDTA) tubes. Pigs were manually restrained on dorsal decubitus at W0, W1 and W6 and using a snout rope at W9. The time of snout restraining was carefully checked not to exceed 2 min to limit pain. When no blood was collected within these 2 min, the sample was considered as missing. Samples were kept on ice, except for blood cell count assessment, and were centrifuged (3000 rpm, 4°C, 15 min). Plasma was aliquoted after collecting and then kept at -80˚C or -20˚C before analyses. Fecal samples were collected one day before the blood sampling performed at W6 to analyze the microbiota composition. Fecal samples were immediately frozen in liquid nitrogen after collecting and then kept at -80ºC until analysis.

Blood analyses

White blood cell count and plasma DAO concentrations were determined from blood collected on EDTA. The other plasma variables were measured from blood collected on heparin. Total number of WBC and the differential count of granulocytes (eosinophils, basophils, and neutrophils) were measured with a hematology automated cell counter calibrated for pigs (MS9®; Melet Schloesing Laboratories, Osny, France). Automated enzymatic methods using a multianalyzer apparatus (Konelab20i, Thermo Electron Corporation, Cergy, France) were used to determine plasma concentrations of dROM and biological antioxidant potential (BAP) (dROM and BAP tests, references MC003 and MC437, respectively; Diacron, Grosseto, Italy). Oxidative stress index was calculated as the dROM to BAP ratio as previously described [13]. Plasma

concentrations of vitamins E and A were assayed by liquid chromatography (HPLC, Waters Alliance) on a dedicated column (Chromsystems, Germany). Quantitative sandwich ELISA tests (Catalog Number MBS9718395) were used to measure DAO concentrations.

Microbiota analysis

Fecal microbiota was analyzed by 16S rRNA gene sequencing as described before [10]. Briefly, DNA was extracted from fecal samples using Quick-DNA Fecal/Soil Microbe 96 Kit (ZymoResearch, Irvine, CA) and PCR amplicons of the V3-V4 region were sequenced by MiSeq Illumina Sequencing. Reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA accession: PRJNA881232). Sequences were analyzed with the 'FROGS' bioinformatic pipeline, following the standard guidelines [33]. Amplicons were selected based on their size (350 – 500 nucleotides). Clustering was performed with Swarm using an aggregation distance of 1. Chimera was removed and operational taxonomic units (OTUs) with an abundance lower than 0.005% of the total of sequences were excluded. The taxonomic affiliation of OTUs was performed with the database 16S SILVA 138.1 with a pintail of 100. The mean number of reads was 15 902 and rarefaction was performed for alpha and beta diversity analysis to 7 975 reads per sample.

Calculations and statistical analysis

The ADG (g/day) and ADFI (g/day) were calculated from the BW gain and the feed consumption, respectively. Feed conversion ratio was calculated as the feed-to-gain ratio (kilogram of feed consumed per kilogram BW).

Statistical analyses were carried out separately for each period using the SAS software (version 9.3, SAS Inst. Inc., Cary, NC). The presence of outliers was evaluated through the residual analysis of data and by daily records of anomalies. Through UNIVARIATE procedure, BoxCox and Shapiro-Wilk tests were used to check homogeneity of the variances and normality of the studentized residuals, respectively. The data were analyzed using linear mixed effects models (MIXED procedure) and were presented as least squares means and pooled residual standard errors. For the post-weaning period, the model included the fixed effects of ED, sex, repetition, and their interactions. For the challenge and finishing periods, the Hyg effect and interactions with ED were included as main effects. The animal was considered as a random error. The individual pig was considered as experimental unit, except for ADFI during the post-weaning where the pen was used as experimental unit. When there was an interaction between ED and Hyg ($P \leq 0.05$), adjusted means were compared using a *post hoc* Bonferroni test. The prevalence of diarrhea was compared between the experimental diets (post-weaning period) and experimental diets and hygiene conditions (challenge period) using a χ^2 test (PROC FREQ, SAS). A difference was considered as significant when $P \leq 0.05$, whereas $0.05 < P \leq 0.10$ was discussed as a trend.

The microbiota analysis was performed using the R software (version 4.2.0) with the phyloseq package (version 1.40.0). Bray-Curtis distances were calculated on the rarefied count matrix and analyzed with PERMANOVA. Microbiota richness, Shannon, and Inverse Simpson α-diveristy indexes were calculated on the rarefied count matrix. When the relative abundance was lower than 0.05%, the OTUs were removed from the statistical analysis. Microbiota

richness, Shannon and Inverse Simpson α-diveristy indexes and bacterial abundances at the phylum, family and genus levels were analyzed by ANOVA with linear mixed models including the fixed effects of experimental diet, sex and their interactions. Random effects included the litter, the pen and the repetition. Bacterial relative abundances were square root transformed before analysis. False discovery rate adjustments were used for multiple testing (family and genus levels).

List of abbreviations

W: Week; AA: amino acids; AAP: blend of functional amino acids and grape polyphenols; BCAA: Branched-chain amino acids; Lys: Lysine; Arg: Arginine; Cys2: Cystine, Gln: Glutamine; CNT: Control; BW: Body weight; ADFI: Average daily feed intake; ADG: Average daily gain; FCR: Feed Conversion Rate; dROM: Diacron-reactive Oxygen Metabolites; BAP: Biological Antioxidant Potential; OSI: Oxidative Stress Index; OTUs: Operational Taxonomic Units; CARRU: Carratelli Unit; HPLC: High Pressure Liquid Chromatography; EDTA: Ethylenediaminetetraacetic Acid; DAO: Diamine Oxidase; WBC: White Blood Cells; ED: Experimental diet; Hyg: Hygiene condition; ED×Hyg: Interaction between ED and Hyg; SEM: Standard error of the mean; P or P-value: Probability value.

Figure Legends

Figure 1. Prevalence of diarrhea in CNT and AAP pigs during the post-weaning period.[1,2]

With diarrhea: Pigs with diarrhea at least once; Without diarrhea: Pigs with no diarrhea (sum of animals with score levels 0 and 1); Total: Total of pigs per diet (CNT and AAP, n = 40). P: Probability value for the effect of groups (with and without diarrhea) for each diet. Values were compared using a χ^2 test.

[1] Post-weaning period: Pigs received a CNT or AAP diet during pre-starter (from week 0 to 1) and starter (from week 2 to 6) phases. The AAP diet consisted of a control standard diet (CNT) supplemented with 0.2% of a blend of functional amino acids (L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine, and L-glutamine) and grape polyphenols.

[2] Data collected three times per week (from week 0 to 6).

Figure 2. Vitamin E (A), haptoglobin plasma concentrations (B), and Oxidative Stress Index (C) at week 9.[1,2]

[1] Pigs received a CNT or AAP diet during pre-starter (from week 0 to 1) and starter (from week 2 to 6) phases. The AAP diet consisted of a control standard diet (CNT) supplemented with 0.2% of a blend of functional amino acids (L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine, and L-glutamine) and grape polyphenols.

[2] After the week 6, CNT and AAP pigs were housed in good (good) and poor (poor) hygiene of housing conditions during 3 weeks (from week 7 to 9).

[3] P-values: Probability values for the effect of experimental diets (ED), hygiene conditions (Hyg) and their interaction (ED×Hyg).

[a,b] Values with different superscripts differed (P≤0.05).

Supplementary information

Additional file 1: Table S1. Fecal microbiota of CNT and AAP pigs at week 6.

Additional file 2: Table S2. Ingredients and composition of the experimental diets.

Declarations

Ethics approval

The experiment was conducted in accordance with the ethical standards of the European Community (Directive 2010/63/EU) and was approved by the Regional ethical committee (CREEA number 07). The experiment approval number is APAFiS #23735-2020012214413172.

Consent for publication

Not applicable.

Availability of data and materials

Individual data used/analyzed in this study are available from the corresponding author on reasonable request.

Competing interests

T. Chalvon-Demersay is employee of METEX ANIMAL NUTRITION.

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Authors' contributions

NLF: coordinated the study; NLF and TCD: conceived the project and animal design; NLF and AZF: participated to sample collection; AZF and NLF: performed the statistics analysis; MB: performed the microbiota analysis; AZF: wrote the original draft; PHRFC, LH, TCD, MB and NLF: revised and edited. All authors read and approved the final version.

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Table 1

Growth performance of CNT and AAP pigs during the total post-weaning period.[1]

Items	Experimental diets[2]		SEM[3]	P-value[4]
	CNT	AAP		
No.[5]	40	40		
Post-weaning, from week 0 to 6				
Initial BW, kg	8.7	8.9	0.12	0.60
Final BW, kg	28.3	28.1	0.33	0.75
ADFI, kg/day	0.806	0.799	0.01	0.48
ADG, kg/day	0.489	0.483	0.01	0.69
FCR	1.68	1.66	0.03	0.89

BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed
conversion ratio.
[1] Values are least square means.
[2] Post-weaning period: Pigs were fed the CNT or AAP diet for 3 weeks after weaning (from week 0 to 6) phases. The AAP diet consisted of a control standard diet (CNT) supplemented with 0.2% of a blend of functional amino acids (L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine, and L-glutamine) and grape polyphenols.
[3] Standard error of the mean.
[4] Probability value for the effect of experimental diets.
[5] Number of animals per group.

Table 2

Growth performance of CNT and AAP pigs housed in good (good) or poor (poor) hygiene of housing conditions for 3 weeks.[1]

Experimental diets[2]	CNT		AAP		SEM[4]	P-value[5]		
Hygiene conditions[3]	Good	Poor	Good	Poor		ED	Hyg	ED×Hyg
No.[6]	20	20	20	20				
Challenge period, from week 7 to 9								
Initial BW, kg	28.5	28.2	27.8	28.4	0.33	0.75	0.80	0.41
Final BW, kg	46.2 a	42.5 b	45.4 ab	44. 9 ab	0.56	0.55	0.03	0.04
ADFI, kg/day	1.846	1.776	1.839	1.945	0.02	0.10	0.67	0.07
ADG, kg/day	0.843 a	0.683 b	0.838 a	0.786 ab	0.02	0.14	<0.01	<0.01
FCR	2.21	2.58	2.21	2.45	0.03	0.60	<0.01	0.18

BW, body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.
[1] Values are least square means.
[2] Pigs were fed the CNT or AAP diet for 6 weeks after weaning (from Week 0 to 6). The AAP diet consisted of a control standard diet (CNT) supplemented with 0.2% of a blend of functional amino acids (L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine, and L-glutamine) and grape polyphenols.
[3] Pigs were housed in good (good) and poor (poor) hygiene of housing conditions for 3 weeks (from week 7 to 9) and were fed the same standard diet.
[4] Standard error of the mean.
[5] Probability values for the effect of experimental diets (ED), hygiene conditions (Hyg) and their interaction (ED×Hyg).
[6] Number of animals per group.

[a,b] Within a row values with different superscripts differed (P≤0.05).

Table 3

Plasma concentrations of indicators of inflammation and oxidative status in CNT and AAP pigs measured before weaning (Week 0) and 1 and 6 weeks after weaning (Weeks 1 and 6).[1]

Items	Experimental diets[2]		SEM[3]	P-value[4]
	CNT	AAP		
No.[5]	40	40		
Week 0				
Haptoglobin, mg/ml	2.91	2.99	0.12	0.70
Vitamin E, µmol/L	7.78	8.23	0.37	0.54
Vitamin A, µmol/L	0.34	0.34	0.01	0.99
Diamine Oxidase, pg/ml	283.7	290.8	4.80	0.11
dROM, CARRU[6]	1000.6	994.6	17.76	0.89
BAP, µmol/L	2442.7	2472.4	23.43	0.54
ISO[7], CARRU/µmol/L	0.41	0.40	0.01	0.23
Week 1				
Haptoglobin, mg/ml	3.52	2.98	0.13	0.03
Vitamin E, µmol/L	3.36	3.61	0.10	0.21
Vitamin A, µmol/L	0.34	0.36	0.01	0.56
Diamine Oxidase, pg/ml	312.8	316.7	6.0	0.54
dROM, CARRU[6]	1073.2	994.9	20.92	0.07
BAP, µmol/L	2327.2	2321.33	27.26	0.96
ISO[7], CARRU/µmol/L	0.40	0.38	0.01	0.06
Week 6				
Haptoglobin, mg/ml	1.49	1.39	0.12	0.65

Vitamin E, µmol/L	1.57	2.00	0.07	<0.01
Vitamin A, µmol/L	0.30	0.35	0.02	0.04
Diamine Oxidase, pg/ml	307.4	287.6	5.9	0.03
dROM, CARRU[6]	970.4	970.9	17.23	0.98
BAP, µmol/L	2826.4	2797.1	20.57	0.48
ISO[7], CARRU/µmol/L	0.43	0.40	0.01	0.09

[1] Values are least square means.
[2] Post-weaning period: Pigs were fed the CNT or AAP diet for 6 weeks. The AAP diet consisted of a control standard diet (CNT) supplemented with 0.2% of a blend of functional amino acids (L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine, and L-glutamine) and grape polyphenols.
[3] Standard error of the mean.
[4] Probability value for the effect of experimental diets.
[5] Number of animals per group.
[6] CARRU (Carratelli Unit, 1 CARRU = 0.08 mg H_2O_2/100mL of sample).
[7] ISO (Oxidative Stress Index) = dROM/BAP

Table 4

Blood and plasma concentrations of indicators of inflammation and oxidative status in CNT and AAP pigs housed in good (good) or poor (poor) hygiene of housing conditions for 3 weeks.[1]

Experimental diets[2]	CNT		AAP		SEM[4]	P-value[5]		
Hygiene conditions[3]	Good	Poor	Good	Poor		ED	Hyg	ED×Hyg
No.[6]	20	20	20	20				
Week 9								
WBC, m/mm^3	18.51	21.49	19.56	21.85	0.48	0.34	<0.01	0.81
Granulocyte, m/mm^3	5.77	8.27	6.77	9.05	0.25	0.14	<0.01	0.47
Vitamin A, µmol/L	0.49	0.48	0.49	0.41	0.02	0.43	0.16	0.24
Diamine Oxidase, pg/ml	310.8	333.4	321.9	315.5	9.06	0.81	0.57	0.31
dROM, CARRU[7]	1035	1150	1036	1148	18.6	0.97	<0.01	0.98
BAP, µmol/L	2823	2836	2707	2800	26.1	0.15	0.26	0.37

WBC, White Blood Cells; dROM, Diacron Reactive Oxygen Metabolites; BAP, Biological Antioxidant Potential.
[1] Values are least square means.
[2] Pigs were fed the CNT or AAP diet for 6 weeks after weaning (Week 0 to 6). The AAP diet consisted of a control standard diet (CNT) supplemented with 0.2% of a blend of functional amino acids (L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine, and L-glutamine) and grape polyphenols.
[3] Pigs were housed in good (good) and poor (poor) hygiene of housing conditions for 3 weeks (from week 7 to 9) and were fed the same standard diet.

[4] Standard error of the mean.
[5] Probability values for the effect of experimental diets (ED), hygiene conditions (Hyg) and their interaction (ED×Hyg).
[6] Number of animals per group.
[7] CARRU (Carratelli Unit, 1 CARRU = 0.08 mg H_2O_2/100mL of sample).

Figure 1

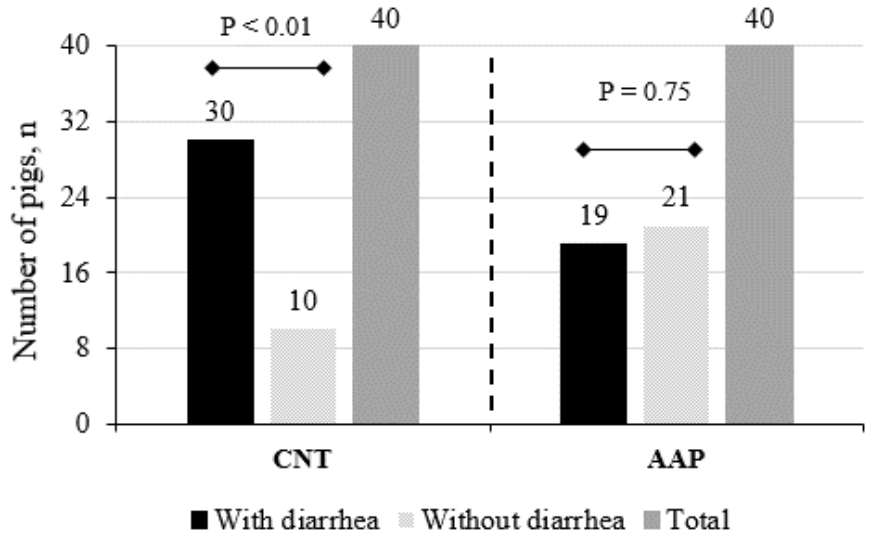
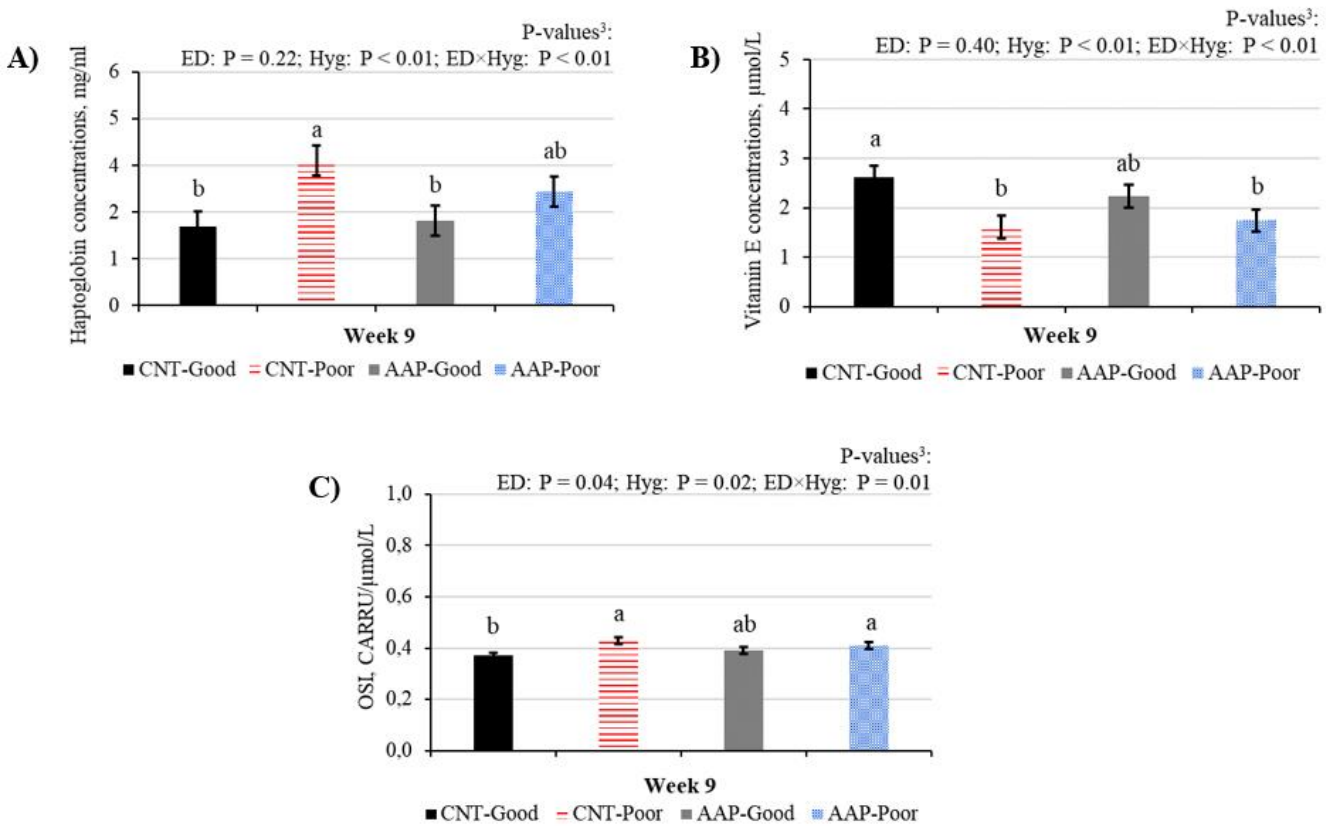

P < 0.01
40
40
P = 0.75
30
19
21
10
Number of pigs, n
40
32
24
16
8
0
CNT
AAP
With diarrhea
Without diarrhea
Total

Figure 2


A)
P-values[3]:
ED: P = 0.22; Hyg: P < 0.01; ED×Hyg: P < 0.01
Haptoglobin concentrations, mg/ml
b
a
b
ab
Week 9
CNT-Good
CNT-Poor
AAP-Good
AAP-Poor
B)
P-values[3]:
ED: P = 0.40; Hyg: P < 0.01; ED×Hyg: P < 0.01
Vitamin E concentrations, µmol/L
a
b
ab
b
Week 9
CNT-Good
CNT-Poor
AAP-Good
AAP-Poor
C)
P-values[3]:
ED: P = 0.04; Hyg: P = 0.02; ED×Hyg: P = 0.01
OSI, CARRU/µmol/L
b
a
ab
a
Week 9
CNT-Good
CNT-Poor
AAP-Good
AAP-Poor

Additional file 1

Table S1. Fecal microbiota of CNT and AAP pigs at week 6.

	Mean		SEM[1]		P-value[2]	P-adjusted[3]
	CNT	AAP	CNT	AAP		
Diversity						
Richness	439.08	434.92	6.79	7.69	0.63	
Shannon	4.51	4.46	0.03	0.04	0.26	
InvSimpson	35.46	32.95	1.50	1.85	0.34	
Phylum (relative abundance)[4]						
Firmicutes	65.24	67.53	1.08	1.21	0.16	
Bacteroidota	33.59	31.14	1.10	1.18	0.12	
Family (relative abundance)[5]						
Prevotellaceae	32.30	29.64	1.14	1.23	0.12	0.58
Lachnospiraceae	22.17	20.82	0.75	0.72	0.16	0.58
Lactobacillaceae	20.32	23.68	1.47	1.82	0.15	0.58
Ruminococcaceae	8.28	7.51	0.31	0.41	0.18	0.58
Clostridiaceae	3.39	3.78	0.32	0.72	0.63	0.87
Butyricicoccaceae	2.86	2.71	0.14	0.14	0.33	0.74
Veillonellaceae	1.84	1.61	0.12	0.11	0.22	0.58
Streptococcaceae	1.81	2.65	0.33	0.32	**0.02**	0.38
Peptostreptococcaceae	1.58	1.55	0.13	0.21	0.94	0.94
Oscillospiraceae	1.47	1.57	0.11	0.13	0.56	0.87
Genus (relative abundance)[6]						
Prevotella_9	23.41	20.83	0.98	1.10	0.06	0.39
Lactobacillus	15.27	17.00	1.09	1.19	0.26	0.74
Limosilactobacillus	4.97	6.57	0.42	0.66	0.07	0.42
Blautia	4.34	3.93	0.26	0.17	0.25	0.74
Faecalibacterium	4.05	3.39	0.24	0.25	0.05	0.39
Roseburia	3.58	3.46	0.22	0.22	0.63	0.95
Prevotella	3.12	2.93	0.19	0.22	0.46	0.94
UCG-008	2.59	2.49	0.14	0.14	0.48	0.95
Clostridium sensu stricto 1	2.43	2.80	0.31	0.65	0.98	0.99
Ruminococcus	1.99	1.74	0.11	0.11	0.20	0.71
Prevotella_7	1.98	2.20	0.35	0.40	0.80	0.95
Subdoligranulum	1.97	2.11	0.07	0.16	0.70	0.95
Dialister	1.84	1.61	0.12	0.11	0.22	0.71
Streptococcus	1.81	2.65	0.33	0.32	**0.02**	0.39
Lachnospiraceae_multiaffiliation	1.78	1.43	0.13	0.14	**0.01**	0.39
Coprococcus	1.22	1.39	0.06	0.06	**0.04**	0.39
Terrisporobacter	1.19	1.21	0.12	0.20	0.83	0.95
Prevotellaceae NK3B31 group	1.02	0.96	0.11	0.11	0.87	0.95

[1] Standard error of the mean.

[2] Probability value for the effect of experimental diets ($P \leq 0.05$ are indicated in bold).

[3] Probability value for the effect of experimental diets adjusted for false discovery rate.

[4,5,6] For each taxonomic rank (phylum, family, and genus), bacterial groups with a relative abundance > 1% are presented.

Additional file 2

Table S2. Ingredients and calculated composition of the experimental diets.[1]

	Pre-starter		Starter	
Experimental diets	CNT	AAP	CNT	AAP
Ingredient composition as-fed basis, %				
Wheat	33.15	33.08	24.40	24.35
Barley	25.19	25.14	25.13	25.08
Soybean, Grain	8.06	8.04	0.00	0.00
Soybean, Meal	0.00	0.00	19.97	19.93
Soybean, Cake	11.06	11.04	0.00	0.00
Soy protein concentrate	2.52	2.51	0.00	0.00
Corn	0.00	0.00	20.10	20.06
Rapeseed	0.00	0.00	3.02	3.01
Beet pulp	0.00	0.00	2.51	2.50
Vegetable oil	0.77	0.77	1.00	1.00
Lactose	13.60	13.57	0.00	0.00
Calcium carbonate	1.04	1.04	1.21	1.21
Monocalcium phosphate	0.69	0.69	0.61	0.61
Salt	0.30	0.30	0.40	0.40
Potato protein concentrate	1.00	1.00	0.00	0.00
L-Lysine[2]	0.57	0.57	0.62	0.62
Methionine[3]	0.24	0.24	0.10	0.10
L-Threonine	0.25	0.25	0.15	0.15
L-Tryptophan	0.09	0.09	0.13	0.13
L-Valine	0.16	0.16	0.04	0.04

Vitamin and mineral premix[4]	0.50	0.50	0.50	0.50
Phytase and organic acids	0.81	0.81	0.11	0.11
Blend of functional AA and grape polyphenols	0.00	0.20	0.00	0.20
Calculated composition[5]				
Net energy, MJ/kg	10.57	10.57	9.55	9.55
Crude protein, %	18.01	18.06	18.40	18.70
Free lysine, %	0.47	0.47	0.33	0.33

[1] Pigs received a CNT or AAP diet during pre-starter (from week 0 to 1) and starter (from week 2 to 6) phases. The AAP diet consisted of a standard diet (CNT) supplemented with 0.2% a blend of functional amino acids (L-arginine, L-cystine, L-leucine, L-valine, L-isoleucine, and L-glutamine) and grape polyphenols.
[2] L-lysine HCL (pre-starter) and liquid lysine; 50.0% of L-lysine (starter).
[3] L-methionine (pre-starter) and DL-methionine (starter).
[4] Mineral vitamin supplement (per kg of diet): Vit. A (2.000.000 UI); Vit. D3 (400.000 UI); Vit. E (4.000 mg); Vit. K3 (870 mg); Vit. B1 (400 mg); Vit. B2 (1.000 mg); Vit. B6 (1.000 mg); Vita. C (8.000 mg); Niacin (4.000 mg); Pantothenic acid (2.000 mg); Folic acid (200 mg); Biotin (40 mg); Vit. B12 (6.0 mg); Copper (4.000 mg); Iodine (120 mg); Manganese (8.000 mg); Selenium (60 mg); Zinc (20.000 mg); Ferrous sulphate (20.000 mg); Choline chloride (160.000 mg); Butylated Hydroxyanisole (75 mg); and Butylated Hydroxytoluene (75 mg).
[5] The calculated values (%) for L-arginine, L-cystine, L-leucine, L-isoleucine, and L-glutamine for CNT diet in both phases were, respectively: 0.04; 0.01; 0.003; 0.004; and 0.05. For AAP diet, the values were, respectively: 0.10; 0.04; 0.02; 0.02; and 0.05. For L-valine the values were in pre-starter phase: 0.18 (CNT diet) and 0.20 (AAP diet); and in starter phase 0.03 (CNT diet) and 0.04 (AAP diet).

CHAPTER 6 – GENERAL OVERVIEW

6.1 Experimental limitations and final considerations

This thesis provides advances in understanding adaptive responses of pigs exposed to environmental challenges (daily cyclic high ambient temperatures and sanitary challenge conditions). In the first study of this thesis work (Chapter 3), we demonstrate that pigs feeding pattern is strongly related to the circadian rhythm. For instance, pigs showed a diurnal feeding pattern characterized by greater probability to start a meal at $22^{o}C_{(06\text{-}10h)}$, followed by $30^{o}C_{(10\text{-}18h)}$ and then $22^{o}C_{(18\text{-}06h)}$. However, there was a considerable and dynamic genetic selection effect able to modulate the pigs feeding behavior responses exposed to daily ambient temperature variations. Pigs selected for improved lean tissue accretion (Line A pigs) had lower probability to start a meal when compared with non-selected animals (Line B pigs), which may partially explain their lower growth performance in high ambient temperature conditions as previously reported (Fraga et al., 2019). Furthermore, we observed that Line A pigs tried to compensate their lower feeding activity in the diurnal period changing part of their feeding behavior (i.e., feed intake and feeder occupancy per meal) to the night. These findings suggest that pigs selected for lean mass deposition are more dependent on behavioral adjustments to maintain homeothermy. To our knowledge, this is the first study in which feeding behavior of pigs exposed to high ambient temperatures are characterized through statistical methodologies of probability. The ambient temperature applied (22ºC and 30ºC) and their variation through 24h-day were according to the meteorological data collected where the experiment was conducted (Jaboticabal, São Paulo, Brazil). However, in tropical regions, the maximum temperature can reach values above to 30ºC and thermal amplitude may be greater than 8ºC. Therefore, caution is required when extrapolating our findings to practical climate tropical conditions. Besides ambient temperature, relative humidity is an important factor that may result in changes of pig's behavior (Kim et al., 2021) and it was not controlled in the current study.

Different experimental models can be used to evaluate the responses of immune challenged pigs. For instance, challenge models based on non-

infectious (administration of LPS) or infectious agents (inoculation with porcine reproductive and respiratory syndrome virus; PRRSV, and oral administration of *Salmonella)* have been used to evaluate the metabolic and physiological changes in pigs exposed to immune challenge. However, in conventional pig production facilities, the animals are exposed to several micro-organisms, not necessary pathogens, resulting in constant and moderate stimulation of immune system (Le Floc'h et al., 2006). Therefore, experimental protocols that consider the continual exposure of pigs to wide range of environment agents (such as microorganism exposition, dust, and gases), as occurs in models of poor hygiene conditions, seem to be a closer representation of practical conditions. Furthermore, poor housing condition is a model that limit growth performance and induce changes in nutrients metabolism without resulting in major clinical signs of diseases (Le Floc'h et al., 2006). Thus, poor hygiene of housing condition was used as a model to study the effects caused by the activation of immune system on metabolism, health, and growth performance of pigs in the last two studies (Chapter 4 and 5) of this thesis work. In the second study (Chapter 4), we compared the effects of exposure to poor hygiene conditions on postprandial plasma concentrations of insulin, energy-related metabolites, and AA in pig lines divergently selected for feed efficiency (more and less efficient lines; LRFI and HRFI, respectively). After nine generation of selection program conducted at INRAE, difference in ADFI between both lines pigs was 165 g/day (Gilbert et al., 2017). Our major and original finding was that pigs from LRFI line had a greater capacity to use AA (in particular, tryptophan and arginine) to support immune responses compared to HRFI line, which may explain their better ability to cope with an immune challenge as previously reported by Chatelet et al. (2018). Furthermore, only pigs from HRFI line had greater plasma concentrations of lysine in poor hygiene of housing conditions, which may be probably a consequence of lower body protein synthesis and/or reduced efficiency of nitrogen utilization for protein retention. These responses are observed in pigs when the immune system is overstimulated (McGilvray et al., 2019). Although we demonstrated important effects on metabolism of sanitary challenged pigs, we used a limited number of animals (6 animals/experimental treatment). Therefore, further studies are still needed to evaluate the potential effects of selection for feed efficiency on metabolite profiles in response to poor hygiene of housing conditions. The

association between nutritional and immune status was highlighted in the third studied of this thesis (Chapter 5). In this study, pigs were fed during six weeks of post-weaning a standard diet supplemented (AAP) or not (CNT) with 0.2% of a blend of AA (glutamine, arginine, cystine, valine, isoleucine, and leucine) and grape extract polyphenols. Our main objective was to evaluate whether AAP diet may improve the pigs' capacity to cope with weaning transition and a subsequent inflammatory challenge caused by poor hygiene of housing conditions in the beginning of growing period, when pigs were not fed anymore the supplemented diet. One week after weaning, AAP pigs had lower plasma concentrations of haptoglobin when compared with CNT pigs. At the end of post-weaning (at week 6), AAP pigs had greater plasma concentrations of vitamin E and A, and lower concentrations of DAO (an indicator of digestive integrity) than CNT pigs. These modulatory effects on health status might explain the lower inflammatory and greater antioxidant response associated with a less impaired growth rate when AAP pigs were housed in poor hygiene conditions in the beginning of growing period. In the current study, the beneficial effects with the supplemented blend cannot be attributed to modulation of gut microbiota composition since no significant differences were observed between AAP and CNT pigs. However, we were expecting to find changes in microbiota profiles since we observed difference of DAO concentrations between AAP and CNT pigs at week 6. Furthermore, Beaumont et al. (2021) reported that supplementation of AA and polyphenols for weaned piglets induced modulation of the gut microbiota by increasing the abundance of *Lactobacillaceae* in the jejunum while decreasing the abundance of the *Proteobacteria* in the caecum. Although we have no conclusive explanation, contrasted result between studies may be related with the gut segment studied (jejunum and caecum versus fecal samples) and material sampling for microbiota analysis (6 weeks versus 2 weeks after the beginning of challenged).

6.2 References

Beaumont M, Lencina C, Painteaux L, Viémon-Desplanque J, Phornlaphat O, Lambert W, Chalvon-Demersay T (2021) A mix of functional amino acids and grape polyphenols promotes the growth of piglets, modulates the gut microbiota in vivo and regulates epithelial homeostasis in intestinal organoids. **Amino Acids** 1-13.

Chatelet A, Gondret F, Merlot E, Gilbert H, Friggens NC, Le Floc'h N (2018) Impact of hygiene of housing conditions on performance and health of two pig genetic lines divergent for residual feed intake. **Animal** 12: 350-358.

Fraga AZ, Campos PHRF, da Silva WC, Caetano RP, Veira AM, dos Santos LS, Hauschild L (2019) Sequential feeding with high-fat/low-crude protein diets for two lines of growing-finishing pigs under daily cyclic high ambient temperature conditions. **Journal of Animal Science** 97: 2493-2504.

Gilbert H, Billon Y, Brossard L, Faure J, Gatellier P, Gondret F, et al (2017) Divergent selection for residual feed intake in the growing pig. **Animal** 11:1427-1439.

Kim YJ, Song MH, et al (2021) Evaluation of pig behavior changes related to temperature, relative humidity, volatile organic compounds, and illuminance. **Journal of Animal Science and Technology** 63: 790.

Le Floc'h N, Jondreville C, Matte JJ, Seve B (2006) Importance of sanitary environment for growth performance and plasma nutrient homeostasis during the post-weaning period in piglets. **Archives of Animal Nutrition** 60: 23-34.

McGilvray WD, Johnson B, Wooten H, Rakhshandeh AR, Rakhshandeh A (2019) Immune system stimulation reduces the efficiency of whole-body protein deposition and alters muscle fiber characteristics in growing pigs. **Animals** 9: 323–339

CHAPTER 7 – PERSPECTIVES

7.1 Perspectives and implications for further research

In this thesis, we evaluated the individual effects of cyclic high ambient temperature and poor hygiene conditions on the different components of adaptive responses of pigs (behavior, physiological and/or metabolic responses). Further research needs to be addressed using experimental models that consider their associated effects (high temperature and sanitary challenge conditions) to mimic the conditions encountered by pigs in practical commercial facilities. The pig feeding behavior assessment (Chapter 3) provides useful information of robustness traits contributing to genetic selection of individuals based on their thermoregulatory responses, productive potential, and well-being. For instance, results generated in our study evidenced that genetic selection for lean tissue accretion affects pigs feeding behavior under daily cyclic high ambient temperature conditions. Pigs selected for lean mass had lower feed intake, feeder occupancy per meal, and probability starting a meal compared with non-selected pigs, which suggests that they were presumably more affected by our experimental conditions as evidenced by their lower growth performance (Fraga et al., 2019). Electronic feeders such as Automated Intelligent Precision Feeders (AIPF), that were used in our study, allow the continuous monitoring of the health status of each individual in real time based on information of feeding behavior (feed intake per meal, feeder occupancy, number of meals, etc). This initiative may contribute to improve pig production by allowing the monitoring of animals and deviations from their typical feeding patterns (Hauschild et al., 2020). Research to explore the use of these technologies for growing-finishing pigs under commercial conditions is encouraged.

Pig diets are usually formulated on the nutritional requirements estimated under ideal production conditions (sanitary rules, ambient temperature, housing conditions, etc). Nutritional programs have limitations in a practical context of production since they do not consider the increased demand of nutrients in situations of immune system activation. Under these conditions, an imbalance between dietary nutrients and those needed for immune and production functions may occur. In the publication nº 2 of this thesis work (Chapter 4), we observed

changes in plasma AA profiles of growing pigs exposed to an immune system stimulation. More precisely, a greater AA utilization in poor hygiene conditions would be related to the better ability of pigs to cope with a challenge. This knowledge may be used by nutritionists to adjust optimal dietary nutrient levels for pigs raised in commercial conditions. Indeed, adapting dietary nutrient concentrations considering the reduced feed intake of pigs is an important step to improve the animal capacity to cope with challenges (Hauschild et al., 2020). It is necessary to find alternatives to antibiotics to improve or maintain the health status of animals in order to allow the development of sustainable production systems. Therefore, in the Chapter 5 of this thesis work, we studied the potential effects of functional AA and grape polyphenols dietary supplementation as a nutritional strategy to improve the pigs' capacity to cope with the weaning transition and with a subsequent inflammatory challenge. Much of the published work has focused on the evaluation of individual AA supplementation during the health challenge. Our results demonstrate that dietary supplementation with a blend of AA and polyphenols may contribute to weaning transition and better prepare them for a subsequent inflammatory challenge in the beginning of growing. Accordingly, an adaptation period with other AA-supplemented diet (L-threonine, DL-methionine, and L-tryptophan) supported gut integrity and reduced pigs' inflammatory responses, which attenuated the negative effects of a subsequent *Salmonella* challenge (Rodrigues et al., 2021). These findings support the development of nutritional strategies potentially applicable in a practical production context to mitigate the effects of challenges. Furthermore, our results reinforce the need for a better understanding of the modulatory effects of AA supplementation and its association with animal health and metabolism. As in both studies (Chapters and 4 and 5) we obtained greater growth performance and health indicators for pigs housed in good than poor conditions, improving housing and management conditions can be considered as important practices to be implemented (or adjusted) in pig production farms.

7.2 References

Fraga AZ, Campos PHRF, da Silva WC, Caetano RP, Veira AM, dos Santos LS, Hauschild L (2019) Sequential feeding with high-fat/low-crude protein diets for two lines of growing-finishing pigs under daily cyclic high ambient temperature conditions. **Journal of Animal Science** 97: 2493-2504.

Hauschild L, Kristensen AR, Andretta I, Remus A, Santos LS, Pomar C (2020) Toward better estimates of the real-time individual amino acid requirements of growing-finishing pigs showing deviations from their typical feeding patterns. **Animal** 14: s371-s381.

Rodrigues LA, Wellington MO, González-Vega JC, Htoo JK, Van Kessel AG, Columbus DA (2021) A longer adaptation period to a functional amino acid-supplemented diet improves growth performance and immune status of Salmonella Typhimurium-challenged pigs. **Journal of Animal Science** 99: skab146.

ANNEX I - TRAINING AND COURSES CARRIED OUT DURING THE COURSE OF THIS THESIS

Classes (UNESP, Jaboticabal)

Animal biometeorology (First semester/2018) ..... 90 hours
Microbiological quality of meat (First semester/2018) ..... 45 hours
Nutrigenomics applied to animal production (First semester/2018) ..... 30 hours
Endocrine and metabolic physiology (Second semester/2018) ..... 90 hours
Physical-chemical analysis of meat (Second semester/2018) ..... 90 hours
Quality meat production (Second semester/2018) ..... 90 hours
Physiology of effort and training (Second semester/2019) ..... 60 hours
Modeling dynamical systems in agricultural sciences
(Second semester/2019) ..... 30 hours
Modelling nutrient digestion and utilization in farm animals
(Second semester/2019) ..... 30 hours
Nutritional strategies to cope with health problem, a focus on nutrients
(First semester/2020) ..... 30 hours
Feed analysis I (First semester/2016)[13] ..... 150 hours
Statistics applied to animal science (First semester/2016)[13] ..... 60 hours
Research methodology in animal science (First semester/2016)[13] ..... 45 hours
Scientific advances in non-ruminants (First semester/2016)[13] ..... 30 hours
Physiology of thermoregulation (Second semester /2016)[13] ..... 75 hours
Microbiology (Second semester /2016)[13] ..... 90 hours
Data analysis in biometeorology (Second semester /2016)[13] ..... 60 hours
Data processing (First semester/2017)[13] ..... 90 hours

[13] Classes carried out during the master's degree whose number of hours were also accounted for the PhD (Unesp Resolution 59, 04-10-2012).

Courses

Statistics courses:

Theorical course in Introduction to Systematic Review and Meta Analysis
University Federal of Viçosa (2020) ……..…… 10 hours
Introductory Course on Data Analysis using R Software
UNESP/Jaboticabal (2017) ……..…… 36 hours
Advanced Course on Data Analysis using SAS Software
UNESP/Jaboticabal (2017) …….. 42 hours

English courses:

Basic and Intermediate levels of English
CCAA English School/Jaboticabal …..……..…… from 2017 to 2020
English conversation (CNA English School/Jaboticabal) ……..…… In progress

French courses:

Méthode de Français – Levels A1 and A2
Aliance Francese/Jaboticabal ..... from 2020 to 2021
Intermediate level of French - Centre International Rennais d'Etudes de Français pour Étrangers (CIREFE, Rennes - 2021/France) ……....…… 40 hours
Méthode de Français – B1 Level
Aliance Francese/Jaboticabal ..... In progress

ANNEX II - SCIENTIFIC PUBLICATIONS AND INDICATORS

Most relevant scientific articles

1. **Fraga, A. Z**.; Campos, P. H. R. F.; Hauschild, L.; Chalvon-Demersay, T.; Beaumont, M.; Floc'h, L. A blend of functional amino acids and grape polyphenols improves the pig capacity to cope with an inflammatory challenge caused by poor hygiene of housing conditions. BMC Veterinary Research, v. 19, p. 1-11 - 2023.
2. **Fraga, A. Z**.; Hauschild, L.; Campos, P. H. R. F.; Valk, M.; Bello, D. Z.; Kipper, M.; Andretta, I. Genetic selection modulates feeding behavior of group-housed pigs exposed to daily cyclic high ambient temperatures. PLoS One., v.17, p.e0258904 - 2022.
3. de Oliveira, M. J. K.; MELO, A. D. B.; Marcal, D. A; Valini, G. A. da C.; Silva, C. A.; Veira, A. M.; **Fraga, A. Z**; Arnaut, P. R.; Campos, P. H. R. F.; Santos, L. S.; Htoo, J. K. K.; Brand, H. G.; Hauschild, L. Effects of lowering dietary protein content without or with increased protein-bound and feed-grade amino acids supply on growth performance, body composition, metabolism, and acute-phase protein of finishing pigs under daily cyclic heat stress. Journal of Animal Science., v.101, p.1 – 2022.
4. **Fraga, A. Z.;** Louveau, I.; Campos, P. H. R. F.; Hauschild, L.; Le Floc'h, N. Selection for feed efficiency elicits different postprandial plasma metabolite profiles in response to poor hygiene of housing conditions in growing pigs. PLoS One., v.16, p.e0246216 - 2021.
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Academic quantitative indicators[14]

Publications in journals with selective editorial policy 12
Publications in technical-scientific journals 2
Published book chapter 1
Abstracts published in event proceedings 75
Lectures 13
Supervised and concluded Monographs 2
Participation in evaluation teams and commissions 4
Participation in events 50

[14] From 2016 to January 2023.