UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

AMINO ACIDS SUPPLEMENTATION FOR GROWING PIGS EXPOSED TO SANITARY CHALLENGES

Graziela Alves da Cunha Valini

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DADOS CURRICULARES DO AUTOR

GRAZIELA ALVES DA CUNHA VALINI – nascida em Porto Feliz, São Paulo, Brasil, no dia 17 de julho de 1994. Ingressou no curso de Zootecnia na Universidade de São Paulo (USP) no ano de 2012, graduando-se em julho de 2017. Durante a graduação, fez estágio na área de produção e nutrição de aves e suínos, participou de projetos de pesquisa, foi membro do Programa de Educação Tutorial (PET), foi membro do grupo Voluntários da Zootecnia (Vzoo) de agosto de 2010 a junho de 2014. Executou um projeto de iniciação científica como bolsista PIBIC de agosto de 2013 a julho de 2014 sob orientação do Prof. Dr. Douglas Emygdio de Faria. Durante o período de agosto de 2014 a julho de 2015 foi bolsista do programa Ciencias sem Fronteiras na Michigan State Universtiy, onde executou um projeto de iniciação científica em comportamento animal sob orientação da Profª. Dr.ª Janice Siegford. Em agosto de 2017 iniciou o curso de Mestrado no Programa de Pós-graduação em Zootecnia, da Universidade Federal de Viçosa/UFV – Campus de Viçosa, como bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), obtendo o título de Mestre em Zootecnia em julho de 2019 sob orientação do Prof. Dr. Gabriel Cipriano Rocha. No mesmo ano, iniciou o curso de doutorado no Programa de Pós-graduação em Zootecnia, da Faculdade de Ciências Agrárias e Veterinárias/UNESP – Campus de Jaboticabal sob orientação do Prof. Dr. Luciano Hauschild. Foi bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, número do processo: 142556/2019-0) no período de agosto de 2019 a janeiro de 2021. Após esse período, foi bolsista da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, número do processo: 2019/20150-3) de janeiro de 2021 a maio de 2024. Durante o doutorado, realizou um ano de doutorado sanduíche no centro de pesquisa Agri-food Canadá (BEPE – FAPESP, número do processo: 2022/02152-1) sob a supervisão da Drª. Aline Remus e do Dr. Candido Pomar. No dia 2 de maio de 2024, Graziela submeteu a presente tese ao comitê para a obtenção do título de doutora em Zootecnia.

AUTHOR'S CURRICULUM DATA

GRAZIELA ALVES DA CUNHA VALINI – born in Porto Feliz, SP, on July 17th, 1994. She joined the Animal Science program at the University of São Paulo (USP) in 2012 and graduated in July 2017. During her undergraduate studies, she interned with poultry and swine production and nutrition and participated in several research projects. She also was a member of Programa de Educação Tutorial (PET), and Voluntários da Zootecnia (Vzoo) from August 2013 to June 2014. She conducted an undergraduate research project (PIBIC scholarship) from August 2013 to July 2014 under Dr. Douglas Emygdio de Faria's supervision. From August 2014 to July 2015, she was granted a scholarship to study abroad (Science without Borders program) at Michigan State University, where she conducted a research project on animal behavior (pigs aggressive behavior characterization) under Dr. Janice Siegford supervision. In August 2017, she began her Master's degree (CNPq scholarship) in Animal Science at Federal University of Viçosa (UFV), under Dr. Gabriel Cipriano Rocha supervision. Her projected focused on feed additives alternatives to antibiotic as growth promoter to piglets under sanitary challenge. In the same year (July 2019), Graziela started her Ph.D. program in Animal Science at Sao Paulo State University (UNESP - Faculty of Agricultural and Veterinary Sciences – Jaboticabal) under Dr. Luciano Hauschild's supervision. In her PhD, she studied the metabolic responses of challenged pigs fed with functional amino acids above the estimated requirement. She was granted two scholarships: the first was granted by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process number: 142556/2019-0) from August 2019 to January 2021; and the second was granted by São Paulo Research Foundation (FAPESP, process number: 2019/20150-3) from January 2021 to May 2024. Additionally, during her Ph.D., she spent one year at the Agri-food Canada research center (BEPE – FAPESP, process number: 2022/02152-1) under the supervision of Dr. Aline Remus and Dr. Candido Pomar. On May 2nd, Graziela submitted the current thesis work for evaluation by the jury committee.

Be kind to each other. Don't be glass half empty or half full people...you can always refill the glass."

Russell Crowe

DEDICATION

To my grandfather, João Alves da Cunha, who inspired and supported me being a "scientist" since I was a little girl.
To my dear brother, Guilherme Alves da Cunha Valini, who left us so suddenly, thank you for laughing with me, supporting and loving me in the most difficult moments

To you, I dedicate it!!

ACKNOWLEDGEMENTS

To God, first and foremost! For life and for all the opportunities and challenges granted to me. I thank my parents, Maria Cristina and Carlos Eduardo, who never spared efforts for the realization of my achievements. I also thank their patience and companionship in every moment. They always have shown me that everything is possible if done with love. Obrigada por serem meu porto seguro aonde quer que eu esteja!

To my brother (*in memory*), Guilherme (sempre em meus pensamentos), and my sister-in-law, Janaína, who have always cheered for my professional and personal growth.

To my grandmother, Ivone, for always being my second mother all these years.

To my fiancé, Pedro, for making my days happier. You have been with me since the beginning of my PhD (even when we were not a couple). You have seen me go through all the possible Ph.D. phases, from “I will revolutionize the swine science” to “I won’t finish it on time” (What 4 years, captain!). But we did it! Thank you for your support, patience, and incentive, for always having my back and cheering me up, and sometimes for getting me a slap on the wrist. Je t'aime beaucoup mon amour!

I express my gratitude to UNESP and the Department of Animal Science for making the Ph.D.'s degree possible. To the National Council for Scientific and Technological Development (CNPq) and São Paulo Research Foundation (FAPESP) for financial support of this project and the scholarships granted.

A special thanks to Professor Luciano Hauschild. Thank you so much for the opportunities you gave me, for trusting in my work, and for everything you shared and taught me.

To my co-advisors, Prof. Paulo Campos and Dr. Diego Melo, for sharing thoughts, your knowledge, and ideas, and also for your words of support in moments of doubt and lack of self-trust.

To Jose Segecic (“Zezinho”), who worked with me on my projects, I always will be grateful to you for your support, knowledge, professionalism, dedication, and positivity! Muito obrigada por tudo Ze!

To Agriculture and Agri-Food Canada (AAFC) and all the employees (special thanks

to Annie Larue, Sophie Horth, Jean Phillipe, and Steve Methot) and researchers (special thanks to Dr. Candido Pomar, Dr. Renee Petri, and Dr. Danyel Dalto) for having me as one of them. To my beloved mentor, Dr. Aline Remus, whom I am proud to call my friend. She taught me some soft skills that are important to be a good scientist and also a good human being: you need to be honest! Be honest with yourself and know your limits. Be honest to your teammates and with the people that surround you…and you also need to have time to relax! Take care of yourself; having time for yourself and friends/family is crucial to being an excellent professional and person. Obrigada por tudo Aline, e obrigada por ser uma inspiração de professional e de pessoa!

To my interns, Larissa, Paulo Henrique, and Willian, thank you for dedicating yourselves to the thesis project and for holding my hand in every moment.

To the colleagues from LABSUI for the assistance in the execution of the project and laboratory analyses, and for all the teachings and friendship. Obrigada por madrugarem comigo para realizarmos todas as nossas coletas!!!

To my AAFC friends, Berta, James, Adriana, Charles and Alex! Without you, the Canadian experiences would not have been so intense and rewarding.

To my longtime friends Amanda, Gabriela, Barbara, and Fabiana, for their support and comprehension in my absences and for cheering me up in the difficult moments. Even when I was far away from Brazil, you made me feel courageous!

I also thank the friends that Jaboticabal has given me: Gabriela, Rafaela, Cleslei, Ismael, Bernardo, Alicia, Lucas, Raully, Jaira, Manoela, Elisa, Livia, and Lorena. Thank you for the support, hugs, studies, and companionship. Thanks for making postgraduate studies lighter and more enjoyable.

“Who receives a benefit with gratitude, pays the first installment of his debt”

Sêneca

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CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Suplementação dietética de aminoácidos no metabolismo e desempenho de suínos em crescimento alojados sob diferentes condições sanitárias"**, protocolo nº 4784/20, sob a responsabilidade do Prof. Dr. Luciano Hauschild, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 10 de dezembro de 2020.

Vigência do Projeto	01/02/2021 a 31/05/2021
Espécie / Linhagem	Suínos
Nº de animais	120
Peso / Idade	6 aos 70 kg
Sexo	Fêmeas
Origem	Granja Comercial Parceira

Jaboticabal, 10 de dezembro de 2020.

Profa. Dra. Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
Tel: 16 3209-7100 - www.fcav.unesp.br

SUPLEMENTAÇÃO DE AMINOÁCIDOS PARA SUINOS EM CRESCIMENTO EXPOSTOS A DESAFIO SANITÁRIO

RESUMO – Desafios sanitários (DS) modificam o desempenho, o metabolismo proteicos, a saúde e o comportamento e bem estar dos suínos. Esta tese foi conduzida para compreender como suínos em crescimento respondem a um modelo de DS (causado por *Salmonella* Typhimurium ou por *Salmonella* Typhimurium e más condições de alojamento), e se, uma estratégia nutricional pode melhorar a capacidade imune dos animais alojados em grupo frente a estes DS aplicados. No primeiro estudo, os resultados revelaram que a inoculação oral de Salmonella *Typhimurium* pode reduzir o desempenho, induzir inflamação intestinal (presença de diarreia) e alterar parâmetros sanguíneos. Num segundo estudo, a suplementação dietética de triptofano (Trp), treonina (Thr) e metionina + cisteína (Met + Cys) melhora a resposta imune, desempenho, e a eficiência de utilização de nitrogênio dos suínos suplementados em comparação com indivíduos não suplementados quando submetidos inoculados com *Salmonella* Typhimurium e alojados em más condições sanitária. Por último, no terceiro estudo, os resultados destacaram que suínos leves e pesados têm respostas diferentes quando submetidos ao mesmo ambiente. As categorias de pesos apresentam padrões de consumo de ração diferente, no qual animais leves são mais irregulares e consomem menos ração em relação a animais mais pesados. Isto pode, em partes, explicar porque alguns indivíduos de uma população são mais resilientes que outros sob DS. Além disso, para animais leves sob DS que receberam a suplementação dietética de Trp, Thr e Met + Cys tiveram um padrão de consumo de ração mais regular do que os não suplementados, enquanto um padrão mais irregular foi observado para os animais pesados suplementados em comparação aos indivíduos pesados não suplementados. Por último, no quarto estudo, a abordagem metabolomica não direcionada mostrou que a suplementação de Trp, Thr e Met para suínos desafiados modulou principalmente o metabolismo antioxidante, anti-inflamatório e mitigou alterações no metabolismo energético em animais desafiados e suplementados quando comparado aos indivíduos desafiados e não suplementados. Esta estratégia nutricional também pode ser, portanto, interessante como uma estratégia nutricional direcionada para categorias suínos sob DS (especialmente para a categoria de peso leve) e sem o uso de antibióticos como

melhoradores de desempenho na dieta. Esses estudos fornecem novos conhecimentos sobre os efeitos do DS e de como uma estratégia nutricional é valiosa para potencializar a resposta imunológica de suínos sob DS e manter o seu desempenho e estado de saúde. Este conhecimento também pode ajudar os produtores de suínos a adaptar as práticas de manejo para melhorar a resiliência dos suínos, reduzindo ao mesmo tempo os antibióticos como melhoradores de desempenho em condições de alojamento intensivas.

Palavras-chave: triptofano, treonina, metionina, saúde, inflamação

AMINO ACIDS SUPPLEMENTATION FOR GROWING PIGS EXPOSED TO SANITARY CHALLENGES

ABSTRACT – Sanitary challenges (SC) modify pigs' growth performance, protein metabolism, health, and behavior. This thesis was conducted to understand how pigs respond to an environmental SC model (caused by *Salmonella* Typhimurium or *Salmonella* Typhimurium and poor housing conditions) and how a nutritional strategy may improve group-house growing pigs' ability to cope with a SC. In the first study, the results revealed that oral inoculation of *Salmonella* Typhimurium could reduce pigs' growth performance, induce inflammation, and impair health status. In a second study, increasing dietary tryptophan (Trp), threonine (Thr), and methionine + cysteine (Met + Cys) to Lys ratio increased supplemented pigs' capacity to support immune responses, improved protein deposition, and nitrogen efficiency compared to non-supplemented pigs under a *Salmonella* Typhimurium oral inoculation and poor housing conditions. In the third study, the findings highlighted that light and heavy pigs had different responses to the same environment, with light pigs having lower total feed intake and shorter meals than heavy pigs. It might help to understand why some pigs in a population have greater coping abilities under SC. Additionally, light pigs supplemented with Trp, Thr, and Met had a more regular feed intake pattern than non-supplemented ones, while a more irregular pattern was observed for supplemented heavy pigs compared to non-supplemented heavy pigs. Lastly, in the fourth study, the untargeted metabolomic approach showed that supplementing Trp, Thr, and Met for challenged pigs mainly modulated antioxidant and anti-inflammatory pathways and reduced energy-related alterations in supplemented pigs compared to challenged and non-supplemented individuals. This nutritional strategy may also be, therefore, interesting as a potential precision nutrition strategy for pigs (especially for light pigs) under SC and without antibiotics as growth promoters. Those studies provide new knowledge on the SC effects and how a nutritional strategy is valuable in pigs' ability to cope with a SC, and maintain their growth performance and health status. This knowledge can also help pig

producers adapt production practices to improve pigs' resilience while reducing antibiotics as growth promoters in intensive housing conditions.

Key-words: tryptophan, threonine, methionine, health, inflammation

PREFACE

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

Journal Publications

1. **Valini, G. A. C**., Arnaut, P. R., Barbosa, L. G., Azevedo, P. H. A., Melo, A. D. B., Marçal, D. A., Campos, P. H. R. F., Hauschild, L. (2023). A simple assay to assess *Salmonella* Typhimurium impact on performance and immune status of growing pigs after different inoculation doses. Microorganisms, v. 11 (2), p.446. doi/10.3390/microorganisms11020446.

2. **Valini, G. A. C**., Arnaut, P. R., Barbosa, França, I., Ortiz, M. T., Oliveira, M. J. K., Melo, A. D. B., Marçal, D. A., Campos, P. H. R. F., Htoo, J. K., Brand, H. G., Hauschild, L. (2023). Increased dietary Trp, Thr, and Met supplementation improves growth performance and protein deposition of salmonella-challenged growing pigs under poor housing conditions. Journal of Animal Science, v. 101, p.1-12. doi/10.1093/jas/skad141.

3. Submitted JAS: **Valini, G. A. C**., Méthot, S., Pomar, C., Hauschild, L., Remus, A. Size matters: lower body weight pigs have a different response to sanitary challenge and amino acids supplementation above the estimated requirements compared to heavy pigs.

4. (Draft): **Valini, G. A. C**., Hauschild, L., Remus, A. Non-target metabolomics profiling indicates the dietary Trp, Thr, and Met supplementation potential to mitigate alterations in protein and energy metabolism in growing pigs housed under a sanitary challenge.

5. Submitted (Microorganisms): Melo, A. D. B., **Valini, G. A. C.**, Neto, J. C. G., Yang, Q., Oliveira, M. J. K., Marçal, D. A., Arnaut, P. R., França, I., Silva, C. A., Korth, N., Pavlovikj, N., Campos, P. H. R. F., Brand, H. B., Htoo, J. K., Benson, A. K., Hauschild, L. Temporal changes in fecal swine microbiome are reflective of sanitary housing conditions despite dietary alterations.

Other papers and abstracts published in conference proceedings

1. Gonçalves, J. P. R., Melo, A. D. B., Oliveira, Yang, Q., M. J. K., Marçal, D. A., Ortiz, M. T., Arnaut, P. R., França, I., **Valini, G. A. C.**, Silva, C. A., Korth, N., Natasha Pavlovikj, N., Campos, P. H. R. F., Brand, H. G., Htoo, J. K., Gomes-Neto, J. C., Benson, A. K., Hauschild, L. (2024). Increased dietary Trp, Thr, and Met supplementation improves the performance of weaned piglets under mixed management and poor housing conditions. Animals (In press).

2. Oliveira, M. J. K., Valk, M., Melo, A. D. B., Marçal, D. A., Silva, C. A., **Valini, G. A. C.**, Arnaut, P. R., Rosa, J. P., Andretta, I., Hauschild, L. (2023). Feeding Behavior of Finishing Pigs under Diurnal Cyclic Heat Stress. Animals, v.13, p. 1-15.

3. Oliveira, M. J. K., Melo, A. D. B., Marçal, D. A., **Valini, G. A. C**., Silva, C. A., Vieira, A. M., Fraga, A. Z., Arnaut, P. R., Campos, P. H. R. F., Santos, L. S., Htoo, J. K., Brand, H. G., Hauschild, L. (2022). 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. 1-16.

4. **Valini, G. A. C.**, França, I., Barbosa, L., Silva, C., Arnaut, P., Ortiz, M., Rosa, J., Melo, A., Marçal, D., Htoo, J., Gonzalez-Veja, J.C., Brand, H., Lanferdini, E., Campos, P., Hauschild, L. (2021). Efeito da suplementação de aminoácidos (Trp, Tre e Met) no desempenho de suínos em condição de desafio sanitário (S. Typhimurium e más condições de higiene). 33ª Reunião CBNA – AVES, SUÍNOS E BOVINOS 2021.

5. Barbosa, L. G., **Valini, G. A. C**., Marçal, D. A., Melo, A. D. B., Hauschild, L. (2021). Efeito da suplementação de triptofano, treonina e metionina sobre o desempenho e composição corporal de suínos em desafio sanitário. XXXIII Congresso de Iniciação Científica da Unesp - FCAV/Jaboticabal.

6. Melo, A. D., **Valini, G. A. C.**, Rosa, J. P., Barbosa, L. G., Azevedo, P. H. A., Arnaut, P. R., Oliveira, M. J. K., Marçal, D. A., Hauschild, L. (2021). Desempenho, incidência de diarréia e leucograma de suínos em crescimento desafiados por Salmonella Typhimurium. 19º Seminário Técnico Científico de Aves e Suínos e 5o.

Congresso de Zootecnia de Precisão – AveSui 2021.

7. **Valini, G. A. C**., França, I., Arnaut, P. R., Ortiz, M. T., Barbosa, L. G., Silva, C. A., Rosa, J. P., Oliveira, M. J. K., Htoo, J., Brand, H., Lanferdini, E., Melo, A. D. B., Marçal, D. A., Campos, P. H. R. F., Hauschild, L. (2022). Dietary Trp, Thr, and Met supplementation attenuates changes in pig metabolism caused by sanitary challenge. SINSUI 2022 – 14º Simposio Internacional de Suinocultura.

8. Arnaut, P. R., **Valini, G. A. C**., Ortiz, M. T., França, I., Rosa, J. P., Silva, C. A., Oliveira, M. J. K., Htoo, J. K., Brand, H. G., Melo, A. D. B., Marçal, D. A., Andretta, I., Hauschild, L. (2022). Aminoácidos funcionais atenuam o impacto do desafio sanitário na exigência de mantença e melhoram a resiliência de suínos. SINSUI 2022 – 14º Simposio Internacional de Suinocultura.

9. Ortiz, M. T., **Valini, G. A. C**., França, I., Oliveira, M. J. K., Silva, C. A., Rosa, J. P., Arnaut, P. R., Melo, A. D. B., Marçal, D. A., Modelo de desafio com Salmonella Typhimurium e condições precárias de alojamento para suínos em crescimento. SINSUI 2022 – 14º Simposio Internacional de Suinocultura.

10. **Valini, G. A. C.**, Oliveira, M. J. K., Barbosa, L. G., Silva, C. A., Rosa, J. P., Arnaut, P. R., Ortiz, M. T., Azevedo, P. H. A., Melo, A. D. B., Marçal, D. A., Htoo, J., Brand, H. G., Lanferdini, E., Campos, P. H. R. F., Hauschild, L. (2022). Dietary supplementation with L-Trp, L-Thr and DL-Met improves the immune status and protein deposition of growing pigs under sanitary challenge. ISEP 2022 – 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition.

11. **Valini, G. A. C**., Arnaut, P. R., Hauschild, L., Remus, A. (2023). Enteric challenge increases amino acids and energetic costs in growing pigs. ASAS Midwest Section 2023.

9. **Valini, G. A. C**., Methot, S., Hauschild, L., Remus, A. (2023). Different initial body weight and dietary supplementation of tryptophan, threonine, and methionine on feeding behavior index of growing pigs under a sanitary challenge. ASAS – CASAS Annual Meeting 2023.

10. **Valini, G. A. C**., Hauschild, L., Remus, A. (2023). Dietary supplementation of tryptophan, threonine and methionine for growing pigs under a sanitary challenge improves the growth and immune status of pigs with lower body weight but not for

heavier pigs. ASAS – CASAS Annual Meeting 2023.

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CHAPTER 1 – GENERAL INTRODUCTION

1.1 Introduction

Pig production and pork meat consumption play an important economic role in agricultural systems worldwide. This has been possible by optimizing the pigs' performance in the different phases of the production cycle. However, selection for traits such as weight gain and lean body mass deposition has been prioritized over traits associated with disease resistance. Besides, world climatic changes may impact pig production, as high temperatures may lead to unbalanced interaction between host and environment, exposing pigs to a higher microbial pressure in the environment (Campos et al., 2017), especially when pigs are group-housed in intensive systems.

As a result, pigs may face health disorders that may increase herd heterogeneity (van der Peet-Schwering et al., 2019) and reduce farm profitability due to impaired feed efficiency and higher production costs with feed and medication (5.6% of total costs) (Larour, 2010). For instance, pigs reared in conventional housing systems with high microbial loads grow 10 to 20% more slowly than pigs kept in ‘clean’ environments (Lee et al. 2005; Renaudeau 2009). Additionally, there is an appeal to reduce the use of antibiotics in order to develop sustainable systems in animal production. The use of antibiotics as growth promoters, for example, has been banned from animal feed in many countries due to their association with the selection of resistant bacteria in both humans and animals. Thus, production animals are assumed to be increasingly exposed to SC conditions.

An alternative to minimize the negative effects of health challenges on animal performance is the supplementation of amino acids (AA) essential for immune response (Kipper et al., 2011). This supplementation is justified by reduced voluntary feed intake (Chatelet et al., 2017) associated with intense muscle catabolism in order to make AA available for immune cell synthesis (Le Floc'h et al., 2004). Growing and finishing pigs kept in poor sanitary conditions (SC) or challenged by Salmonella Typhimurium showed improved feed efficiency when fed a diet with higher concentrations of AA (+20% on methionine (Met), threonine (Thr) and tryptophan (Trp) levels; van der Meer et al., 2016; Rodrigues et al., 2021) and on weight gain (+20% on Thr level; Wellington al., 2019), respectively.

Similarly, in the post-weaning phase, piglets housed in poor SC, when fed a diet with higher Trp intake (+17%), showed increased feed consumption (Le Floc'h et al., 2009). Despite growing evidence that some AA requirements are affected by immune system activation, most results were obtained for post-weaning piglets housed individually or in small groups supplemented with Trp, Thr, or Met alone for a short period of time (one to two weeks of SC; Trevisi et al., 2015; Jayaraman et al., 2016; Kahindi et al., 2018; Capozzalo et al., 2020). Thus, it can be questioned to what extent these results can be extrapolated to a commercial condition where pigs are constantly exposed to multiple antigens without severe clinical signs of disease or mortality (van der Meer et al., 2016), and which may require at the same time the supplementation of more than one of these AA.

Given the great socioeconomic importance of pig production in Brazil and worldwide, studies aimed at evaluating nutritional strategies for pigs under SC in the growing phase can help develop technologies to advance sustainable pig production. Therefore, this thesis was performed to improve our understanding of how dietary Trp, Thr, and Met supplementation above requirements may influence growing pigs' coping abilities under SC.

For this purpose, we decided to apply Salmonella Typhimurium oral inoculation, and no cleaning routine + manure spreading on the pen floor as the SC model, mimicking a commercial condition where pigs are constantly exposed to antigens/pathogens. In turn, responses of challenged pigs fed with dietary amino acid supplementation were evaluated through changes in performance, feeding behavior, immunological parameters, herd variability (heavy vs. light pigs), and metabolism through metabolomics analysis. It should be noted that assessing the SC impact over time allowed a better understanding of how pigs cope with SC and provides useful information based on their productive potential and well-being.

This thesis is composed of 8 chapters. A brief literature review followed by the main objectives and hypotheses are presented in chapter 2. Chapters 3, 4, 5, and 6 present the results (in the format of journal manuscripts) from the studies carried out during the Ph.D. course. Chapter 3 aimed to determine the dosage for oral gavage inoculation with *Salmonella* Typhimurium required to assess immunological, physiological, and growth performance alterations in pigs. Chapter 4 was performed

to evaluate the effects of dietary Trp, Thr, and Met + Cys supplementation on growth and immune system activation of growing pigs challenged with Salmonella Typhimurium and group-housed under poor housing conditions. Chapter 5 evaluated the effect of initial body weight (light vs. heavy) on the growing pigs' capacity to cope with a sanitary challenge and how Trp, Thr, and Met supplementation above NRC requirements influence the immune response and growth composition within bodyweight categories. Chapter 6 explored the systemic metabolome of pigs differing in health condition, induced by oral Salmonella Typhimurium and poor housing condition, and the impact of dietary supplementation of Trp, Thr, and Met above NRC requirements on plasma metabolites of growing pigs under a sanitary challenge. Chapter 7 contains the general discussion, experimental strengths, and limitations and, discusses the perspectives and implications for further research. Finally, in Chapter 8, the final considerations and conclusions of this thesis are presented.

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CHAPTER 2 – LITERATURE REVIEW

2.1 Metabolic and physiological responses of pigs under sanitary challenge conditions

Pigs respond to SC by activating the immune system (Badaoui et al., 2013), which includes neuroendocrine and metabolic adjustments to coordinate physiological responses to inflammation in an attempt to restore animal homeostasis. Among these, it is highlighted the reduction of voluntary feed consumption (Chatelet et al., 2017) and intense muscle catabolism to have AAs available for immune cell synthesis (Le Floc'h et al., 2004). Even with no severe clinical signs, SC pigs may show reduced appetite and growth compared to non-challenged pigs (Valini et al., 2023). These modifications may reduce farm profitability. For example, SC-growing pigs had lower carcass yield and economic losses of up to $26.10 per pig (Cornelison et al., 2018).

The voluntary feed intake reduction in challenged pigs may occur due to the increase of signaling agents such as prostaglandin (PGE2), which reduces gastrointestinal tract peristalsis and gastric emptying (Exton et al., 1995; Plata-Salamán, 1998), thereby decreasing appetite. In addition, PGE2 and cytokines (such as Il-1β) stimulate leptin production by adipocytes, which induces to an inflammatory state, reduces feed intake, and increases energy expenditure (Finck et al., 1998). As a result, there is an increased muscular catabolism and nutrient partitioning, especially AAs (Wellington et al., 2018), and also energy demand (Huntley et al., 2017) from growth to immune response (Reeds and Jahoor, 2001), impacting protein deposition rate and animal growth.

Cytokines produced in the inflammatory process may inhibit muscle protein synthesis (Cooney et al., 1994) and induce muscle protein degradation (Zamir et al., 1994). For example, piglets under a SC had increased plasma alpha1-acid glycoprotein (an indicative of cytokine release) and reduced protein deposition and nitrogen retention by 28% and 20%, respectively, compared to non-challenged pigs (Williams et al., 1997a, b).

Besides, other physiological responses/mechanisms occur to adapt, maintain the individuals' integrity, and recognize and interrupt pathogens proliferation inside the body. Chemical-mechanical barriers are the first protection against pathogens. These

include skin, stomach pH, bile secretion, and mucus (Riera et al., 2016). For example, pigs in poor SC increase mucus and immunoglobulins production (Celi et al., 2017), contributing to intestinal integrity and preventing pathogens translocation through the gut mucosa.

In addition, two defense mechanisms are triggered under a SC: innate and acquired immunity. These mechanisms are influenced by the type of stimulus and the immune and nutritional status of the pigs (Le Floc'h et al., 2014; Lu et al., 2017). The innate immune system consists, beyond the physical barriers. It also includes mononuclear phagocytes (e.g. monocytes and macrophages), dendritic cells, polymorphonuclear granulocytes (such as neutrophils, eosinophils, and basophils), mast cells, natural killer cells, platelets and humoral factors (lysozymes, C-reactive proteins, and interferons). This system is responsible for the rapid response to invading pathogens, and it is essential in guiding the adaptive immune response; however, it lacks specificity and memory effect. As an immediate response to SC (innate immunity), there is an increase in plasma concentrations of cytokines, glucocorticoids (Campos et al., 2017), and acute phase proteins (haptoglobin) (Cruvinel et al., 2010), changes in insulin sensitivity, and lower circulating levels of thyroid hormones (Castro et al., 2013).

Another clinical sign of infection and inflammation of the innate response is hyperthermia (fever). Pro-inflammatory cytokines released by phagocytes, such as interleukins (IL) IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α), act as pyrogenic cytokines that stimulate the synthesis of PGE2, which is the central mediator of the febrile response (Netea et al., 2000). When fever occurs, there is an increase in energy consumption by the immune system. For a pig weighing 80 kg, each degree Celsius increase in body temperature is associated with about a 10% increase in basal metabolism (Black and Pluske, 2011).

Depending on the severity and type of SC, innate immunity does not fully clear the infection, and acquired immune system activation occurs. It requires the T and B lymphocyte proliferation (Riera et al., 2016), which demandes a surplus of AAs (from the diet or body reserves) for their synthesis (Li et al., 2007). For example, a higher lymphocyte proliferation was found in 49-day-old piglets challenged with Salmonella enteritidis after 12 days of inoculation (Volf et al., 2012). Indeed, the acquired

immune system may take several days to weeks to develop, but it creates a long-term memory mechanism that helps pigs recover after prolonged exposure to SC (Nicholson, 2016).

Tissue injury and inflammation may also activate another physiological mechanism through the sympathetic nervous system and the corticotropic axis. Cytokines may induce the production of corticotropin-releasing hormone at the neural system level (Uehara et al., 1989) and cortisol release. The augment in cortisol may impair other anabolic hormones, such as insulin, and insulin-like growth factor 1 (Balaji et al., 2000), impacting protein metabolism and nutrient partitioning (Mormede et al., 2007) (Figure 1). For example, Campos et al. (2019) observed changes in the metabolism of growing pigs after a SC, such as increased plasma concentrations of glucose, non-esterified fatty acids, and lower postprandial concentrations of AA, as for example, Trp.

Finally, the presence of cytokines in the central nervous system may alter pigs' behavior and cognition (Colditz, 2002), contributing to reduced intake and growth. The cytokines may prompt the catecholaminergic anorectic neurotransmitter release (Plata-Salamán, 1997) or block neuropeptide Y, a powerful stimulant of feed intake (Sonti et al., 1996). The magnitude of the feed intake reduction may depend on the SC type/class and its severity (time and/or pathogen dosage). At a subclinical level, up to 80% reduction in voluntary feed intake may occur (Kyriazakis et al., 2008), whereas in a more pronounced SC, the voluntary feed intake can be close to zero.

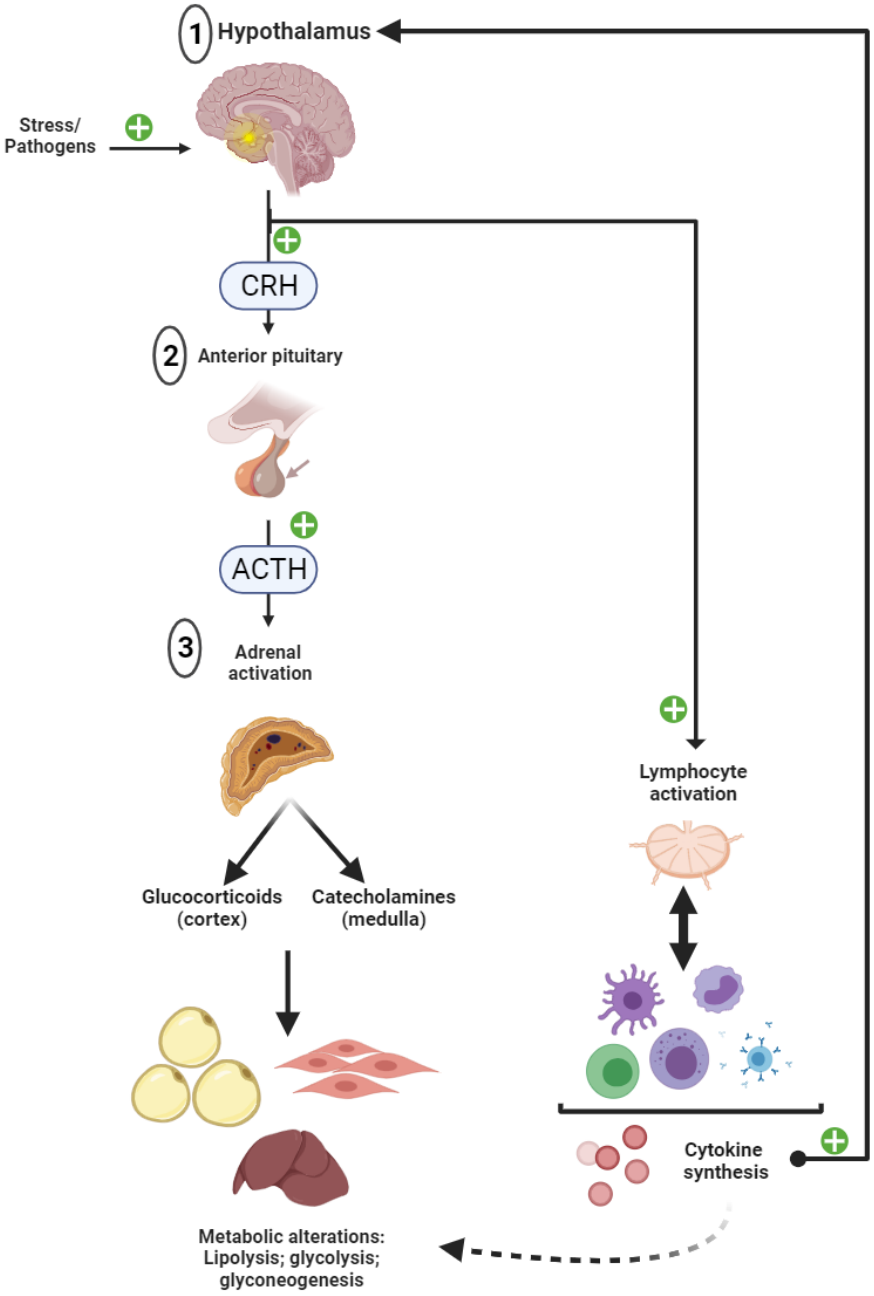

Figure 1: Schematic representation of physiological responses under inflammation/sanitary challenge

2.2 Sanitary challenge models – characterization

In both domestic and laboratory animals, the results evidencing the physiological and metabolic changes of immune activation come from experimental SC models. These SC models' most commonly observed results include reduced feed intake, protein synthesis, increased energy expenditure, and body temperature

(Black, 2009). However, the duration of these responses depends on the SC type and intensity and pigs coping ability to stimulate their immune response and eliminate pathogens (Sandberg et al., 2007; Kyriazakis and Doeschl-Wilson, 2009).

In the literature, some models include the inoculation of live microorganisms (E. coli (ETEC) and Salmonella spp.), chemical substances (bacterial lipopolysaccharide; LPS), or models that mimic the deterioration of housing conditions (Le Floc'h et al., 2006). These models are important for elucidating the interactions between pathogens and animal homeostasis, but all these approaches have limitations. Thus, the choice of the SC model will depend on the research objectives, costs, biosecurity, phase of pig production, type of response (acute or chronic), and feed additive for testing, among others.

For a long time, researchers have used LPS as an SC model in farm animals (Yang et al., 2008). LPS is a molecule on the outer surface of gram-negative bacteria, which causes immune stimulation (Chapman et al., 2005). It is well documented that LPS administration increases body temperature (Parrott and Vellucci, 1998), decreases feed intake (Webel et al., 1997), alters plasma concentration of acute phase proteins, activates the hypothalamic-pituitary-adrenal axis (Webel et al., 1997) and inhibit the somatotropic axis (Johnson, 1997). Consequently, LPS impairs growth and increases economic losses (Liu et al., 2003). Although the use of the LPS model has allowed researchers to comprehend some physiological and metabolic, this approach has limitations. The LPS model usually provides short-time immune system activation rather than a chronic response (Balaji et al., 2000). It results from the pigs' capacity to develop immune tolerance to multiple subsequent LPS administration (Kegley et al., 2001; Liu et al., 2003). Thus, other SC models have been proposed, with the oral inoculation of live pathogens.

Enterotoxigenic *Escherichia coli*, or ETEC, is the main pathogen cause of diarrhea in nursery pigs, with a mortality rate of up to 30% (Rhouma et al., 2017). In addition, the K88 fimbrial antigen is the most frequently isolated in piglets and is associated with colibacillosis. Therefore, an oral inoculation with ETEC K88 has been widely used as a SC model for post-weaning diarrhea (Wellock et al., 2009). ETEC attaches to the gut mucosa, modifies intestinal morphology (reduced villus height and increased crypt depth), and leads to nutrient malabsorption, diarrhea and decreased

performance (Trevisi et al., 2009). Other effects observed by ETEC are increased serum concentrations of urea (Kiarie et al., 2009), haptoglobin (Lee et al., 2012) and pro-inflammatory cytokines (TNF-α and IL-6) (Li et al., 2015), increased rectal temperature (Trevisi et al., 2015).

Although this model is widely used to elucidate the SC effect on pigs for a longer period of time (chronic response) than LPS, the ETEC impact on pigs' performance is still controversial in the literature, which is the main limitation. The pigs' age and weight at the time of challenge may influence the effectiveness of this SC model. There is a higher expression of ETEC receptors (for fimbriae F4 and F18) in the small intestine during the first three weeks of age. As a result, the higher infection rate of ETEC occurs mainly during the neonatal period but also at weaning (Luppi, 2017).

After this period, the immune system matures and is well-developed between 6 to 8 weeks of age (Stokes et al., 2004). At this stage, pigs have fewer ETEC receptors and greater immune competence to respond to infections. Therefore, another SC model with live pathogens for growing pigs was established to evaluate the interactions between immune system activation and depressed growth: oral inoculation of *Salmonella spp*.

Salmonellosis in pigs is one of the top 10 most common diseases in the growing and finishing phases (Haley et al., 2012). It is estimated that a large proportion of the herd can be affected by Salmonella spp., between 25% and 50% of the pig herd (Davies et al., 1997; Funk et al., 2001). The most common is *Salmonella* Typhimurium (ST), which has a great capacity to persist in the environment for long periods. Pigs challenged with ST have diarrhea, anorexia, and lethargy. In addition, ST triggers increased serum haptoglobin concentrations (Turner et al., 2002), rectal temperature, gut pro-inflammatory cytokines synthesis (Collado-Romero et al., 2010; Santos et al., 2001), and growth performance is generally reduced (Turner et al., 2002; Wellington et al., 2019). The dose of ST used varies from 10^7 to 10^9 CFU, as it was previously reported that growing-finishing phase pigs could shed high concentrations of ST in the environment (between 10^5 and 10^8 CFU/g of feces). However, such a range of doses results in different outcomes. Some authors report models with mild infectious outcomes (Walsh et al., 2012; Moura et al., 2021; Davis

et al., 2022); while other studies showed clinical responses (Balaji et al., 2000; Spiehs et al., 2008), and even responses that overwhelmed treatment capacity (Casey et al., 2007), therefore being a disadvantage of this SC model.

Due to the ST and ETEC model disadvantages, and because pigs in conventional housing systems may be exposed to several pathogens at the same time; another SC model was proposed by Le Floc'h and collaborators (2006): the deterioration of housing conditions. Pigs are raised in facilities without disinfection and cleaning before arriving or while allotted within it. This SC model increases the environment microbial pressure, which stimulates the immune system while impairing the growth performance of the animals without major signs of illness (prostration, dehydration, vomiting, and feed intake close to zero).

The main responses observed in animals housed in poor hygienic conditions are increased serum haptoglobin concentrations (Le Floc'h et al., 2006, 2009), reduced villus height, and increased intestinal crypt depth (Jayaraman et al., 2016), decreased feed intake, weight gain and low feed efficiency (Le Floc'h et al., 2006, 2009; Kahindi et al., 2014; van der Meer et al., 2016). This SC model may be applied to large herds and is less costly than the other models. However, its major limitation is standardizing the housing condition and determining the main causative agent for any immunological response (impacting its reproducibility over time and among laboratories).

Considering these SC models available in the literature (their mechanisms to induce an immune system activation, impairment in metabolism and growth performance, and limitations), we decided to use ST inoculation and no cleaning routine throughout the experimental period as the SC model. The choice was based on the following criteria: the production phase of interest (growing pigs, in which pigs are susceptible/predisposed to ST infection), the type of response to be evaluated (chronic response/long-term response), and mimic a commercial condition where pigs are constantly exposed to antigens/pathogens.

Due to the wide range of ST doses used in the literature (varies from 10^7 to 10^9 CFU), and outcomes (mild infectious to severe clinical signs and death), in the first study of this thesis (chapter 3), we aimed to determine the dosage for oral gavage inoculation with ST required to assess immunological, physiological, and

growth performance alterations in pigs.

However, due to the lack of effect on growth parameters after 14 days of trial and the small alterations in blood parameters, in the second study of this thesis (chapter 4), we decided to increase the ST dose inoculated (from 10^8 to 10^9) and include manure spreading (from a commercial pig herd) in the pen floor as part of the SC model. These modifications aimed to enhance and recycle the pathogenic pressure and prolong the immune system activation of pigs SC throughout the trial (28 days).

2.3 Amino acid supplementation for pigs under sanitary challenge

Pigs raised in conventional commercial production systems are exposed to various SC, mainly through inhalation or ingestion of antigens and pathogens, which may induce an immune response. In this context, there might be an increase in nutrient requirement estimated at around 5-7% in the maintenance requirement for monogastric animals (Kim and Pluske, 2016). Consequently, immune system activation can affect several processes involving AAs and proteins, based on their roles as precursors of energy and their functions beyond nutritive utilization (protein building blocks) during immune system activation (Chalvon-Demersay et al., 2021). Therefore, under such conditions, AAs are good candidates for feeding adjustments to regulate key metabolic pathways to improve animals' health and growth (Wu, 2007).

However, when formulating diets, the effects of SC on animal metabolism and nutritional requirements are rarely considered. This raises the question of whether the current AA recommendations for growing-finishing pigs estimated to maximize the growth performance of healthy pigs (NRC, 2012) would be adequate for pigs under a SC. Thus, by not considering the use of AA in immune system activation, nutritional programs may have some limitations in a practical production context. Under these conditions, an increase in dietary concentrations of some AAs may be a strategy to rebalance immune and production functions.

Additionally, when AAs in free form are supplemented in the diet, they appear in peripheral plasma more rapidly than AAs arising from intact proteins (Morales et al., 2015). Yen et al. (2004) reported peak concentrations of Lys and Thr in arterial plasma and arterial portal of growing pigs one hour after the provision of free AAs in

the diet (postprandial), whereas the peak for AAs from intact proteins occurred only after 2.5 hours. Therefore, the supply of AAs in different forms can be a physiological basis for preventive or therapeutic nutritional intervention through the diet (Le Floc'h et al., 2018).

Some AAs have been adopted as a nutritional strategy to attenuate the effects of SC on pigs' growth due to their functions in modulating immune responses (Wu, 2007). For example, studies have highlighted the immune function of Met (Sun et al., 2016; Zhou et al., 2016), whose metabolism is essential in the redox system (Sierzant et al., 2019).

Oxidative stress results from an imbalance between the endogenous production of reactive oxygen species (ROS) and antioxidant defenses (Wu et al., 2004). ROS production is a mechanism some immune cells use (e.g., macrophages) to exert their cytotoxic function. As a result, ROS may increase oxidative stress and overwhelm the antioxidant defenses (Li et al., 2007). To improve the antioxidant capacity under an immune system activation, a significant proportion of sulfur AAs, such as Met, is redirected and retained in non-protein compounds, such as glutathione (Sierzant et al., 2019). Le Floc'h et al. (2006) observed a decrease in plasma concentration of sulfur AAs and glutathione in weaned piglets subjected to poor SC. In another study, challenged growing-finishing pigs had higher blood concentrations of glutathione from the conversion of Met and cysteine (Rakhshandeh and de Lange, 2010) compared to non-challenged pigs. Therefore, methionine supplementation may contribute to antioxidant capacity, to neutralize the ROS by some immune cells.

Another important AA for SC pigs is Trp. When there is an immune system activation, gamma interferon activates the enzyme indoleamine 2,3-dioxygenase (IDO), whose function is the catabolism of Trp into kynurenine (Le Floc'h et al., 2010; Le Floc'h et al., 2004). The IDO activation and the Trp metabolites production regulate the T cell proliferation, coordinating the long-lasting immune activation (Popov and Schultze, 2008), and immune tolerance (Sharma et al., 2007). The oxidative stress may also depress plasma Trp and increase plasma kynurenine (Mor et al., 2021). Such modifications are expected to affect Trp availability for growth. Therefore, Trp supplementation may benefit pigs under SC (Le Floc'h et al., 2018).

For example, the dietary supplementation with 1 g of Trp/kg above the requirements improved growth response in weaned pigs predisposed to E. coli compared to non-susceptible pigs (Trevisi et al., 2009).

Finally, Thr is another functional AA for pigs under SC caused by enteric pathogens. Usually, enteric pathogens stimulate the innate immune system and gut barrier permeability, which results in leaky gut and diarrhea (Campbell et al., 2013; Wang et al., 2015). In this sense, an adequate Thr supply may contribute to the maintenance of intestinal functionality (Ruth and Field, 2013), as it prevents inflammations and pathogen translocation (Pluske et al., 2017). Thr is the major constituent of mucus glycoproteins (10-13%; Pluske et al., 2018) and immunoglobulin A (7-11%; Sandberg et al., 2007). Therefore, Thr may benefit pigs by reducing the gut inflammatory response (Trevisi et al., 2009) through mucus synthesis, and bacteria binding to the mucosal surface (Rakhshandeh et al., 2013). As examples, the exposure of Thr-deprived intestinal cells for 2 hours to a culture medium induced a compensatory increase in IL-8, mucin 2 and immunoglobulin A mRNA expression during E. coli challenge. However, this increase was suppressed upon Thr supplementation in an in vitro study (Zhang et al., 2017). In vivo, growing and finishing pigs under a SC had improved feed efficiency and weight gain when fed diets supplemented with Thr (+10% in Thr levels; Jayaraman et al., 2015; +20% in Thr level; Wellington al. 2019, respectively).

It is shown that nutritional status and the immune system are linked. Thus, the dietary Trp, Thr, and Met supplementation may counterbalance the reduced nutrient availability and reduced feed intake during a SC. To the best of our knowledge, however, only one study evaluated the effect of dietary AA supplementation on growing pigs (van der Meer et al., 2016) during a long period of time (more than two weeks). Additionally, it is still questioned if this nutritional strategy can be extrapolated to a commercial condition where pigs are group-housed and constantly exposed to multiple antigens without severe clinical signs of disease or mortality (van der Meer et al., 2016), and which may require at the same time the supplementation of more than one of these AA. Furthermore, few studies have evaluated AA supplementation on the efficiency of nutrient utilization for lean deposition. The data available in the literature are from mathematical equations (Koopman et al., 2012;

McGilvray et al., 2019) or slaughterhouses (van der Meer et al., 2016), which allow only a single measurement throughout the experimental period. Thus, the use of absorptiometry (DXA) equipment is interesting, as it allows several measurements in the same animal and with good precision in the estimation.

Therefore, in this thesis, one of the studies (chapter 4) was performed to evaluate the effects of dietary Trp, Thr, and Met + Cys supplementation on growth, protein deposition, and immune system activation of growing pigs challenged with *Salmonella* Typhimurium and group-housed under poor housing conditions.

2.4 Behavioral modifications in sanitary challenge pigs and amino acid supplementation effects

Dietary protein deficiencies or AA imbalances may influence animals' behavior in intensive production systems. Relative shortages of one or more AAs may increase the exploratory (Jensen et al, 1993) and aggressive behaviors toward the pen mates (Jericho and Church, 1972). For example, the dietary Trp concentration effect on feeding and aggressive behavior has been associated with its role as a precursor of serotonin (5-hydroxytryptamine; 5-HT). Lower brain stimulation of 5-HT receptors reduced orexigenic factor production, which decreased pigs' feed intake (Collin et al., 2002). Additionally, lower levels of 5-HT stimulated aggressive behavior in piglets ("tail biters") (Ursinus et al., 2014). Therefore, the dietary AA levels may impair pigs' ability to cope with stressful conditions.

Sanitary challenges may also lead to AA imbalance by redirecting AAs towards the immune system activation (such as Trp). Consequently, it may contribute to behavior changes (van der Meer et al., 2017). For instance, weaned piglets housed in poor SC spent more time with exploratory behavior than with voluntary feed intake (Pastorelli et al., 2012). This explained 10% of the reduction in feed intake (Pastorelli et al., 2012). The modifications in feeding and exploratory behavior may be related to the feed aversion developed by pigs due to their association between feed ingestion and post-ingestion effects, such as abdominal pain (Day et al., 1998).

Accordingly, the dietary supplementation of some AAs, such as Trp, Thr, and Met may reduce behavioral alterations. Growing pigs under poor SC had greater

competition for the feeder (+25%) and a higher incidence of stereotyped/aggressive behaviors (ear and tail biting; van der Meer et al., 2017). However, dietary supplementation of Trp, Thr, and Met (+20% of the ratio recommended by the NRC, 2012) reduced ear biting frequency (-16%) and mounting behavior of growing pigs under poor SC (van der Meer et al., 2017). The reduced agonistic behavior may be associated with meeting the requirements of these AAs to support the immune, decreasing the exploratory chewing behavior towards pen mats. It also reflected positively on performance, with higher average daily gain and gain-to-feed ratios (van der Meer et al., 2016).

Nevertheless, information on the behavior of growing submitted to SC is still scarce (Wechsler and Lea, 2007; Pastorelli et al., 2012). Studies recording feeding and aggressive behaviors can be an important tool to detect and better understand the relationship between SC, physiological responses, and nutritional requirements of SC pigs. Therefore, in one of the studies in this thesis (chapter 5), we analyzed the feeding behavior of growing pigs as an approach to evaluate pigs' capacity to cope with a SC and the effect of dietary Trp, Thr, and Met supplementation above NRC requirements on pigs' coping abilities.

2.5 Individual variability and amino acids supplementation for pigs under a sanitary challenge

The practical application of phase feeding programs involves feeding pigs a unique diet formulated to meet the requirements of the most demanding pigs and maximize weight and lean tissue gain (Remus et al., 2021). For this purpose, it is assumed that the population's growth response is equal for all pigs (Wellock et al., 2004). However, pigs at the same age show an in-between-animal variation, which leads to an increase in pigs’ growth performance variation and economic gains.

Moreover, individual pigs’ requirements vary over time, and the nutrient requirements (especially AA) can be altered by several factors that contribute to increasing the variation in the response of individual animals (Remus et al., 2021). One of these factors is environmental stressors (Pomar et al., 2015; van der Peet-Schwering et al., 2019), as individuals within a population may respond differently to environmental stimuli. Challenging sanitary conditions can result in reduced intestinal

absorption of AA, stimulation of endogenous protein losses in the gut, and oxidation of AA for immune system response, which negatively impacts growth, and changes AA requirements (van der Peet-Schwering et al., 2019).

The extent of the immune stimulation impact on the variability depends, amongst others, on pigs' ability to cope with a SC, which may be affected by body weight (BW) and body composition. Based on resource allocation theory (van der Waaij, 2004), a higher percentage of lean body mass is expected to improve an animal's capacity to handle immune stimulation, redirecting nutrients and body protein reserves to support immune system activation (Spurlock, 1997). Therefore, heavier pigs may have a better coping ability than lighter mates.

Additionally, lighter pigs display lower feed intake and limited physical capacity to ingest nutrients when compared to heavy pigs (higher BW; Aymerich et al., 2020; Ribeiro et al., 2016), which may increase their probability of being susceptible to a sanitary challenge (Laghouaouta et al., 2021). Besides, when compared to heavier pigs, lighter pigs have an increased propensity to be affected by stressors (Njoku et al., 2021), which may be associated with altered nutrient metabolism and increased intestinal inflammation in pigs. Therefore, the requirements, especially for AA, of the lightest pigs under a SC may vary greatly from heavier mates.

As a result, the dietary AA supplementation of Trp, Thr, and Met as a nutritional strategy to improve the growth performance of pigs under a SC may have different effects on pigs with different BW categories. As the positive effects of these AA are mainly associated with improved intestinal mucosa integrity, antioxidant defense, and immune molecule synthesis (Le Floc'h et al., 2018), they may have a higher impact on the growth performance of pigs with an increased propensity to be affected by stressors.

To the best of our knowledge, however, there is no study in the literature regarding variability and coping characteristics of pigs with different BW under a SC and supplemented with Trp, Thr, and Met above NRC requirements during the growing or finishing phase. Besides, as the growing–finishing phase is the most expensive period of the pig's life (approximately 65% of the total cost; SIP Consultors, 2018), understanding, addressing, and maybe, reducing the inter-individual variability is crucial for optimizing pig nutrition and health management

strategies. Therefore, in one of the studies in this thesis (chapter 5), we aimed to evaluate the effect of initial BW (low vs high) on the growing pigs' capacity to cope with a sanitary challenge and how Trp, Thr, and Met supplementation above NRC requirements influence the immune response and growth composition within BW categories.

2.6 Conclusions of the literature review

Based on this literature review, it is clear that under a sanitary challenge, pigs alter their physiology (e.g., increased rectal temperature and altered fecal score), behavior (e.g., feeding behavior pattern deviations), and metabolism (e.g., protein metabolism) as mechanisms to reestablish homeostasis and maintain growth. However, depending on the microorganism and its pathogenicity and how long pigs are exposed, these responses may not be enough, and negative effects on pigs' well-being and performance are observed. These negative responses may occur because, in commercial production facilities, pigs receive a diet formulated to contain the nutrients required to express their growth potential in an ideal condition without accounting for the interactions between pigs and the environment. Thus, the current feed formulation and the genetic selection for feed efficiency may contribute to pigs' susceptibility to sanitary challenges under intensive production systems. Therefore, the conventional pig production system has to find new strategies to overcome this and other challenges (e.g., environmental impact and feed costs) while meeting the pork demands worldwide. The literature review also showed how nutrition interacts with health/immune system responses and how a nutritional strategy (through AA supplementation) may benefit pigs under a sanitary challenge. However, a large variation in the performance and immune response among animals receiving the same treatment exists, suggesting that there are intrinsic animal factors affecting those responses and it should be investigated and accounted for. Some additional gaps and questions still remain for future research: How can the AA supplementation concept be in a commercial system where pigs face several environmental challenges simultaneously? How does the AA supplementation concept apply in sustainable swine production systems with co-product utilization in feed formulation? What is the AA supplementation's financial impact on the long-term (slaughter)?

2.7 Hypotheses and objectives

Our main hypothesis was that supplementing Trp, Thr, and Met + Cys above NRC requirements attenuates the effect of a sanitary challenge on growing pigs by improving antioxidant capacity, regulating immune cell proliferation, reducing the synthesis of pro-inflammatory molecules, and maintaining gut mucosa integrity.

Additionally, we hypothesized that pigs of the same age but different BW (light versus heavy) have different responses to Trp, Thr, and Met supplementation under a sanitary challenge by differences in feeding patterns and body reserves utilization to support immune system activation.

To evaluate our hypotheses, we set the following objectives:

- Develop a sanitary challenge model to activate the immune system and evaluate growing pigs' feed intake, weight gain and feed efficiency responses.
- Evaluate the effect of dietary Trp, Thr, and Met + Cys supplementation on protein deposition, feed intake, weight gain, and nitrogen utilization efficiency of challenged growing pigs.
- Assess the impact of dietary Trp, Thr, and Met + Cys supplementation on serum levels of haptoglobin, albumin, and urea in challenged-growing pigs.
- Study the effect of initial BW (light vs. heavy) on feeding behavior and the coping capacity of growing pigs under a sanitary challenge.
- Determine the plasma metabolites and metabolic pathways affected by dietary acid supplementation strategy and the sanitary challenge of challenged growing pigs.

2.8 References

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CHAPTER 3 – A simple assay to assess Salmonella Typhimurium impact on performance and immune status of growing pigs after different inoculation doses

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Abstract: *Salmonella* Typhimurium is the most frequent serovar in pigs, and it causes infections in humans. However, the dosage used for experimentation is not well defined. The present study aimed to evaluate a dosage for oral inoculation with *Salmonella* Typhimurium to assess immunological, and growth performance alterations in pigs. Gilts were randomly allocated into one of three experimental treatments: no *Salmonella* Typhimurium inoculation (Basal), or oral inoculation of 1×10^8 or 1.5×10^8 colony forming units of *Salmonella* Typhimurium. Growth rate, rectal temperature, fecal *Salmonella* shedding were recorded, and blood samples were taken. Inoculated pigs shed the bacteria for up to 7 days, but no difference between groups were observed. No difference was observed for rectal temperature, body weight, average daily feed intake. However, a reduction in average daily gain (-17 and -22%) and feed efficiency (-14 and -20%) were observed in pigs inoculated with 1×10^8 and 1.5×10^8 colony forming units, respectively. The hemoglobin and hematocrit concentrations increased in challenged pigs compared to Basal pigs. The oral dosage of 1.5×10^8 colony forming units of *Salmonella* Typhimurium is a suitable to induce pigs' immune system activation and access *Salmonella* impact on pig performance.

Keywords: growth, immunologic response, sanitary challenge, swine.

1. Introduction

The optimal growth performance of growing-finishing pigs can be affected by a wide range of environmental and sanitary factors. In commercial production systems, highly intensified production, management conditions, and the assumed lower capacity of modern genotypes to adapt to environmental and sanitary challenges are likely to predispose pigs to recurrent immune responses [1].

Salmonella enterica serovar Typhimurium (ST) is the most common serovar in pigs worldwide [2], which has zoonotic importance in humans [3] and a great capacity to persist in the environment for long periods [4]. In this sense, pigs often become carriers of ST persisting in the gut and gut-associated lymphoid tissues [5]. Pigs with salmonellosis manifest diarrhea, anorexia, and lethargy. In addition, growth performance is generally reduced in pigs affected by an ST infection, in part resulting from the redistribution of nutrients [6] to support the synthesis of immunological molecules, maintenance of the intestinal barrier, and oxidative status.

However, studies using ST as a challenge model in growing pigs are scarce, and its deleterious effects depend on the inoculum dosage, strain virulence and host health status. The dose of ST used in studies usually varies from 107 to 109 colony-forming units (CFU), as it was previously reported that growing-finishing phase pigs can shed high concentrations of ST in the environment (between 105 and 108 CFU/g of feces) [7, 8]. However, such a range of doses results in different outcomes. Some authors report models with mild infectious outcomes [9, 10, 11]; while other studies showed clinical responses [12, 13], and even responses that overwhelmed treatment capacity [14].

Thus, before proposing strategies to mitigate the negative impact of ST, it is important to determine a suitable challenge model regarding its practicality, inoculum dosage and immunological and physiological alterations without inducing a severe illness. Understanding the challenge impact on the immune system and performance responses may aid future researchers in choosing a more efficient attenuating strategy for ST -challenged pigs. Therefore, this study aimed to determine the dosage for oral gavage inoculation with ST required to assess immunological, physiological, and growth performance alterations in pigs.

2. Materials and Methods

2.1 Animals, housing and management

All experimental procedures in this trial followed the Brazilian National Council of the Control of Animal Experimentation guidelines and were reviewed and approved by the Ethical Committee on Animal Use of São Paulo State University (protocol no. 4784/20).

The study was conducted at the Swine Research Facility of São Paulo State University (FCAV/UNESP, Jaboticabal, Brazil) in two similar open-sided nursery buildings. Before the pigs'

arrival, both buildings were cleaned and disinfected with iodine 2.6% iodophor (dilution 1:250, Biocid, Pfizer, Brooklyn, New York) as instructed by the manufacturer. Additionally, prior to animals allocation (day -7), surface drag swabs were taken in both buildings to screen for Salmonella spp. The swabs were serially diluted in buffered peptone solution (1:10) until they reached a final concentration of 10-6. From each dilution, 0.1-mL was plated on brilliant green agar (CM0263, Oxoid, Basingstoke, Hampshire, England), and incubated at 37 °C for 24 h. No Salmonella spp. presence was detected in the facilities.

Thirty crossbred female pigs (Pietran × [Large White × Landrace]) with an initial average body weight (BW) of 27.3 ± 5.1 kg were used in a 14-day trial. The pigs were individually housed in suspended pens with fully slatted plastic floors (1.4 × 1.5 m). Each pen was equipped with a one-sided self-feeder and a nipple drinker that allowed ad libitum access to feed and water. Before the study started, rectal swabs were collected individually to verify that pigs were free of detectable *Salmonella spp* shedding. Rectal swabs were diluted, plated, and incubated using the same surface drag swab protocol. All pigs were negative for *Salmonella spp*.

Throughout the trial, all pigs were fed a mashed standard corn-soybean meal-based diet, formulated to meet the nutrient requirements according to the NRC [15]. The standard diet was formulated with 2,548 kcal/kg of net energy and a standard ileal digestible lysine content of 9.9 g/kg. The standard ileal digestible methionine + cysteine, threonine, tryptophan, and valine contents expressed as a percentage of digestible lysine were 56, 59, 17 and 68%, respectively, according to the amino acid profile [15].

2.2 Experimental design

On d 0, pigs were randomly assigned to one of three treatments according to BW with ten replicates of one animal per treatment. Treatments consisted of no ST inoculation (Basal), 1×10^8 CFU of ST inoculation, and 1.5×10^8 CFU of ST inoculation.

The Basal group was inoculated by oral gavage with 5-mL of brain heart infusion (BHI, CM 1135, Oxoid, Thermo Fisher Scientific, Hampshire, England) broth solution without ST and kept in a separate facility to avoid cross-contamination. The ST-challenged groups received an oral inoculum with 5-mL of BHI broth solution containing 1×10^8 CFU of ST or 1.5×10^8 CFU of ST. For inoculation, one oral dose of *Salmonella enterica* subsp. enterica serovar Typhimurium, isolated from a farm outbreak, and selected for antibiotic resistance to nalidixic acid (Nal+) was used. The inoculum was prepared from a sample of Salmonella Typhimurium (RLO971/09) and filed at the Laboratory of Ornitopathology of the Department of Veterinary Pathology (FCAV/UNESP, Jaboticabal-SP). The oral inoculum was prepared 48 h before incubation at 37 °C in buffered peptone water and diluted with sterile phosphate-buffered saline to reach 10^8 CFU. The gilts were fasted for six hours and had no water consumption for 1-hour prior to inoculation.

After inoculation, no cleaning routine was adopted in the ST-challenged facility to mimic a commercial housing condition, and to assist in exacerbating the inflammatory response and maintaining the challenge as long as possible. On the other hand, the Basal group facility was cleaned twice a day, and it was pulverized with a bleach solution (1:10) once a week as part of the biosecurity protocol. During the experimental period, the team members were required to wear clean and disinfected clothing, and footwear was cleaned with a bleach solution (1:10) when entering the facility.

2.3 Data and sample collection

Feed provided and feed wastage were collected and weighed daily to determine the average daily feed intake (ADFI). The BW was recorded weekly to estimate the average daily gain (ADG) and feed efficiency (F:G).

The rectal temperature (RT) and the fecal score (FS) were measured daily in all pigs from 0 to 7 days postinoculation (dpi). The RT was recorded using a digital thermometer (Accumed-Glicomed, Rio de Janeiro, Brazil), and the FS was attributed to each pig with the following scoring system: normal consistency feces were given a score of 0, semisolid feces were given a score of 1, and watery

feces were given a score of 2. The sum of feces with scores of 1 and 2 was used for the analysis of the incidence of diarrhea.

Furthermore, at 1, 3, 7, and 14 dpi, fresh fecal samples (10 g) were collected from individual pigs for ST quantification. Fecal samples were serially diluted in buffered peptone solution (1:10) until they reached a final concentration of 10^{-6}. From each dilution, 0.1-mL was plated on brilliant green agar containing Nal+ (25 μg/mL). Plates were incubated at 37 °C for 24 h. In the absence of bacterial growth on agar plates, an equal volume of Rappaport broth (CM0669, Oxoid, Thermo Fisher Scientific, Hampshire, England), prepared at double concentration, was added to conic tubes containing the homogenized sample in PBS (1:10). The sample tubes were incubated at 37 ºC for 24 h and plated again in Nal+ at 37 ºC for 24 h. The number of colonies per gram of sample was transformed into log10 for further analysis.

Blood samples were taken at the jugular vein from all pigs at 0, 7, and 14 dpi. The pigs were fasted for six hours before blood sampling. For each sampling day, one 4-mL EDTA tube per animal was filled for blood cell count (Vacuplast; Cral, São Paulo, Brazil). Blood samples were stored on ice and transported to the laboratory, where the complete blood count was performed using a Microcell counter (Horiba Micros-60; Horiba ABX SAS, Montpellier, France).

2.4 Statistical analysis

Data were tested for normality using the UNIVARIATE procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) and the Shapiro-Wilk test. Studentized residuals were used to identify outliers (> 3 standard deviations from the mean). Data were analyzed using the GLIMMIX procedure of SAS as a randomized complete design. The data collected over time were included in the analysis as a repeated effect, and each pig was considered the experimental unit. Differences between means were determined using the Tukey's post-hoc test, except for the incidence of diarrhea. For this analysis, a chi^2-test was used to compare treatments. The significance level adopted for all analyses was 5% ($p < 0.05$), and a trend toward significance was considered at $p \leq 0.10$.

3. Results

3.1. Salmonella Typhimurium fecal shedding, rectal temperature, and fecal score

Pigs started shedding ST within 1 dpi (10 out of 20 pigs; Figure 1) and persistently shed the bacteria until the end of the first week of the study. The concentrations of Salmonella in the feces ranged from 2 to 6 log10 CFU/g of feces for the 1×10^8 CFU group (6 out of 10 pigs) and from 2 to 4.5 log10 CFU/g feces for the 1.5×10^8 CFU group (4 out of 10 pigs) at 1 dpi. The number of pigs shedding ST dropped markedly until 7 dpi (2 out of 20 pigs), however, pigs maintained higher colony counts, ranging from 4.7 to 6 log10 CFU/g. After 7 dpi, ST dropped in all samples, and it was not detectable on plates at 14 dpi. The Basal group remained negative until the end of the trial.

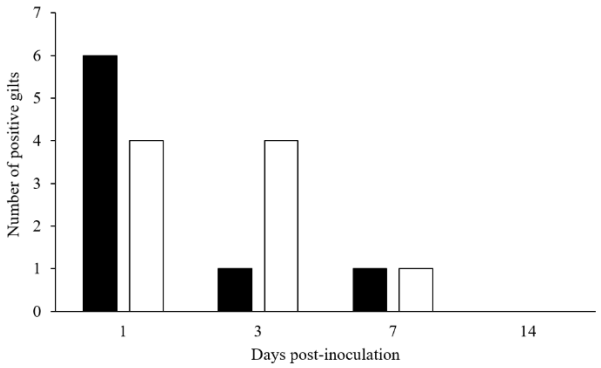

Figure 1. Number of pigs shedding fecal *Salmonella* Typhimurium after 1, 3, 7, and 14 days post-inoculation. (■) Pigs orally challenged with 1×10^8 CFU of *Salmonella* Typhimurium; (□) Pigs orally challenged with 1.5×10^8 CFU of *Salmonella* Typhimurium.

No significant differences were detected in rectal temperature between treatments ($p > 0.10$; Table 1). However, infection with ST caused a febrile response in 35% of the ST-challenged gilts (7 out of 20 pigs; 3 pigs in the 1×10^8 CFU group and 4 pigs in the 1.5×10^8 CFU group), with rectal temperatures above 39.5 ºC.

Table 1. Rectal temperatures (ºC) of growing pigs orally challenged or not with Salmonella Typhimurium.

Days post-inoculation	Inoculation level, CFU			SEM	*P*-value
	0 (Basal)	1×10^8	1.5×10^8		
1	39.1	39.2	39.2	0.05	0.71
2	39.2	39.3	39.2	0.05	0.58
3	38.9	39.0	39.0	0.06	0.17
4	39.2	39.0	39.1	0.05	0.11
5	39.1	38.9	38.9	0.04	0.11
6	39.1	39.1	39.1	0.04	0.99
7	38.9	38.9	39.0	0.05	0.80

SEM, Standard error of the mean.

In Basal pigs, normal fecal consistency (score 0) was observed in 70% (7 out of 10 pigs) of the animals from 1 to 4 dpi and in 80% of the animals from 5 to 7 dpi. Transitory diarrhea was observed in both ST-challenged groups, with semiliquid to liquid feces. Characteristic salmonellosis mild diarrhea (score 1) with the presence of mucus, started at 1 dpi, and a few episodes of severe diarrhea (score 2) ocurred. Only 1 ST-challenged gilt in the 1×10^8 CFU group scored 2 for 2 consecutive days, and 1 pig in the 1.5×10^8 CFU group scored 2 for 1 day in the overall week. At 1 dpi, a higher frequency of diarrhea was observed in the 1.5×10^8 CFU group ($p < 0.01$) than in the 1×10^8 CFU group. However, there was an increase in the percentage of pigs with diarrhea in the 1×10^8 CFU group, with a tendency of a higher number of pigs with diarrhea at 5 dpi compared to the 1.5×10^8 CFU group ($p = 0.08$). At 6 dpi, a tendency toward a reduction in the percentage of pigs with diarrhea was observed in the 1×10^8 CFU group compared to the 1.5×10^8 CFU group ($p = 0.08$; Table 2). After a week of challenge (7 dpi), no difference in the percentage of pigs with diarrhea was observed between groups ($p = 0.74$), however, half of the 1×10^8 CFU pigs and 40% of the 1.5×10^8 CFU pigs still showed mild diarrhea.

Table.2. Incidence of diarrhea[1] after *Salmonella* Typhimurium inoculation.

Days post-challenge	Inoculation level, CFU		*P*-value
	1×10^8	1.5×10^8	
1	20%	50%	<0.01
2	50%	40%	0.74
3	90%	60%	0.13
4	70%	50%	0.32
5	80%	50%	0.08
6	60%	70%	0.08
7	50%	40%	0.74

[1]The percentage of diarrhea was calculated as a proportion of the total number of pigs showing feces scored as 1 and 2 per treatment from 1 to 7 dpi.

3.2. Hematological parameters

There was no significant ($p > 0.10$) treatment effect on the white blood cell (WBC) count at 7 and 14 dpi (Table 3). However, there were two blood parameters that showed significant differences ($p < 0.05$) between treatments. For hemoglobin (HGB), at 7 and 14 dpi, oral ST-challenge increased HGB values in challenged pigs compared to Basal pigs ($p < 0.05$), with no significant difference between the dosages administered ($p > 0.10$). In addition, the hematocrit (HTC) concentrations were affected by the ST-challenge. At 7 dpi, there was a greater HTC concentration in the 1×10^8 CFU group than in the Basal group ($p < 0.05$), and no significant difference between the dosages administered ($p > 0.10$). On the other hand, at 14 dpi, the oral ST-challenge increased HTC values in both the 1×10^8 and 1.5×10^8 CFU groups compared to the Basal group ($p < 0.05$), with the 1.5×10^8 CFU group showing a higher numeric HTC concentration than the 1×10^8 CFU group ($p > 0.10$).

Table 3. Blood parameters in growing pigs orally challenged or not with *Salmonella* Typhimurium.

	Inoculation level, CFU			**SEM**	***P*-value**
Item, mm³	0 (Basal)	1×10^8	1.5×10^8		
		Leucocytes			
7 dpi	18,320	21,080	19,530	1683	0.37
14 dpi	18,510	18,650	18,430	1273	0.73
		Lymphocytes			
7 dpi	8,543	11,788	9,427	1360	0.39
14 dpi	9,658	10,490	10,732	727	0.73
		Monocytes			
7 dpi	1,110	1,345	1,186	133	0.39
14 dpi	1,045	1,115	1,103	78	0.96
		Neutrophils			
7 dpi	7,368	7,507	8,551	760	0.85
14 dpi	5,758	6,535	6,256	565	0.33
		Hematocrit			
7 dpi	35.4^{b}	37.0^{ab}	37.7^{a}	0.64	0.03
14 dpi	35.1^{b}	36.9^{a}	39.3^{a}	0.68	<0.05
		Hemoglobin			
7 dpi	10.5^{b}	11.1^{a}	11.0^{ab}	0.18	0.05
14 dpi	10.5^{b}	11.2^{a}	11.3^{a}	0.20	<0.01

SEM, Standard error of the mean.

[a,b] Within a row, means not sharing the same superscript letter differ, P<0.05.

3.2. Growth performance

All pigs were healthy and had similar growth performance in the preinoculation period (data not shown). In the first week postinoculation, there was no effect ($p > 0.10$) of ST challenge on BW and ADFI (Table 4). However, a steep reduction in ADG (-17 and -22%; $p < 0.01$) and G:F (-14 and -20%; $p < 0.01$) was observed in the first week postinoculation in both ST-challenged groups compared to the Basal pigs, with no significant difference between the ST dosages ($p > 0.10$). However, for the experimental period (0 to 14 dpi), no significant differences were observed in BW, ADFI, ADG, and G:F between treatments ($p > 0.10$).

Table.4. Growth performance of growing pigs orally challenged or not with *Salmonella* Typhimurium.

	Inoculation level, CFU			**SEM**	***P-value***
Item	0 (Basal)	1×10^8	1.5×10^8		
BW, 0 dpi	27.25	27.23	27.42	1.66	0.99
BW, 7 dpi	32.95	31.94	31.90	1.80	0.89
BW, 14 dpi	38.10	37.79	37.74	2.04	0.99
0 to 7 dpi					
ADG, kg	0.81[a]	0.67[b]	0.64[b]	0.03	<0.01
ADFI, kg	1.55	1.52	1.54	0.07	0.95
G:F, kg/kg	0.52[a]	0.45[b]	0.42[b]	0.02	<0.01
0 to 14 dpi					
ADG, kg	0.84	0.75	0.74	0.04	0.26
ADFI, kg	1.66	1.63	1.68	0.07	0.86
G:F, kg/kg	0.46	0.47	0.44	0.01	0.22

SEM, Standard error of the mean
[a,b] Within a row, means not sharing the same superscript letter differ, P<0.05.

4. Discussion

The administration of 10^8 CFU was used to more closely mimic the fecal-oral transmission of Salmonella organisms often found in intensive swine production conditions. These findings suggest that both inoculum concentrations used could produce a moderate immune response in pigs.

Studies performing ST challenge on weaning pigs often report the occurrence of clinical symptoms such as fever and diarrhea [14, 16, 17]. However, in this study the ST inoculation doses evaluated did not increase rectal temperature. These doses may have been insufficient to overwhelm intestinal and mesenteric lymph node immunity, and thus, system migration and acute systemic inflammation may not have occurred. This would also explain the lack of an effect on the white blood cell count observed in the current study. The low dosage of oral *Salmonella* Typhimurium used might have resulted in lower colonization of the mesenteric lymph nodes, spleen, and liver, which resulted in a mild and self-limiting immune response in the gut epithelium [18]. As a result, lower immune cell transmigration into the intestinal lumen might have occurred to avoid epithelial barrier disruption and pathogen translocation. The same results were reported by Davis et al. [19] in younger pigs inoculated with 10^8 CFU of ST in a 14-day trial.

Although no differences were observed in WBC counts, blood parameters showed significant differences between treatments. HTC and HGB were greater in the 1.5×10^8 CFU group than in the Basal and 1×10^8 CFU groups. Increases in HTC and HGB concentrations could indicate the level of dehydration, since both indices are based on whole blood and are dependent on plasma volume. As challenged pigs showed an increased incidence of diarrhea after ST inoculation, the augmentation in

HTC and HGB concentrations can be associated with fecal water losses. The presence of diarrhea is a characteristic sign of salmonellosis in pigs. This occurs due to the ability of *Salmonella enterica* serovar Typhimurium to induce the production of proinflammatory mediators in the gut barrier, altering chloride channel function and disrupting tight junctions, resulting in gut leakage and diarrhea [20].

Furthermore, higher HTC and HGB concentrations have been described as indicators of decreased performance [21] since both are related to impaired metabolism over the course of diarrhea observed in challenged pigs. Under conditions of water restriction, growth may be impaired. First, the digestive processes of organic macronutrients (carbohydrates, proteins, and lipids) may have been compromised by the lower activity of hydrolase enzymes [22]. In addition, reduced water content is reported to inhibit the incorporation of amino acids into proteins [23], causing a decrease in protein accretion in the tissues. In this way, the increased HTC and HGB values observed at 7 and 14 dpi may partially explain the reduced growth performance observed after 7 dpi.

Additionally, regarding growth performance, the gastroenteritis disorder caused by ST may have reduced nutrient digestion and absorption [24], lowering nutrient availability for growth. Thus, gut barrier disruption may explain the ST impact on growth performance observed in the first week. In addition, the absence of ADFI reduction in both ST groups observed in this study confirms that ST affects pigs' performance indirectly via nutrient utilization efficiency rather than via a reduction in voluntary feed intake. Meanwhile, 1.5 × 108 CFU pigs had a more pronounced reduction in ADG and G:F when compared to the Basal group, which can be related to the higher dose concentration.

However, a negative effect on performance from 0 to 14 dpi was observed in either ST-challenged group. The lack of effect on growth parameters might be associated with the inoculating concentration used in the trial where only 10% of the pigs (2 out of 20 pigs) were shedding ST at 7 dpi, and all inoculated pigs were negative at 14 dpi. Moreover, unlike field conditions, in this trial, pigs were housed individually in pens with fully slatted floors. This housing system prevented challenged pigs from re-infected by the fecal-oral cycle, especially long-term carrier pigs that can, continuously or intermittently, shed ST in feces as a way of environmental contamination [25].

Taken together, the results demonstrate that both concentrations of the oral ST challenge model were able to affect pig performance and blood parameters. However, the oral dosage of 1.5 × 108 CFU of Salmonella Typhimurium is a more suitable dosage to induce pigs immune system activation and impair growth performance.

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CHAPTER 4 – Increased dietary Trp, Thr, and Met supplementation improves growth performance and protein deposition of salmonella-challenged growing pigs under poor housing conditions

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Lay summary

Immune system activation alters pigs' physiology and metabolism, increasing maintenance requirements and reducing voluntary feed intake and weight gain. Dietary functional amino acid supplementation (tryptophan, threonine, and methionine) is a strategy to support the immune system activation for immune components production, maintenance of the gut barrier integrity, and reduction of the oxidative status. Additionally, amino acid supplementation may mitigate growth performance losses. In this context, this study was conducted to investigate the effect of diets with or without tryptophan, threonine, and methionine supplementation on the performance and immune system activation of growing pigs under a sanitary challenge. The amino acid supplementation mitigated the immune system activation of challenged growing pigs, and improved growth performance when compared to pigs fed diets with no supplementation. The functional amino acid supplementation may be an efficient nutritional strategy to optimize health and growth performance of immune challenged pigs.

Teaser text

The impaired growth performance and protein deposition of pigs under sanitary challenge can be attenuated by dietary tryptophan, threonine and methionine supplementation. The amino acid supplementation mitigates immune system activation and improves the efficiency of nitrogen utilization, increasing pigs’ resilience with no in-feed antibiotics in a group-housed system.

Abstract: Highly intensified rearing conditions and precarious sanitary management predispose pigs to immune system activation, altered amino acid (AA) metabolism, and decreased growth performance. Thus, the main objective of this study was to evaluate the

effects of increased dietary tryptophan (Trp), threonine (Thr), and methionine + cysteine (Met + Cys) supplementation on performance, body composition, metabolism, and immune responses of group-housed growing pigs under challenging sanitary conditions. A hundred and twenty pigs (25.4 ± 3.7 kg) were randomly assigned to a 2 × 2 factorial arrangement, consisting of two sanitary conditions (SC, good (GOOD) or salmonella-challenge and poor housing condition (ST + POOR)) and two diets (D, control (CN) or supplemented with AA (Trp, Thr, and Met + Cys:Lys ratios 20 % higher than those of the CN diet (AA+)). Pigs were followed during the growing phase (25 to 50 kg) and the trial lasted 28 days. The ST + POOR SC pigs were challenged with *Salmonella* Typhimurium and raised in a poor housing condition. The ST + POOR SC increased rectal temperature, fecal score, serum haptoglobin, and urea concentration ($P < 0.05$) and decreased serum albumin concentration ($P < 0.05$) compared with GOOD SC. Body weight, average daily feed intake, average daily gain (ADG), feed efficiency (G:F), and protein deposition (PD) were greater in GOOD SC than in ST + POOR SC ($P < 0.01$). However, pigs housed in ST + POOR SC fed with AA+ diet had lower body temperature ($P < 0.05$), increased ADG ($P < 0.05$) and nitrogen efficiency ($P < 0.05$), and a tendency for improved PD and G:F ($P < 0.10$) compared with CN diet fed pigs. Regardless of the SC, pigs fed AA+ diet had lower serum albumin ($P < 0.05$) and tended to decrease serum urea levels ($P < 0.10$) compared with CN diet. The results of this study suggest that the ratio of Trp, Thr, and Met + Cys to Lys for pigs are modified by sanitary conditions. Furthermore, supplementation of diets with a blend of Trp, Thr, and Met + Cys AA improves performance, especially under salmonella-challenge and poor housing conditions. Dietary Trp, Thr, and Met supplementation can modulate immune status and influence resilience to sanitary challenges.

Keywords: amino acids, body composition, immune system, inflammation, swine

Abbreviations

AA, amino acids

AA+, supplemented diet

ADFI, average daily feed intake

ADG, average daily gain

BL, body lipid

BP, body protein

BW, body weight

CFU, colony forming units

CN, control diet

D, diet

DXA, Dual-energy X-ray absorptiometry

G:F, gain:feed ratio

GOOD, good sanitary condition

LD, lipid deposition

Met + Cys, methionine + cystine

PD, protein deposition

ST + POOR, *Salmonella* Typhimurium challenge and poor housing condition

SC, sanitary condition

SID, standardized ileal digestive

ST, *Salmonella* Typhimurium

Thr, threonine

Trp, tryptophan

1. Introduction

In commercial pig production, growing pigs often face non-ideal housing conditions (e.g., increased animal density, batch mixing, and poor hygiene management) which facilitates disease transmission through increased microbial pressure in the environment (Campos et al., 2017) and affects pig health, and productivity (Jayaraman and Niachoti, 2017). The degradation of housing hygiene is known to induce chronic immune system overstimulation (Le Floc'h et al., 2014). For instance, pigs reared in conventional housing systems with high microbial loads grow 10 to 20% more slowly than pigs kept in 'clean' environments (Lee et al. 2005; Renaudeau 2009).

During exposure to a sanitary challenge, pigs respond with a cascade of metabolic alterations, including anorexia, increased breakdown and decreased synthesis of skeletal muscle proteins, and increased nutrient utilization for immune system functioning, such as hepatic synthesis of acute phase proteins and immune cells proliferation (Le Floc'h et al., 2004; Campos et al., 2014). Hence, growth and tissue accretion are reduced (Campos et al., 2019a), and amino acid (AA) requirements may change during a chronic immune system activation (van der Peet-Schwering et al., 2019). This raises the question of whether the current AA recommendations for growing-finishing pigs estimated to maximize the growth performance of healthy pigs (NRC, 2012) would be adequate for pigs under a sanitary challenge.

Some AA have been adopted as a nutritional strategy to attenuate the effects of sanitary challenges in pigs growth performance and health. These AA are called functional AA due to their functions beyond nutritive utilization (meeting growth requirements) during immune system activation, benefiting gastrointestinal integrity (Chalvon-Demersay et al.,

2021), and enhancing immune responses (Rodrigues et al., 2021). For example, in growing pigs with chronic immune system activation, increased dietary supplementation of tryptophan (Trp), threonine (Thr), and methionine + cysteine (Met + Cys) has improved protein deposition (van deer Meer et. al., 2016) and body weight gain (Rodrigues et al., 2021a). Furthermore, higher body nitrogen retention and feed efficiency have been observed with Trp supplementation for pigs under lipopolysaccharide challenge (de Ridder et al., 2012). In addition, sulfur AA (Met + Cys) supplementation increased protein deposition rate (+ 14 %) in lipopolysaccharide-challenged pigs (Kim et al., 2012). Besides, higher levels of dietary Thr resulted in a linear increase in body protein deposition (+ 32 %) and average daily gain (+ 12 %) in immune system-stimulated pigs (McGilvray et al., 2019; Wellington et al., 2019).

Despite growing evidence that some AA requirements are affected by immune system activation, most results were obtained for post-weaning piglets housed in small groups supplemented with Trp, Thr, or Met alone for a short period of time (one to two weeks of challenge). Additionally, in these studies, pigs were repeatedly challenged with lipopolysaccharide (McGilvray et al., 2019; Kim et al., 2012) or Escherichia coli inoculation (Kahindi et al., 2018; Capozzalo et al., 2017a,b), which may lead to severe clinical responses and mortality (high dose) or immune tolerance over time (low doses). Thus, it can be questioned to what extent these results can be extrapolated to a commercial condition where pigs are constantly exposed to multiple antigens without severe clinical signs of disease or mortality (van der Meer et al., 2016), and which may require at the same time the supplementation of more than one of these AA.

Moreover, there is limited information about immune system overstimulation on AA requirements of growing pigs, especially for pigs housed in large groups and exposed to different challenges during a long period of time. Therefore, this study was performed to

evaluate the effects of dietary Trp, Thr, and Met + Cys supplementation on growth and immune system activation of growing pigs challenged with *Salmonella* Typhimurium and group-housed under poor housing condition. It was hypothesized that increased dietary supplementation of Thr, Trp, and Met + Cys above requirement improves performance and reduces inflammation in growing pigs challenged with *Salmonella* Typhimurium and group-housed under poor housing condition.

2. Materials and Methods

2.1. Animals, housing, and management

All experimental procedures applied in this trial followed the Brazilian National Council of the Control of Animal Experimentation (CONCEA) and were reviewed and approved [protocol no. 4784/20] by the Ethical Committee on Animal Use (CEUA) of Faculdade de Ciências Agrárias e Veterinárias (FCAV/UNESP – Jaboticabal, SP, Brazil).

One hundred and twenty female pigs [Pietrain × (Large White × Landrace)] with an initial body weight (BW) of 25.4 ± 3.7 kg were used in a 28-d trial. Pigs were housed in the facilities of the Swine Research Laboratory at São Paulo State University (Unesp; Jaboticabal, SP, Brazil). Animals were identified and assigned to one of two similar environmentally controlled growing-finishing rooms (0.9 m^2/pig) with full concrete floors. Both rooms were cleaned and disinfected before the animals' allocation. Fourteen days before the study started, rectal swabs were individually collected to screen *Salmonella spp*. presence and only negative pigs were included in the trial.

In each room, the ambient temperature was controlled through an automated evaporative pad cooling system (Big Dutchman, Araraquara, SP, Brazil) and exhaust fans. The temperature was set at 22 ºC and recorded every 30 min using two data loggers (Hobo, Onset Computer Corporation, Bourne, MA, United States) located in the middle of the pen.

Artificial lights were used to maintain a 12 h photoperiod (0700 – 1900 h). Additionally, each room was equipped with four Automatic and Intelligent Precision Feeders (University of Lleida, Lleida, Spain) and six ball bite drinkers to allow ad libitum access to feed and water, respectively. Pigs had an individual radio frequency ear tag (Allflex, Joinville, SC, Brazil) attached in the right ear to access the feeders.

Each feeder consisted of a single space feeder that delivers volumetric amounts of up to four diets stored in independent feed containers located on the top of the feeder. The feeder identified the pig as its head entered the feeder and then provided the assigned experimental diet (see experimental diets sub-section) in response to each animal request. This allowed the pigs to be housed in the same pen, and any pig could access any of the feeders and receive the prescribed diet according to its experimental group (Pomar et al., 2011).

At d 0, pigs were blocked by BW and were randomly assigned to one of four experimental groups in a 2 × 2 factorial arrangement. Pigs were allocated in two sanitary conditions (SC): good (GOOD) or challenged with *Salmonella* Typhimurium and raised under poor housing condition (ST + POOR); and were fed two diets (D): control diet (CN), with the AA profile according to NRC (2012) or supplemented diet (AA+), with the AA profile containing 20 % higher standardized ileal digestible (SID) Trp:Lys, Thr:Lys, and Met + Cys:Lys ratios than the CN. Each room represented a SC. Thus, the study was composed of four experimental groups with 30 pigs each.

2.2. Sanitary challenge

A sanitary challenge was used to induce an immune response in pigs under the ST + POOR SC. At d 0, all the 60 pigs in the ST + POOR SC room were inoculated via oral gavage with 5 mL of brain heart infusion (CM 1135, Oxoid, Thermo Fisher Scientific, NH, England) broth solution with 2×10^9 colony forming units (CFU) of *Salmonella enterica* subsp.

enterica serovar Typhimurium (ST) selected for antibiotic resistance to nalidixic acid (25 μg/mL). The inoculum was prepared according to the recommendations of Wood et al. (1991) and Oliveira et al. (2010) from an original strain of *Salmonella* Typhimurium (RLO971/09) filed at the Laboratory of Ornitopathology, Department of Veterinary Pathology, Unesp, Jaboticabal, SP, Brazil. The oral inoculum was prepared in brain heart solution cultured in stationary conditions overnight at 37 ºC, and adjusted by spectrophotometry (OD600) to reach the desired concentration of 2×10^9 CFU. After this procedure 1ml of the inoculum was added to 4ml of brain heart infusion broth, to increase the inoculant volume for a better oral gavage procedure.

In both SC, the gilts were fasted for six hours and had no water consumption for one hour prior to inoculation. After the ST inoculation, fresh manure from a commercial pig farm was spread on the ST + POOR SC pen floor Besides, during the 28-d trial, the ST + POOR SC was not cleaned, and no hygiene protocol was applied as described by Li et al. (2017). The objective of combining ST inoculation, manure spreading, and no hygiene protocol in the ST + POOR SC was to enhance and recycle the pathogenic pressure in the barn, which may prolong the challenge through a chronic immune system activation of pigs and keep the deterioration in housing hygiene throughout the 28-d trial.

Otherwise, in the GOOD SC, pigs received a placebo inoculum via oral gavage with 5 mL of brain heart infusion broth solution without ST, and manure was not spread on the floor. The GOOD SC room was cleaned twice a day with a water jet stream, and potassium monopersulfate (1:200; Virkon; Lanxess, Colony, Germany) was diluted and applied in the facility once a week as part of the biosecurity protocol to improve the hygiene condition. The team members were also required to wear clean clothing and clean footwear with a bleach solution (1:10) when entering the building. Regardless of SC, pigs did not receive any

medication or preventive treatment.

2.3. Experimental diets

Experimental corn-soybean meal-based diets were formulated using the reported nutrient content and analyzed AA content of ingredients to meet or exceed the nutrient requirements for 25 to 50 kg gilts according to NRC (2012) and AMINODat 6.0 (Tables 1 and 2, respectively). In the CN diet, AA profile met the SID AA requirements according to NRC (2012), while in the AA+ diet, AA profile contained 20 % higher SID Trp, Thr, and Met + Cys to Lys ratios. No in-feed antibiotics as growth promoters were used before or throughout the trial. The diets were steam pelleted (2.5 mm) and provided ad libitum through the feeders.

2.4. Data collection

All animals were used for growth performance and fecal score evaluation and fecal ST quantification (n = 30 pigs/experimental group). Additionally, a group of 80 pigs (n = 20 pigs/experimental group) with the closest BW to the average of its experimental group were selected for the evaluation of body composition, nitrogen balance, rectal temperature, and blood sampling.

2.4.1. Growth performance, body composition and nitrogen balance

Individual BW was recorded weekly, and individual daily feed intake (ADFI, kg/d) was measured by the feeders system. Average daily gain (ADG, kg/d) and ADFI were used to calculate feed efficiency (G:F, kg/kg). Total body lean, and fat mass were measured on d -1 (pre-challenge) and at 28-days post challenge (dpc) by dual-energy X-ray absorptiometry (DXA; GE 205 Lunar Prodigy Advance; GE Healthcare, Madison, WI, United States). Animals were fasted for six hours before being sedated by intramuscular injection of xylazine (1.5 mg/kg) and ketamine (15 mg/kg). Pigs were scanned in the prone position using the total

body scanning mode in the manufacturer-provided software (Lunar enCORE software, version 8.10.027; GE Healthcare).

The DXA body lean and fat mass values were converted to its protein and lipid chemical equivalent, as proposed by Pomar and Rivest (1996). Total body protein (BP) and lipid content (BL) from each scanned pig were calculated as the difference between the respective body constituents estimated from DXA readings at the beginning and end of the experimental period. Afterwards, average daily protein (PD) and lipid deposition (LD) were calculated by dividing the corresponding variables by the duration of experimental period (28-d).

Nitrogen excretion (g/d) was obtained for each scanned pig by subtracting the nitrogen retention (PD divided by 6.25) from the nitrogen intake (estimated by [CP in the diet multiplied by the DFI] divided by 6.25). Nitrogen efficiency (%) was calculated by dividing the nitrogen retention by the nitrogen intake.

2.4.2. Rectal temperature, fecal score and fecal Salmonella Typhimurium shedding

For the *Salmonella* spp. presence before the beginning of the trial, the rectal swabs were serially diluted in phosphate-buffered saline (1:10) until they reached the final concentration of 10^{-6}. From each dilution, 0.1 mL was plated on the brilliant green agar (CM0263, Oxoid, Basingstoke, NH, England), and incubated at 37 ºC for 24 h.

The rectal temperature was measured from 0 to 7-dpc with a digital thermometer (Accumed-Glicomed, RJ, Brazil) in both SC. In addition, the fecal score was performed individually in fecal samples collected from ST + POOR SC pigs at 5, 7, 14, and 21-dpc. Normal consistency feces received a score 0, semisolid feces a score 1, semisolid-watery feces score of 2, and watery feces given a score of 3. Furthermore, at 2, 5, 7, 14, 21, and 28-dpc, fresh fecal samples (10 g) were collected by rectal stimulation from individual pigs to

evaluate fecal ST shedding. Fecal samples were serially diluted in phosphate-buffered saline (1:10) until they reached final concentration of 10^{-6}. From each dilution, 0.1 mL was plated on brilliant green agar containing nalidixic acid. Plates were incubated at 37 °C for 24 h. The number of CFU per gram of fecal sample was transformed into log10 for further analysis. In the absence of ST growth, an equal volume of Rappaport-Vassiliadis broth double concentrated (CM0669, Oxoid, Basingstoke, NH, England) was added to tubes containing the respective homogenized sample in phosphate-buffered saline for ST enrichment. The samples were incubated at 37 ºC for 24 h and plated in green brilliant agar containing nalidixic acid, and evaluated for the presence or absence of ST. In the case of positivity after enrichment, the sample was considered with a bacterial load of 2 log10, for calculation purposes.

2.4.3. Blood sampling and analysis

Blood samples were collected from pigs at 7 and 28-dpc from the jugular vein after six hours of fasting. The pigs were contained with snout rope and the sampling procedure was performed in less than two minutes to avoid pain and interference on blood parameters. Per sampling day, two 8-mL serum tubes (BD Vacutainer; NJ, EUA) were collected per animal for haptoglobin, albumin, total protein, urea, and creatinine concentrations. Blood samples in serum tubes were allowed to clot for one hour, after which serum was collected after centrifugation for 10 minutes at 3000 × g at 4 ºC (Novatecnica, NT 835, Piracicaba, SP, Brazil) and stored at −80 °C for further analysis. Serum levels of haptoglobin and albumin were accessed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The molecular weight and protein fraction concentrations were determined by computer densitometry (Shimadzu 9301 PC, Shimadzu Corp, Kyoto, Japan) using a simple scanner. Biomarkers were used for protein identification (Sigma Marker, Sigma-Aldrich Biotechnology LP, Germany). For the densitometric evaluation of protein bands, reference

curves were made from the reading of the standard marker. Afterwards, serum haptoglobin and albumin concentrations were corrected by total serum total protein analysis. Serum concentrations of total protein, urea, and creatinine were determined by the biuret method with commercial reagents (Labtest; Labtest Diagnostica SA, Lagoa Santa, MG, Brazil) and performed by semi-automatic spectrophotometry (Labmax Plenno, Labtest Diagnostica SA; Lagoa Santa, MG, Brazil).

2.5. Statistical analyses

Data were tested for normality using the UNIVARIATE procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) and the Shapiro-Wilk test, and the studentized residual was used to identify outliers (> 3 standard deviations from the mean). Data were analyzed using the GLIMMIX procedure of SAS and presented as least-squares means. Experimental SC, D, and their interactions (SC × D) were included as fixed effects, while the blocks of BW were included as a random variable effect in the statistical model. Additionally, the initial BW was used as a covariate for body composition analysis. The data collected over time were included in the analysis as a repeated measure in time, and each pig was considered as an experimental unit. As two different rooms were used to allocate each condition (GOOD and ST + POOR SC), it might result in pseudo-replicates. However, housing animals in two different barns was established to avoid cross-contamination between sanitary conditions and have different confounding factors. Differences between means were determined using the Tukey *post hoc* test, except to analyze the percentage of positive pigs for fecal ST shedding and fecal scores. For the percentage of positive pigs for fecal ST shedding, a chi-square test was used to compare days and diets. The Cochran-Mantel-Haenszel test was used to assess fecal score changes over time and D effect on fecal score severity at each day of measurement. Additionally, an ordinal logistic analysis was performed according the Proportional Odds

Model with the LOGISTIC procedure of SAS to assess the D effect on fecal score severity. The categorical indicator (fecal score) was assigned as the response, and day and D were assigned as the categorical predictors. The significance level adopted for all analysis was 5 % ($P \leq 0.05$), and a trend towards significance was considered at $0.05 < P \leq 0.10$

3. Results

Before the beginning of the trial (d -14 to d 0), there was no effect of the rooms on the pigs' growth. Pigs housed in both facilities (representing GOOD and ST + POOR SC) had similar BW (25.3 ± 0.21 kg vs 25.5 ± 0.21 kg, respectively) and ADFI (0.96 ± 0.02 kg vs 0.98 ± 0.02 kg, respectively), which allowed comparisons between SC during the experimental period. Throughout the experimental period, four animals were removed. In the GOOD SC, one pig died, and another pig showed dyspnea, which was treated with tulathromycin. In the ST + POOR SC (after the sanitary challenge), two pigs presented fever, excessive weight loss, and no voluntary feed intake for three consecutive days. They were treated with enrofloxacin to respect animal welfare principles and removed from the experiment. In addition, ST + POOR SC pigs were lethargic and showed reduced rates of feed intake after the challenge; however, signs of recovery (increased voluntary feed intake, and no visible lethargy) were observed after 5-dpc.

3.1. Rectal temperature and fecal score

After 24 hours of challenge, rectal temperature increased in ST + POOR SC compared to GOOD SC ($P < 0.05$; Fig.1a). The rectal temperature in pigs of the ST + POOR SC reached its maximum (39.6 ºC) at 3-dpc. From 4 to 6-dpc, rectal temperature of ST + POOR SC pigs decreased, remaining higher than pigs of the GOOD SC ($P < 0.05$). At 7- dpc, rectal temperature of pigs in ST + POOR SC was similar to pigs in GOOD SC and to the pre-challenged period ($P > 0.10$). Regarding AA supplementation, during 7-dpc, there was a diet

effect for rectal temperature ($P < 0.05$). Pigs fed CN diet had higher rectal temperature than the ones fed AA+ at 3-dpc (39.7 ± 0.12 ºC vs. 39.4 ºC ± 0.12) and 4-dpc (39.7 ± 0.11 ºC vs. 39.5 ± 0.11 ºC). However, no significant differences were observed afterwards (Fig. 1b).

The ST + POOR SC negatively affected the fecal score and it resulted in transitory diarrhea, with higher mean fecal score at 5-dpc when compared to 7, 14 and 21-dpc ($P < 0.05$; Fig. 2a). Afterwards, a decrease in the mean fecal score was observed with the lowest mean fecal score at 21-dpc (Fig. 2a). Regarding dietary treatments, there was a difference in fecal score between diets only at 14-dpc, with higher mean fecal in pigs fed CN compared to AA+ diet ($P < 0.05$; Fig. 2b). Additionally, at 14-dpc, pigs fed AA+ diet had a higher probability (three times) to show a less severe score compared to pigs fed CN diet ($P < 0.05$; Table 3).

3.2. Fecal Salmonella Typhimurium shedding

Throughout the trial, pigs raised in the GOOD SC remained negative for ST shedding, having no cross-contamination between rooms. In ST + POOR SC, ST colony detection persisted until the end of the trial (24,4 %; 14 out of 58 pigs), however the percentage of positive pigs and fecal ST shedding continuously reduced from 2 to 28-dpc, regardless of the dietary treatment ($P < 0.05$; Fig. 3a). From 2 to 14-dpc, there was no difference between dietary treatment on the percentage of positive pigs and fecal ST shedding ($P > 0.10$). Meanwhile, there was a significant decrease in the percentage of positive pigs for ST shedding at 21 and 28-days (22 and 24 %, respectively), with a higher percentage of positive pigs when fed CN (31 %; 14 out of 28 pigs) compared to AA+ diet (14 %; 4 out of 28 pigs; $P < 0.05$). The same pattern was observed for the ST shedding. Pigs fed CN diet kept higher ST colony counts (from 2 to 4.3 log CFU/g) than pigs fed with AA+ at 21 and 28-dpc (from 0.5 to 4 log CFU/g; $P < 0.05$; Fig. 3b).

3.3. Blood parameters

No interactions between SC × D were observed for any blood parameter ($P > 0.05$; Table 4) at 7 and 28-dpc. However, at 7-dpc, serum total protein, and urea concentrations were affected by SC, with pigs in the ST + POOR SC presenting higher urea levels, and lower total protein concentrations than GOOD SC pigs ($P < 0.05$). Additionally, at 7 and 28-dpc, the ST+POOR SC pigs had increased haptoglobin and decreased albumin serum concentrations compared to pigs housed in GOOD SC ($P < 0.05$). No D effect ($P > 0.10$) was observed for haptoglobin or total protein serum concentrations at 7 and 28-dpc. Meanwhile, serum albumin and urea were modified by D at 7 and 28-dpc. Pigs fed AA+ diet had higher ($P < 0.05$) serum albumin concentration, and tended to reduce urea levels ($P < 0.10$) than those fed the CN diet. No SC or D effect was observed on serum creatinine concentration at 7 and 28-dpc ($P > 0.10$).

3.4. Growth performance and body composition

In the first week post challenge, ST + POOR SC pigs had lower ($P < 0.05$) performance than those in GOOD SC (Table 5). Pigs in the ST + POOR SC had a reduction of 22 %, 37 %, 28 %, and 4 % on ADFI, ADG, G:F, and BW, respectively, compared to pigs in GOOD SC ($P < 0.05$). There were no D effects on growth performance from 0 to 7-dpc ($P > 0.10$).

From 0 to 14-dpc, there was a tendency for interaction between SC × D for ADFI and ADG ($P < 0.10$), and a SC effect on BW ($P < 0.05$). Pigs housed in GOOD SC fed with CN diet had higher consumption ($P < 0.10$) than pigs in ST+POOR SC, regardless dietary treatment. On the other hand, ST + POOR SC pigs fed AA+ diet had greater ADG (+ 24 %) than those fed CN diet ($P < 0.10$), whereas GOOD SC pigs had similar ADG irrespective of diet ($P > 0.10$). At the same time, ST + POOR SC pigs had a lower BW (- 6 %) compared to GOOD SC ($P < 0.05$).

Interactions between SC × D for ADFI and ADG ($P < 0.05$), and trends for BW and G:F ($P < 0.10$) were observed until the third-week post-challenge (0 to 21-dpc). Pigs housed in GOOD SC fed CN diet had greater ADFI ($P < 0.05$) than ST + POOR SC fed CN or AA+ diet. Nevertheless, ST + POOR SC pigs fed AA+ diet had a greater ADG (+ 22 %), G:F (+ 19 %) and higher BW (+ 7 %) compared to pigs fed CN diet.

Considering the entire experimental period (0 to 28-dpc), interaction between SC × D was observed for ADG ($P < 0.05$), and a trend for BW, ADFI, and G:F ($P < 0.10$). Pigs housed under ST + POOR SC fed AA+ diet had higher ADG (+ 17 %) and a trend for higher final BW (+ 7 %) compared to those fed CN diet. Pigs housed in GOOD SC fed CN diet had higher ADFI ($P < 0.05$) than pigs in ST + POOR SC. In the meantime, pigs fed AA+ diet showed improved G:F compared to the CN diet in both conditions ($P < 0.10$). However, pigs under ST + POOR SC had a greater difference in G:F compared to GOOD SC pigs due to AA supplementation (+ 14 vs. + 5 %, respectively).

Regarding body composition, a tendency of SC × D interaction ($P < 0.10$) was noticed (Table 5) for BP and PD. At 28-dpc, AA+ affected BP content ($P < 0.10$) of pigs under a sanitary challenge. Under ST + POOR SC, pigs fed AA+ diet had higher BP content compared to pigs fed CN diet (+ 7 %) and were similar to pigs housed in GOOD SC. Besides, for the overall growing period (0 to 28-dpc), ST + POOR SC pigs fed AA+ had higher PD (+ 17 %) compared to ST + POOR SC pigs fed CN diet (+ 7 %) and similar PD to GOOD SC pigs ($P < 0.10$). At the same time, no SC × D interaction ($P > 0.05$) for BL content was observed. There was a significant effect of SC ($P < 0.05$), with GOOD pigs showing higher BL mass (+ 9 %) and LD (+ 25 %) compared to ST + POOR SC pigs.

For nitrogen balance, no SC × D interaction was observed for nitrogen intake ($P > 0.10$) (Table 5); however, a SC effect was observed ($P < 0.05$). Pigs raised under ST + POOR

SC presented a reduced nitrogen intake compared to GOOD SC. Nevertheless, there was a SC × D interaction ($P < 0.05$) for nitrogen efficiency. The ST + POOR SC pigs fed AA+ diet had greater nitrogen efficiency than ST + POOR pigs fed CN (+ 9 %) and it was similar to GOOD SC pigs. Meanwhile, no differences between experimental groups were found for nitrogen excretion ($P > 0.10$).

4. Discussion

This study aimed to evaluate whether increasing dietary Trp, Thr, and Met to Lys ratio would attenuate the negative impacts of sanitary challenge (induced by *Salmonella* Typhimurium challenge and poor housing condition) on growth performance and mitigate the chronic immune system activation of group-housed pigs. Our major and original finding is that increasing dietary Trp, Thr, and Met + Cys to Lys ratio improves protein deposition and nitrogen efficiency of group-housed growing pigs under a sanitary challenge.

These results go in line with the hypothesis that enteric challenges modify animal metabolism and limit the growth potential expression of pigs by inducing an immune response. Under such conditions, functional AA are good candidates for feeding adjustments because they can regulate key metabolic pathways to improve animals' health, and growth (Wu, 2007). Accordingly, Trp, Thr, and Met have been applied as functional AA due to their key roles in modulating immune responses (Wu, 2007). Hence, increased requirements of Trp (Le Floch'h et al., 2008), Thr (Ren et al., 2014), and Met (Kim et al., 2012) have been observed in pigs under sanitary challenges. Recent findings have shown that dietary supplementation of Trp, Thr, and Met can mitigate the negative effects of chronic system activation on growth performance (van der Meer et al., 2016; Rodrigues et al., 2021a). However, long-term studies are scarce, and there is limited information about the impact of immune system overstimulation and AA dietary supplementation on body protein deposition

(Litvak et al., 2013), especially when pigs are housed in large groups.

In this context, threonine, an important AA for intestinal mucosal integrity (Ruth and Field, 2013), may benefits pigs by reducing the gut inflammatory response (Trevisi et al., 2009) through mucus synthesis, and bacteria binding to the mucosal surface. Methionine may contribute to antioxidant capacity, as a substrate to glutathione synthesis (Sierzant et al., 2019), to neutralize the reactive oxygen species produced by some immune cells to exert their cytotoxic functions during an immune response. Tryptophan may also attenuate gut inflammation (Rodrigues et al., 2021a) and modulate gut microbiota through indole components, which have an anti-inflammatory function and bacteriostatic properties on gram-negative enterobacteria, especially against *Salmonella* and *Shigella* genus (Gao et al., 2018).

4.1. Response of pigs to Salmonella Typhimurium challenge and poor housing condition

The presence of feces with watery consistency observed in ST + POOR SC pigs demonstrates that the degradation of sanitary conditions affects gut integrity. Pathogens' attachment and colonization of the gut mucosa stimulate the innate immune system and increase cell permeability, which results in leaky gut and diarrhea (Campbell et al., 2013; Wang et al., 2015). In addition, the febrile response in ST + POOR SC pigs points out that the sanitary challenge applied was sufficient to overwhelm intestinal immunity resulting in a systemic inflammatory response. When an infection occurs, a series of innate immune cells are activated (e.g., monocytes and macrophages), triggering the synthesis of pro-inflammatory cytokines (e.g., IL-1β and TNF-α) (Netea et al., 2000), which are translated to the brain where fever and sickness behavior (e.g., feed intake reduction) are elicited. Thus, lower ADFI and higher body temperature in ST + POOR SC pigs observed in the first week could have been a mechanism to reduce the pathogenicity caused by infections.

Different from fever, acute phase proteins remain for a longer period in the

bloodstream during infections (Asai et al., 1999), and their decrease is correlated with the resolution of the inflammatory response (Eckersall, 2000). Serum haptoglobin and albumin are positive and negative acute-phase proteins, respectively, and have been directly associated with pigs' health status (van der Meer et al., 2016; Kampman-van de Hoek et al., 2016). Indeed, higher haptoglobin and reduced albumin serum concentrations observed in ST + POOR SC pigs until 28-dpc, confirm an immune system overstimulation throughout the experimental period. Collectively, these results indicate that the sanitary challenge model successfully infected the pigs, which can be characterized by good indicators such as diarrhea (Wessels et al., 2021), fever (Rodrigues et al., 2021 a), and higher serum levels of haptoglobin (Parois et al., 2017).

Under a sanitary challenge, alteration in nutrient utilization and protein metabolism occurs, with the redirection of dietary and body nutrient reserves to support the immune system (Campos et al., 2014; 2019a). Since the muscle AA profile differs from the immune component's profile, an AA imbalance may occur in immune system-stimulated pigs (Reeds et al., 1994). Therefore, a proportion of the mobilized AA not used for immune response and protein synthesis is converted into urea and excreted, negatively affecting nitrogen efficiency. Although no significant differences in nitrogen excretion were observed between experimental groups, there was a reduction in ADFI and nitrogen intake, which may have contributed to this result. If there was no such reduction in nitrogen intake, the nitrogen excretion might be higher for ST + POOR SC pigs. Nevertheless, pigs under ST + POOR SC had higher urea serum concentrations (at 7 and 28-dpc) and lower nitrogen efficiency utilization than GOOD SC pigs, which may indicate an AA imbalance (Heo et al., 2009).

Moreover, the sanitary challenge affected the growth rate. The reduced ADG and BW observed in ST+POOR SC pigs are partially due to the lower ADFI, which agrees with

previous studies (Le Floc'h et al., 2009; van der Meer et al., 2016 Rodrigues et al., 2021a). On the other hand, the G:F was also affected. At 28-dpc, the reduction in G:F of ST + POOR SC pigs shows that the ADG (- 19 %) was influenced more than just by the reduction in ADFI (- 10 %) when compared to GOOD SC. This can be explained by the redirection of AA and energy from growth to the immune system (Le Floc'h et al., 2004). Under enteric infections (such as caused by *Salmonella* Typhimurium) and poor housing conditions, intestinal integrity is impaired, which may result in increased endogenous losses and poor digestion (Coop and Kyriazakis, 1999), leading to reduced AA and energy availability. In addition, the reduction in ADG may also result from the metabolic cost associated with fever (heat production; Campos et al., 2019b) and cells and immune component synthesis. This finding is similar to what was reported in growing challenged pigs' studies (Pastorelli et al., 2012a; van der Meer et al., 2016) and in meta-analytical approaches (Pastorelli et al., 2012b; Rodrigues et al., 2021b), suggesting that the major percentage of the ADG and BW loss were due to a disturbance in metabolism and nutrient utilization or greater maintenance requirement.

Different sanitary challenge models were used to assess their effects on the pigs' growth performance and metabolism. Le Floc'h et al. (2006) observed an increase of 259 % in the haptoglobin serum concentration and a decrease of 8 % in the ADG when evaluating the impact of poor housing conditions. Furthermore, upon evaluating the effect of *Salmonella* Typhimurium inoculation in weaned pigs, Rodrigues et al. (2021a) observed an increase of 28 % in haptoglobin, increased rectal temperature, and a reduction of 35 % in the ADG in the first week post-challenge. These findings are similar to the results observed in this trial. However, we are not aware of any other sanitary challenge model which has evaluated the effect of poor cleaning routine and ST inoculation in growing group-housed pigs concomitantly. Taken together, the results observed herein demonstrate that the sanitary

challenge model was able to impact the pig' physiological (such as body temperature, fecal score, body weight) and protein metabolic parameters, proving to be a reliable method to validate the pigs' response during immune system overstimulation.

4.2. Effect of Trp, Thr, and Met supplementation on rectal temperature, fecal score and fecal Salmonella Typhimurium quantification

In the current study, increasing dietary supplementation of Trp, Thr, and Met + Cys reduced the rectal temperature of ST + POOR SC pigs fed AA+ diet when compared to pigs fed CN diet. Interestingly, this result was not observed in previous studies (Jayaraman et al., 2017; Wellington et al., 2019; Rodrigues et al., 2021a,b), which may be due to the sanitary challenge model applied and the higher number of experimental units used for this analysis (n = 20 pigs/experimental group). The faster rectal temperature decrease in ST + POOR SC pigs fed AA+ compared to the CN diet suggests that Trp, Thr, and Met + Cys supplementation may have attenuated inflammation by increasing gut integrity (Ruth and Field, 2013). Indeed, the lower mean fecal score, the reduced number of positive pigs for fecal ST shedding, and the low ST quantification in ST + POOR SC pigs fed an AA+ diet indicate that these AA may have contributed to improving intestinal health by reducing bacterial establishment and colonization in the gut lumen (Faure et al., 2006; Gao et al., 2018).

4.3 Effect of Trp, Thr, and Met supplementation on serum metabolites

Regardless of the SC, pigs fed AA+ diet had higher albumin and a trend for reduction in urea serum concentration at 7 and 28-dpc. Albumin is the major blood protein and has high cysteine and threonine content in its composition (Reeds and Jahoor, 2001; Remus et al., 2019). Therefore, increasing AA dietary levels may have modulated albumin synthesis in the liver. Additionally, albumin is an important protein carrier for steroids, fatty acids, and hormones and it can serve as an anti-oxidant by preventing irreversible oxidative losses

(Roche et al., 2008) by capturing free AA and transporting them to peripheral tissues for protein synthesis (De Feo et al., 1992;). Thus, the higher albumin content in pigs fed AA+ may have contributed to maintaining AA supply for peripheral tissues, and pigs efficiency of AA utilization. Indeed, this was observed in pigs fed AA+, as a tendency for lower serum urea concentration, which is an indicator of AA utilization.

4.4 Effect of Trp, Thr, and Met supplementation on growth performance, body composition, and nitrogen balance

The increased ADG and nitrogen efficiency and the tendency to increase in BW, G:F, and PD in ST + POOR SC fed AA+ pigs compared to ST + POOR SC pigs fed CN diet confirms the hypothesis that the dietary AA+ profile better matches the AA requirements for growth and nutrient utilization in pigs under sanitary challenge. The AA+ diet may have reduced body nutrient mobilization in pigs for immune system activation resulting in more nutrients available for growth performance. This may be confirmed by the fact that ST+POOR SC pigs fed AA+ diet had a better performance than ST + POOR SC pigs fed CN diet, and at the same time, they had similar BW, BP, and PD and nitrogen efficiency compared to pigs housed in GOOD SC at 28-dpc. Overall, these positive results of Trp, Thr, and Met + Cys supplementation on ADG, BW, G:F and nitrogen efficiency is in agreement with previous studies (van der Meer et al., 2016; Rodrigues et al., 2021a). However, the magnitude of the AA supplementation effect on performance varies between studies, which might be related to the type of sanitary challenge model applied, animal density per pen, age/phase, and challenge duration.

In addition, it should be noticed that the improved ADG response without a significant increase in ADFI also suggests that AA supplementation improved pigs' capacity to maintain productivity in a challenging environment. Although the ADG of ST + POOR SC pigs was

lower than those in GOOD SC, pigs fed AA+ showed greater ADG compared with pigs fed CN diet in ST+POOR SC (from 14 until 28-dpc), which demonstrates that Trp, Thr, and Met + Cys supplementation supports pigs' recovery after a sanitary challenge.

5. Conclusion

The Trp, Thr, and Met + Cys supplementation, 20 % above NRC (2012) requirements, for growth mitigates the immune system activation and increases growth performance, protein deposition, and nitrogen efficiency of *Salmonella* Typhimurium-challenged growing pigs under poor housing conditions.

Conflict of interest statement

The authors declare no conflicts of interest.

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Table 1. Ingredient and nutrient composition of experimental diets[1] (%, as-fed basis).

Item	CN	AA+
Centesimal composition		
Corn	74.32	74.32
Soybean meal	22.08	22.08
Limestone	0.73	0.73
Maltodextrin	0.50	0.50
Dicalcium phosphate	0.53	0.53
Salt	0.45	0.45
Kaolin	0.30	0.05
Soybean oil	0.10	0.10
L-Lysine (60%)[2]	0.480	0.480
DL-Methionine (99 %)[2]	0.090	0.201
L-Threonine (98.5 %)[2]	0.070	0.183
L-Tryptophan (98 %)[2]	0.010	0.045
L-Valine (98 %)[2]	0.0002	0.0002
Vit + min premix[3]	0.150	0.150
Antifungal	0.100	0.100
Choline chloride	0.060	0.060
Phytase[4]	0.005	0.005
Calculated chemical composition[5]		
Net energy, kcal/kg	2,550	2,559
Crude protein, %	16.54	16.73
Total nitrogen, %	2.65	2.68
Lysine:Crude protein	5.92	5.85
SID[6] Lysine, %	0.98	0.98
SID Methionine + Cysteine, %	0.55	0.66
SID Methionine, %	0.32	0.43
SID Threonine, %	0.59	0.70
SID Tryptophan, %	0.17	0.20
SID Valine, %	0.67	0.67
SID Arginine, %	0.95	0.95
SID Isoleucine, %	0.59	0.59
SID Leucine, %	1.28	1.28
SID Histidine, %	0.38	0.38
SID Phenylalanine, %	0.70	0.70
Calcium, %	0.66	0.66
STTD[7] Phosphorus, %	0.31	0.31
Chloride, %	0.34	0.34
Potassium, %	0.64	0.64
Sodium, %	0.19	0.19

[1]CN, control diet (basal AA profile); AA+, supplemented diet (supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio).

[2]Amino acids (BioLys, MetAmino, ThreAmino, TrypAmino, ValAmino, respectively) provided by Evonik Nutrition & Care GmbH (Hanau-Wolfgang, Germany).

[3]Mineral premix supplied (per kg of diet): Manganese (40 mg); copper (15 mg); iron (24.93 mg); cobalt (0.168 mg); iodine (1.416 mg); and zinc (74.971 mg). Vitamin premix supplied (per kg of diet): Folic Acid (0.32 mg); D-pantothenic acid (14.8 mg); Biotin (0.04 mg); Niacin (28 mg); Selenium (0.25 mg); Vit. A (6000 IU); Vit. B_1 (1.2 mg); Vit. B_{12} (22 mcg); Vit. B_2 (4.4 mg); Vit. B_6 (1.4 mg); Vit. D_3(1400 IU); Vit. E (26 IU); and Vit. K_3 (2.16 mg).

[4]Phytase provided by Cargill and contained 500 FTU/ton.

[5]Nutrient content of diets based on estimated nutrient contents of ingredients, according to AMINODAT 6.0.

[6]SID, standardized ileal digestible.

[7]STTD, standardized total tract digestible.

Table 2. Analyzed crude protein and total amino acid contents of experimental diets[1] (as-fed basis).

Item	CN	AA+
Crude Protein, %	16.00	17.00
Total amino acid[2], %		
Lysine	1.09 (1.07)	1.15 (1.07)
Methionine + Cysteine	0.56 (0.61)	0.71 (0.72)
Methionine	0.32 (0.34)	0.46 (0.45)
Threonine	0.68 (0.67)	0.83 (0.78)
Valine	0.79 (0.75)	0.79 (0.75)
Arginine	1.01 (1.01)	1.04 (1.01)
Isoleucine	0.68 (0.65)	0.69 (0.66)
Leucine	1.50 (1.44)	1.49 (1.43)
Histidine	0.45 (0.43)	0.45 (0.43)
Phenylalanine	0.82 (0.78)	0.82 (0.78)

[1]CN, control diet (basal AA profile); AA+ , supplemented diet (supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio).

[2]Calculated values are given in parentheses.

Table 3. Logistical regression analysis of the fecal score[1] probability (%) of growing pigs challenged with *Salmonella* Typhimurium and raised under poor housing condition.

Days post-challenge	CN[2]			AA+			Estimate	Odds ratio[3]	RSD[4]	P-value
	0	1	2	0	1	2				
5	11.3	39.9	48.8	9.7	37.2	53.1	-0.17	0.84	0.51	0.74
7	42.4	33.3	24.4	41.9	33.4	24.8	-0.02	0.98	0.49	0.96
14	31.4	34	34.5	57.6	30.3	15.1	1.08	2.96	0.51	0.03
21	57.8	33.7	8.5	68.5	26	5.5	0.46	1.59	0.54	0.39

[1]As few fecal samples were categorized liquid feces (score 3) after 7-dpc, fecal samples categorized as scores 2 and 3 were grouped, and categorized as score 2.

[2]CN, control diet (basal AA profile); AA+, supplemented diet (supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio).

[3]Odds ratio = AA+/CN.

[4]Residual SD.

Table 4. Blood parameters of growing pigs fed control or supplemented diet above the requirement raised under good sanitary condition or challenged with *Salmonella* Typhimurium and raised under poor housing conditions for 28 days.

	GOOD[1]		ST+POOR		RSD[2]	P-value		
Item	CN	AA+	CN	AA+		SC	D	SC × D
7 days post-challenge								
Haptoglobin, g/L	0.27	0.40	0.86	0.89	0.13	<0.01	0.31	0.50
Total protein, g/dL	6.60	6.65	6.15	6.31	0.26	<0.01	0.48	0.70
Albumin, g/L	41.63	43.13	35.77	38.44	1.87	<0.01	0.05	0.57
Urea, mg/dL	17.05	17.11	21.63	18.23	2.03	0.03	0.08	0.20
Creatinine, mg/dL	0.83	0.88	0.95	0.88	0.18	0.56	0.90	0.52
28 days post-challenge								
Haptoglobin, g/L	0.22	0.24	0.35	0.39	0.07	<0.01	0.50	0.78
Total protein, g/dL	5.76	5.71	5.73	5.85	0.14	0.47	0.63	0.31
Albumin, g/L	34.54	35.70	31.36	34.49	1.54	0.01	0.01	0.26
Urea, mg/dL	17.03	16.78	20.08	16.63	1.83	0.15	0.09	0.12
Creatinine, mg/dL	1.28	1.31	1.29	1.32	0.05	0.80	0.28	0.83

[1]GOOD, good sanitary condition; ST+POOR, salmonella challenge and poor housing condition; CN, control diet (basal AA profile); AA+, supplemented diet (supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio).

[2]Residual SD

Table 5. Growth performance, body composition and nitrogen balance of growing pigs fed control or supplemented diet raised under good sanitary condition or challenged with *Salmonella* Typhimurium and raised under poor housing conditions for 28 days.

	GOOD[1]		ST + POOR		RSD[2]	P-value		
Item	CN	AA+	CN	AA+		SC	D	SC × D
Initial conditions								
BW, kg	25.42	25.36	25.36	25.48	0.58	0.92	0.91	0.77
Body protein, kg	3.38	3.37	3.38	3.39	0.02	0.32	0.87	0.48
Body lipid, kg	4.92	4.95	4.88	4.86	0.08	0.18	0.93	0.58
0 to 7 days post-challenge								
Final BW, kg	29.45	29.19	27.60	28.22	0.95	<0.01	0.72	0.36
ADG, kg	0.58	0.56	0.32	0.39	0.09	<0.01	0.65	0.26
ADFI, kg	1.20^{x}	0.94^{x}	0.74^{y}	0.79^{y}	0.06	<0.01	0.62	0.08
G:F, kg/kg	0.54	0.57	0.37	0.44	0.09	0.01	0.32	0.65
0 to 14 days post-challenge								
Final BW, kg	34.02	33.92	31.06	32.58	1.08	<0.01	0.20	0.14
ADG, kg	0.61^{x}	0.61^{x}	0.41^{z}	0.51^{y}	0.05	<0.01	0.08	0.07
ADFI, kg	1.16^{x}	1.09^{xy}	0.96^{z}	1.00^{yz}	0.02	<0.01	0.59	0.06
G:F, kg/kg	0.52	0.56	0.40	0.49	0.03	<0.01	<0.01	0.20
0 to 21 days post-challenge								
Final BW, kg	40.19^{x}	40.02^{x}	35.91^{y}	38.30^{x}	1.37	<0.01	0.11	0.07
ADG, kg	0.70^{a}	0.69^{a}	0.50^{c}	0.61^{b}	0.05	<0.01	0.05	0.03
ADFI, kg	1.34^{a}	1.26^{ab}	1.12^{c}	1.17^{bc}	0.02	<0.01	0.69	0.05
G:F, kg/kg	0.52^{x}	0.55^{x}	0.43^{y}	0.51^{x}	0.03	<0.01	<0.01	0.08
0 to 28 days post-challenge								
Final BW, kg	47.98^{a}	47.84^{a}	42.18^{c}	45.12^{b}	1.60	<0.01	0.08	0.06
ADG, kg	0.81^{a}	0.80^{a}	0.60^{c}	0.70^{b}	0.04	<0.01	0.04	0.03
ADFI, kg	1.45^{x}	1.38^{xy}	1.24^{z}	1.29^{yz}	0.07	<0.01	0.80	0.08
G:F, kg/kg	0.55^{y}	0.58^{x}	0.47^{z}	0.54^{y}	0.01	<0.01	<0.01	0.06
Final body protein, kg	7.24^{x}	7.27^{x}	6.46^{y}	6.95^{x}	0.22	<0.01	0.03	0.06
Final body lipid, kg	7.38	7.32	6.51	6.84	0.24	<0.01	0.31	0.16
Protein deposition, g/d	137^{x}	139^{x}	109^{y}	127^{x}	7.71	<0.01	0.03	0.07
Lipid deposition, g/d	87	84	57	70	8.60	<0.01	0.32	0.11
Nitrogen intake, g/d	37	36	32	35	0.52	<0.01	0.43	0.20
Nitrogen excretion, g/d	15	14	15	14	0.39	0.48	0.75	0.73
Nitrogen efficiency, %	60^{a}	60^{a}	55^{b}	59^{a}	0.02	<0.01	0.03	0.03

[1]GOOD, good sanitary condition; ST+POOR, salmonella challenge and poor housing condition; CN, control diet (basal AA profile); AA+, supplemented diet (supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio).

[2]Residual SD

For body composition, initial BW as a covariate was significant for all variables, $P \leq 0.05$.

[a,b]Different lowercase letters indicate the difference between experimental groups by the Tukey test ($P \leq 0.05$).

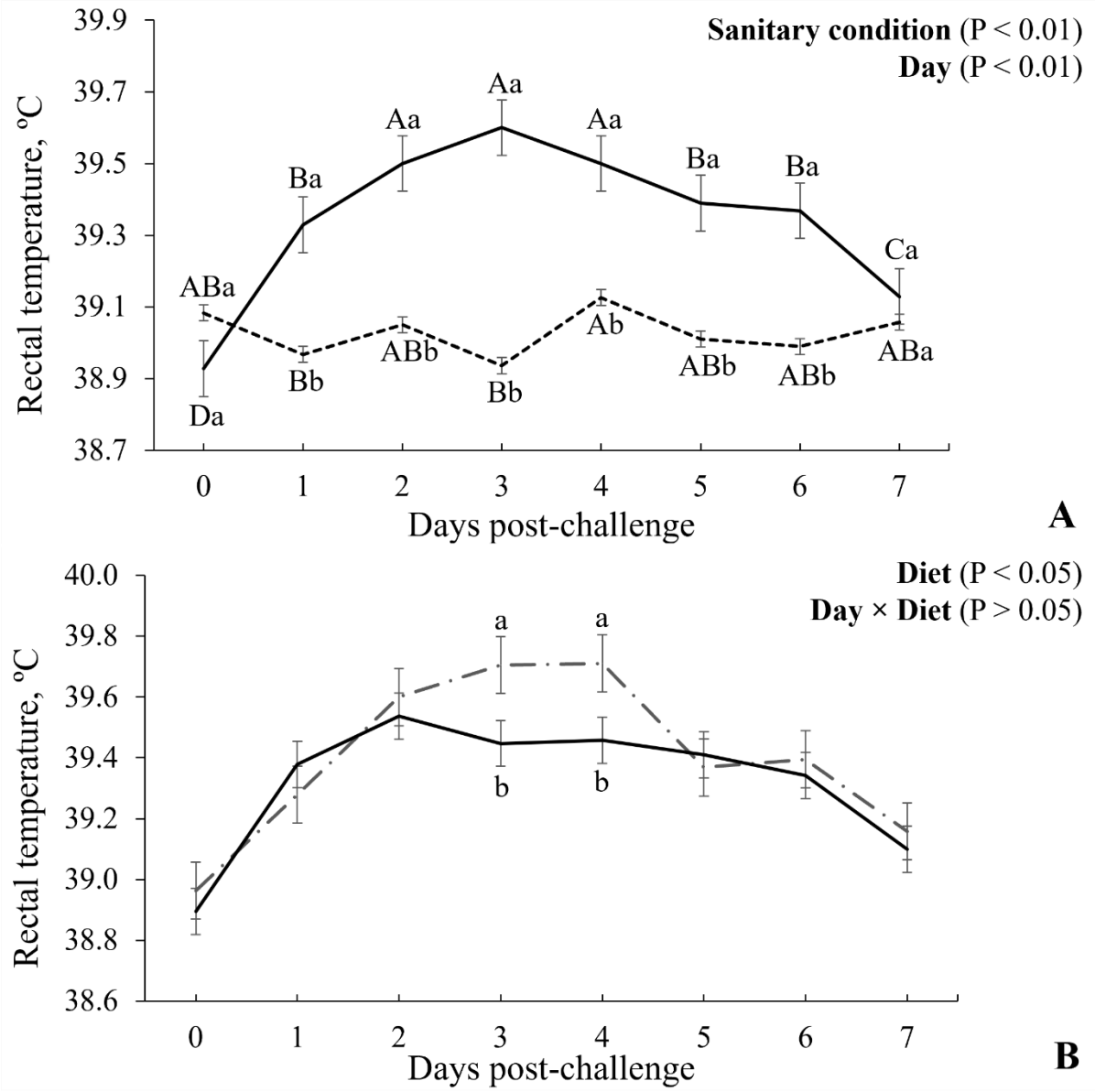
[x,y]Different lowercase letters indicate the tendency between experimental group by the Tukey test ($0.05 < P < 0.10$).

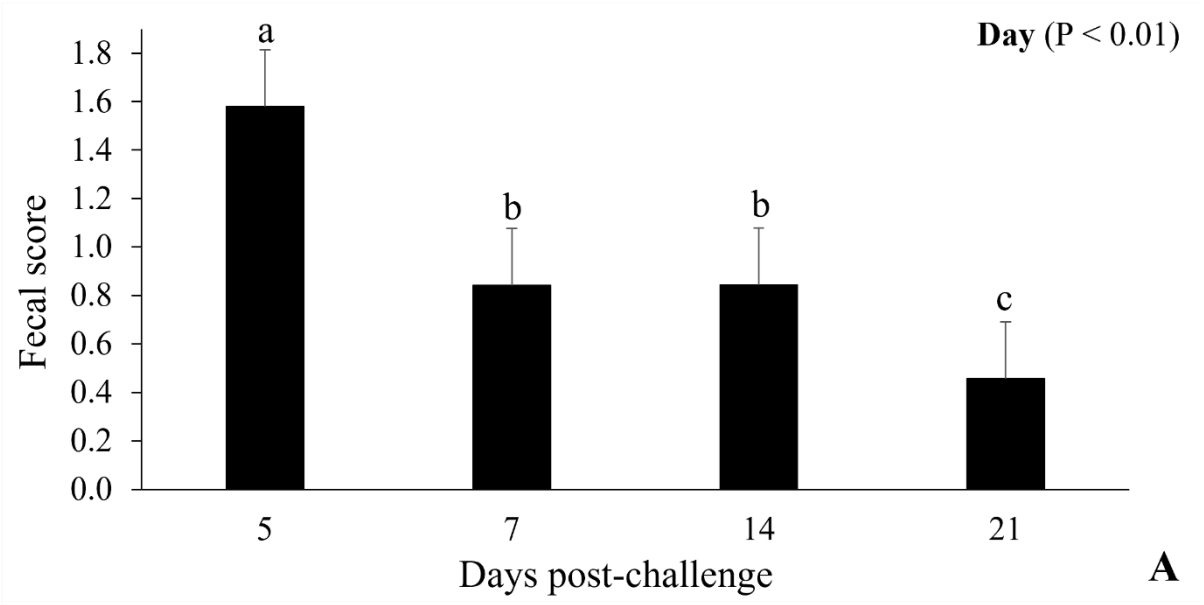
Figure legend

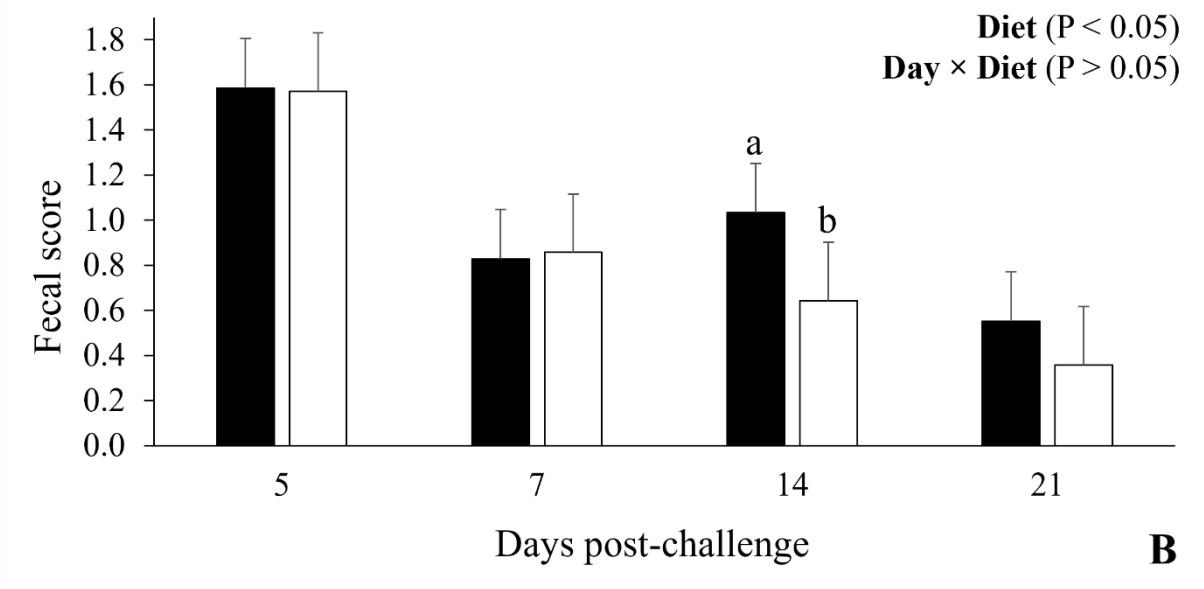
Figure 1. Rectal temperature (A) of pigs in good sanitary condition (— —) or challenge with *Salmonella* Typhimurium and raised under poor housing condition (——). Different lowercase letters indicate difference between sanitary conditions, and different uppercase letters indicate difference between days for the same sanitary condition by the Tukey test ($P < 0.05$). Rectal temperature (B) of pigs housed in POOR sanitary condition fed with control (CN: control diet with basal AA profile (− · −)) or AA supplemented (AA+: supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio (——)). Different lowercase letters indicate differences between diets by the Tukey test ($P < 0.05$).

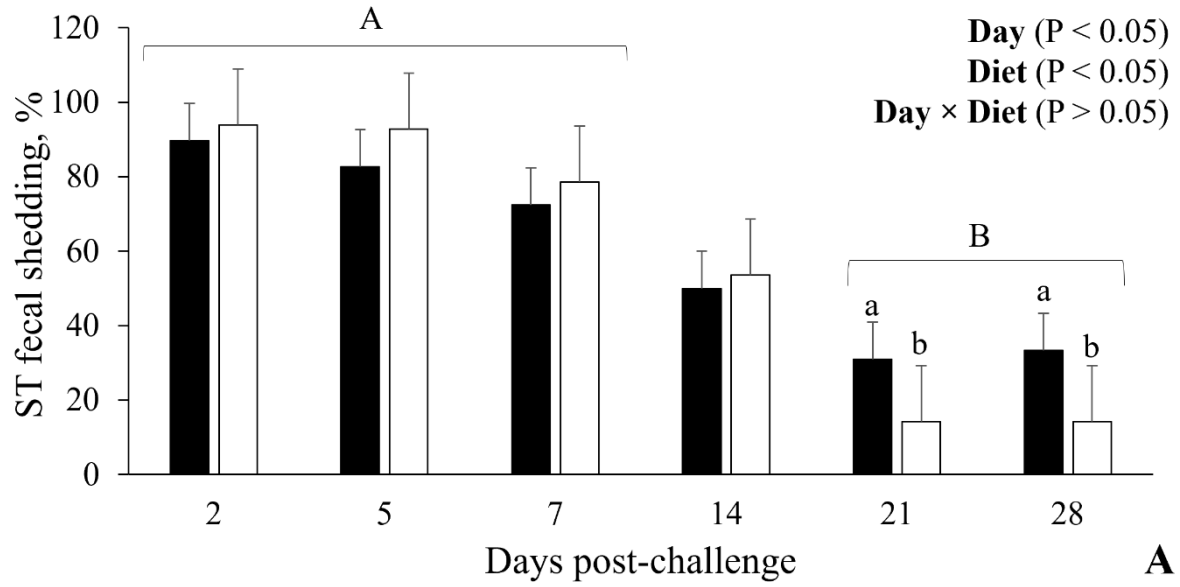
Figure 2. Post-challenge mean fecal score of pigs challenged with *Salmonella* Typhimurium raised under poor housing conditions (A) fed with control (CN: control diet with basal AA profile ■) or AA supplemented diet (AA+: supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio □) (B). Normal, semisolid, semisolid-watery, and watery feces were given the scores 0, 1, 2 and 3, respectively.

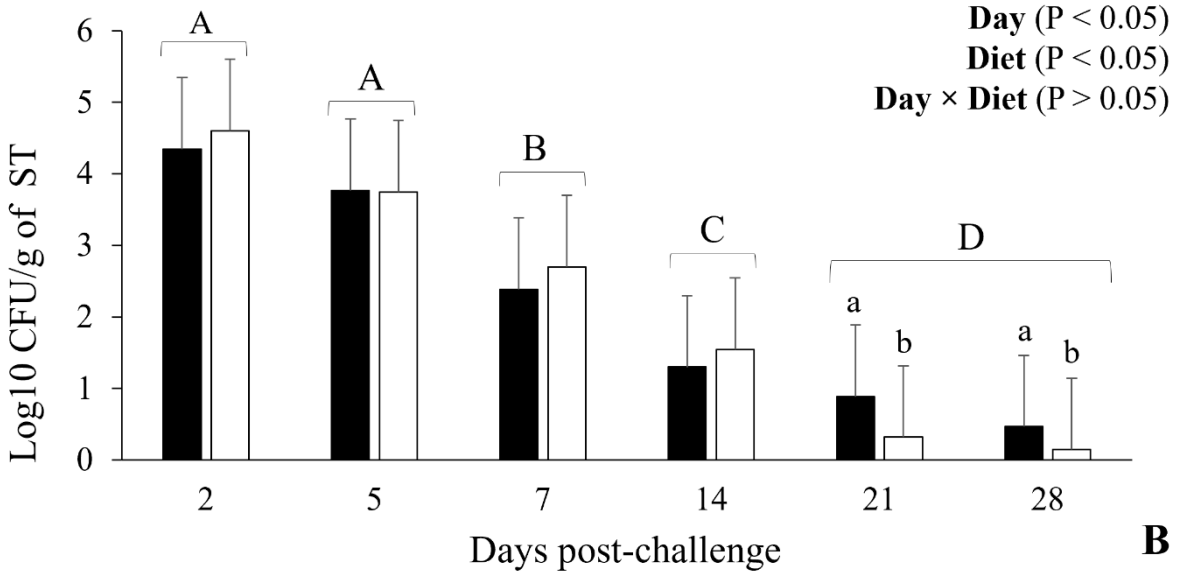
Figure 3. Fecal *Salmonella* Typhimurium shedding percentage (A) of pigs challenged with *Salmonella* Typhimurium and raised under poor housing conditions, fed with control (CN – control diet with basal AA profile ■) or AA supplemented diet (AA+ – supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio □). Fecal *Salmonella* Typhimurium shedding quantification (B) of pigs challenged with *Salmonella* Typhimurium and raised poor housing conditions, fed with control (CN – control diet with basal AA profile ■) or AA supplemented diet (AA+ – supplemented AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio □). Different uppercase and lowercase letters indicate the difference between days and between dietary treatments by the Tukey test ($P < 0.05$), respectively.


Sanitary condition (P < 0.01)
Day (P < 0.01)
Rectal temperature, ºC
39.9
39.7
39.5
39.3
39.1
38.9
38.7
Aa
Aa
Aa
Ba
Ba
Ba
Ca
ABa
Bb
ABb
Bb
Ab
ABb
ABb
ABa
Da
0
1
2
3
4
5
6
7
Days post-challenge
A
Diet (P < 0.05)
Day × Diet (P > 0.05)
Rectal temperature, ºC
40.0
39.8
39.6
39.4
39.2
39.0
38.8
38.6
a
a
b
b
0
1
2
3
4
5
6
7
Days post-challenge
B


Day (P < 0.01)
a
b
b
c
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fecal score
5
7
14
21
Days post-challenge
A


Diet (P < 0.05)
Day × Diet (P > 0.05)
a
b
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fecal score
5
7
14
21
Days post-challenge
B


ST fecal shedding, %
120
100
80
60
40
20
0
A
B
a
b
a
b
Day (P < 0.05)
Diet (P < 0.05)
Day × Diet (P > 0.05)
2
5
7
14
21
28
Days post-challenge
A


Log10 CFU/g of ST
6
5
4
3
2
1
0
A
A
B
C
D
a
b
a
b
Day (P < 0.05)
Diet (P < 0.05)
Day × Diet (P > 0.05)
2
5
7
14
21
28
Days post-challenge
B

CHAPTER 5 – Size matters: lower body weight pigs have a different response to sanitary challenge and amino acids supplementation above the estimated requirement compared to heavy pigs

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Lay summary

An immune challenge impacts pig welfare and may decrease growth and protein deposition. These may happen due to the different nutrient requirements of immune-challenged pigs from those of non-challenged. The dietary supplementation of tryptophan, threonine, and methionine has been proven to be a strategy to mitigate performance losses by supporting the immune component production, maintaining the gut barrier integrity, and reducing the oxidative status. However, individuals within a population with similar age and genetics have distinct responses to dietary strategies due to different coping abilities to an immune challenge, which may be due to body weight/body composition and feeding behavior patterns. In this context, this study investigated the effect of body weight (light vs heavy) and the tryptophan, threonine, and methionine supplementation on feeding behavior and the coping capacity of growing pigs under an immune challenge. Heavy pigs had greater feed intake regularity and coping abilities over time when compared to light pigs. However, increasing the amino acid level in the diet improved feed intake regularity in light pigs. The amino acid supplementation may be a potential precision nutrition strategy for light pigs by improving feed intake regularity over time, reducing the probability of being susceptible to an immune challenge.

Teaser text

Heavy pigs have greater total intake, meal duration, and feed intake constancy than light pigs under an immune challenge. The amino acid supplementation improves light pigs' feed intake regularity and robustness under an immune challenge.

Abstract: The immune response varies between pigs, as not all animals have the same

response toward a stressor. This variation may exist between individuals due to body weight (BW) or body composition, which may impact coping capacity to an immune challenge (IC). The tryptophan (Trp), threonine (Thr), and methionine (Met) requirements might also play a considerable part in supporting the immune system activation while reducing variation between pigs; however, it has yet to be reported. This exploratory study investigated the effect of initial BW (light vs. heavy) and supplementation of Trp, Thr, and Met above NRC requirements on feeding behavior and the coping capacity of growing pigs under an IC. Sixty gilts were categorized into two groups according to BW: light (22.5 ± 0.8 kg) and heavy (28.5 ± 1.1 kg). Both BW groups were group-housed for 28 d trial in good or poor sanitary conditions (SC). Pigs within poor SC were orally inoculated with 2×109 colony units of Salmonella Typhimurium, and fresh manure from a pig farm was spread on the floor. Pigs within good SC were not inoculated, nor was manure spread. Two diets were provided within each SC: control (C) or supplemented (S) with Trp, Thr, and Met at 120% of NRC recommended levels. A Principal Component Analysis was performed in R and a feeding behavior index was calculate in SAS. Results showed that light and heavy pigs were clustered separately on d 0, where light pigs had a positive correlation with body lipid percentage (r = 0.83) and heavy pigs had a positive correlation with body protein percentage (r = 0.75). After the IC, cluster configuration changed, with diets influencing light pigs more than heavy pigs within poor SC. On d 14, light pigs fed S diet in poor SC were clustered separately from light pigs fed C diet, whereas light pigs fed S and C diets in good SC were clustered together. For feeding behavior, in both analyzed periods (period 1 - d 7 to 14 and period 2 - d 21 to 28), light pigs had lower total feed intake and shorter meals than heavy pigs ($P < 0.10$) independent of the SC. Furthermore, light pigs fed S diet had a more regular feed intake pattern than those on the C diet, while a more irregular pattern was observed for heavy pigs

fed S diet than C diet at period 2. These findings suggest that supplementing Trp, Thr, and Met above requirements may be a nutritional strategy for light pigs under IC by improving feed intake regularity and reducing the probability of being susceptible to IC.

Keywords: tryptophan, threonine, methionine, feeding behavior, salmonella

Abbreviations

AA, amino acids

AIPF, automatic intelligent precision feeders

BL, body lipid percentage

BP, body protein percentage

BW, body weight

C, control diet

D, diet

DPC, days post-challenge

DXA, Dual-energy X-ray absorptiometry

FI, feed intake

GOOD, good sanitary condition

IIFI, index of irregularity of feed intake

IC, immune challenge

Lys, lysine

Met + Cys, methionine + cystine

PCA, principal component analysis

POOR, poor sanitary condition

S, supplemented diet

SC, sanitary condition

ST, Salmonella Typhimurium

Thr, threonine

Trp, tryptophan

1. Introduction

Immune system activation and inflammation modify the pigs' metabolism and nutrient utilization. For instance, increased energy expenditure, body temperature, increased acute phase protein synthesis, and reduced protein deposition are commonly observed in immune-stressed pigs (Black et al., 2009; Le Floc'h et al., 2018). As a result, specific nutrients, such as amino acids (AA), are shifted from protein deposition towards the immune-stressed tissues and the proliferation of cells involved in inflammation (Le Floc'h et al., 2004). Therefore, the nutrient requirements of immune-challenged pigs differ from those of non-challenged (Bikker et al., 2006), and providing diets accounting for the higher nutritional needs of immune-challenged pigs can reduce their recovery time, improve growth performance, and reduce nutrient imbalances.

One potential nutritional strategy to support pigs' immune responses to cope with a sanitary challenge is dietary AA supplementation such as Trp, Thr, and Met. Their positive impact on growth and health is associated with improved intestinal mucosa integrity, antioxidant defense, and immune molecule synthesis (Le Floc'h et al., 2018). Indeed, supplementing Trp, Thr, and Met + Cys:Lys above the NRC growth requirements has been reported to improve immune status and growth performance in normal birth weight and growing pigs housed under sanitary challenges (van der Meer et al., 2016; Rodrigues et al., 2022; Valini et al., 2023).

Immune system stimulation can not only decrease growth performance but also increase growth variability (e.g., final body weight (BW), feed efficiency) among pigs (van

der Peet-Schwering et al., 2019). However, the extent of the impact of immune system stimulation on growth depends, among others, on pigs' ability to cope with an immune challenge (IC), which may be affected by initial BW and feeding behavior patterns. For example, light pigs (lower BW) display lower feed intake (FI) and limited physical capacity to ingest nutrients when compared to heavy pigs (higher BW; Aymerich et al., 2020; Ribeiro et al., 2016). Furthermore, light pigs have a higher propensity to be affected by stressors (Njoku et al., 2021) and have an increased probability of being susceptible to IC (Laghouaouta et al., 2021).

Thus, dietary AA supplementation may affect pigs within different BW categories differently. It has been hypothesized that pigs with similar age but higher BW and FI capacity might better cope with an IC by potentially redirecting nutrients and body protein reserves to support the activation of the immune system. Additionally, it was hypothesized that supplementing Trp, Thr, and Met + Cys above requirements (e.g., NRC, 2012) may attenuate inflammation in light pigs with a minor effect on heavy pigs. Therefore, this study aimed to evaluate the effect of BW (light vs. heavy) and Trp, Thr, and Met supplementation on feeding behavior and the coping capacity of growing pigs under an IC.

2. Materials and Methods

2.1. Dataset description

The data used in this study originated from a trial (Valini et al., 2023) conducted at the Swine Research Facility of UNESP, Jaboticabal, SP, Brazil. Data used in this study came from 30 light (22.5 ± 0.8 kg) and 30 heavy (28.5 ± 1.1 kg) high-performance gilts [Pietrain × (Large White × Landrace)] raised during a 28 d sanitary challenge trial. These pigs were selected to address variation in growth and body composition among pigs under an IC, which might be associated with the different coping abilities and feeding behavior patterns of pigs

with different BW but with the same genetic background and age.

Data from all the pigs (n = 10 pigs/treatment) were used for feeding behavior analysis (see section 2.4). A subsample of 5 pigs per treatment was selected to evaluate growth and body composition, rectal temperature, and blood sampling (sections 2.2 and 2.3). Detailed information on animals, housing, inoculation/challenge model protocol, and diets can be found in Valini et al. (2023). All experimental procedures followed the ethical principles in animal research from the Brazilian National Council of the Control of Animal Experimentation (CONCEA) and were reviewed and approved [protocol no. 4784/20] by the Ethical Committee on Animal Use (CEUA) of Universidade Estadual Paulista "Júlio de Mesquita Filho", FCAV/UNESP, Jaboticabal, SP, Brazil.

Briefly, two experimental environmentally controlled growing-finishing buildings were used to simulate the two different sanitary housing conditions (SC): good or poor, in which pigs were group-housed on a single solid concrete floor pen (0.9 m2/pig). Two buildings were necessary to ensure safety protocols, avoiding undesirable bacterial contamination between SC. In both buildings, the humidity was approximately 60%, and the temperature was maintained at 24 ± 2°C with a controlled 12 h daily light period. Each SC had four automatic Intelligent Precision Feeders (AIPF; Exafan, San Mateo de Gallego, Spain) and six ball-bite drinkers. Pigs were fed ad libitum and had unrestricted access to water throughout the experiment.

The IC in poor SC was partly induced by oral inoculation. On d 0, pigs were orally inoculated with a 5 mL solution containing Salmonella enterica serovar Typhimurium (ST; 2 × 109 colony forming units; Valini et al., 2023) selected for antibiotic resistance to nalidixic acid (25 µg/mL). Fresh manure from a commercial pig farm was also spread on the barn floor, and no daily cleaning was applied in the poor SC throughout the trial to increase the

pathogenic pressure. Meanwhile, the good SC pigs were inoculated with a saline solution without ST (to induce the same handling stress in both SC), and no manure was applied on the floor. The good SC was cleaned twice daily, and potassium monopersulfate (1:200; Virkon; Lanxess, Colony, Germany) was pulverized weekly.

Within each SC, two dietary treatments were applied. The control diet (C) was formulated according to NRC (2012), while the supplemented diet (S) contained 20% higher standardized ileal digestible (SID) Trp:Lys, Thr:Lys, and Met + Cys:Lys concentrations compared to C diet (Tables 1 and 2). Both diets had the same ingredient composition besides DL-Methionine, L-Threonine, and L-Tryptophan. Corn and soybean meal were the primary energy and protein ingredient providers. Diets were animal protein-free and were formulated using the NIRS analyzed AA content of ingredients to provide the nutrient requirements for 25 to 50 kg of BW, according to NRC (2012). No antibiotics in-feed as growth promoters were used throughout the trial.

2.2. Growth and body composition

The AIPFs measured individual daily FI while the individual BW was obtained by weighting the pigs before challenge (d -1) and at 7, 14, and 28 days post-challenge (dpc). The percentage change in relative feed intake (FI %) for each pig during the initial seven dpc was computed as the percentage difference from the average of the two days preceding the challenge. Besides, the individual relative BW change (%) in the first seven dpc was calculated as the percentual difference from the BW on d 7 and d -1. The total body lean content was measured on d -1 and at d 28 with a dual-energy X-ray absorptiometry device (DXA, GE Lunar Prodigy Advance; GE Healthcare, Madison, WI). Pigs were scanned in the prone position using the full-body scanning mode (GE Lunar enCORE, version 8.10.027; GE

Healthcare). Anesthesia was induced by intramuscular injection of xylazine (1.5 mg/kg) and ketamine (15 mg/kg). The DXA body lean and fat masses were converted to their protein and lipid chemical equivalent, respectively, as proposed by Pomar and Rivest (1996). The body protein (BP) and lipid (BL) masses at 14 dpc were determined by calculating the product of the daily protein and lipid deposition rates (g/d; obtained by dividing the difference between final and initial measurements by the number of experimental days) and multiplying it by 14. This value was then added to the initial BP and BL masses. Moreover, the BP and BL relative percentage to BW were calculated as the proportion of the measured BW at 14 and 28 dpc.

2.3. Rectal temperature and blood parameters

Individual rectal temperature was measured daily from 0 to 7 days post-challenge with a digital thermometer (Accumed-Glicomed, RJ, Brazil). Blood samples were taken before challenge (d -1) and on 14 and 28 dpc from a jugular vein puncture into 4 mL vacutainer tubes coated with EDTA (Vacuplast; Cral, São Paulo, Brazil) and on tubes containing no additive (BD; New Jersey, EUA). Pigs were fasted for six hours before blood sampling. One blood sample collected in EDTA tubes was immediately stored on ice and transported to the laboratory for lymphocyte count (Horiba Micros-60; Horiba ABX SAS, Montpellier, France). The blood samples collected into additive-free tubes were allowed to clot for one hour. Afterward, the tubes were centrifugated for 10 min at 3,000 × g at 4 ºC. The serum was separated and stored at -80 ºC. Albumin, urea, and triglycerides were quantified by the biuret method with a commercial reagent (Labtest Diagnostica, Lagoa Santa, MG, Brazil) and performed using a semi-automatic spectrophotometer (Labmax Plenno, Labtest Diagnostica, Lagoa Santa, MG, Brazil).

2.4. Feeding behavior

The assessment of the index of irregularity of FI (IIFI) was calculated using the real-time information recorded AIPFs (individual meal FI, initial and final meal time) as proposed by Salgado et al. (2021). Data collected on days with animal handling (i.e., weighted, blood sampled, or scanned) were not used for calculations. Errors in the data, such as visits to feeders without intake, were removed, and visits at intervals less than 2 min apart by the same pig were combined into one meal (Remus et al., 2020).

For each pig, a monotonically increasing step function was obtained using the relative cumulative FI and relative time per period (period 1: 7 to 14 dpc, and period 2: 21 to 28 dpc). These periods were selected as they represented the acute (Broz et al., 2012) and chronic phases of an ST infection. Afterward, a linear regression model was fitted between the relative cumulative FI and relative time. Then, the areas between the step function and the regression line were added to obtain the IIFI for each pig within periods. In addition to the IIFI, the number of visits, the total intake (g), and meal duration (min) were calculated for each pig within periods.

2.5. Statistical analyses

2.5.1. Rectal temperature, relative FI, and BW changes: first-week post-challenge

Data were analyzed as a completed randomized design within each SC (n = 5 pigs/treatment) using the MIXED procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC). The model included the BW groups and diets for FI% and BW% changes. Rectal temperature was analyzed as repeated measures in time.

2.5.2. Principal Component Analysis (PCA)

Associations between the quantitative variables (BW, BL, BP, albumin, urea, triglycerides, lymphocytes, and the IIFI) of the sampled pigs were investigated using a PCA

performed in R (version 4.3.1; R Foundation for Statistical Computing, Vienna, Austria) with the factorextra package (v1.0.7; Kassambara and Mundt, 2020) for each time point (0, 14, and 28 dpc). The differences between groups were indicated by constructing confidence ellipses (95% confidence interval).

2.5.3. Feeding behavior

Data was analysed as a complete randomized design with a 2 x 2 x 2 factorial arrangement within each period (period 1: 7 to 14 dpc, and period 2: 21 to 28 dpc). The model included the SC, BW groups, and diets as fixed effects using the MIXED procedure in SAS (n = 10 pigs/treatment). The SC was added to the model to verify if there were deviations in the feeding pattern of pigs (using good SC as a reference population) since there is no literature regarding feeding pattern modification between SC. Although having only room to represent each SC condition (good or poor) results in pseudo-replicates, housing pigs separately avoided cross-contamination between SC and permitted a clear overview of the SC impact on pigs. Besides, during the adaptation period (14 d), there was no room effect on pigs' growth, which allowed comparisons between SC during the experimental period as previously reported (Valini et al., 2023). The individual pig was the experimental unit for all the analyses, and the significance level adopted was 10 % ($P \leq 0.10$).

3. Results

3.1. Rectal temperature

There were no interactions between BW groups, diets, and days within each SC ($P > 0.10$). Besides, no differences were found for BW groups or diets in both SC ($P > 0.10$). However, a day effect was observed in both SC ($P < 0.10$), as previously reported (Valini et al., 2023). Under good SC, the rectal temperature remained constant throughout the first week (Fig. 1A), while under poor SC, the rectal temperature increased and had the maximum value

at 4 dpc (average of 39.7 °C) and started decreasing from 5 dpc and returned to the basal rectal temperature at 7 dpc (Fig.1B).

3.2. Relative feed intake and body weight changes

No interactions were observed between BW groups and diets within each SC ($P > 0.10$; Fig. 2 A, B). However, a diet effect was observed in good SC. Pigs fed the C diet had a 10% and 16% increase in relative FI and BW changes, respectively, while pigs fed the S diet had a 9% reduction in relative FI change and a 10% increase in relative BW change at 7 dpc ($P < 0.10$; Fig. 2 C, D). No differences were observed within poor SC for BW groups or diet ($P > 0.10$; Fig. 2 E, F).

3.3. Principal component analysis (PCA)

3.3.1 PCA: initial conditions

At day 0, the first two principal components (PC) of the PCA accounted for 61% of the total variance (PC1 = 36.5%; PC2 = 24.5%), and heavy and light pigs were clustered in two separate groups (Fig. 3). The PC1 had a positive strong correlation with BL percentage of BW (r = 0.85), but was negatively correlated with BW (r = -0.95), and BP (r = -0.75). The PC2 had negative correlations with albumin (r = -0.67), urea (r = -0.82) and triglycerides (r = -0.70) concentrations.

3.3.2 PCA: Good SC

After the challenge, at 14 and 28 dpc, the first two PC of the PCA accounted for 54.9% (PC1 = 33.7%; PC2 = 21.2%) and 52.3% (PC1 = 30.6%; PC2 = 22.0%) of the total variance (Fig. 4), and light pigs fed C or S diet were clustered separately from heavy pigs fed C or S diet. At 14 dpc, PC1 had a strong positive correlation with BL (r = 0.80) but was negatively correlated with BW (r = -0.87), BP (r = -0.52), and serum albumin concentration (r = -0.66). The PC2 had a strong positive correlation with the IIFI (r = 0.75) and serum urea

concentration (r = 0.76) and a moderate correlation with BP (r = 0.51). At 28 dpc, PC1 had a strong negative correlation with BW (r = -0.72), serum albumin (r = -0.84), and urea (r = -0.82) concentrations, whereas PC2 had a strong positive correlation with BL (r = 0.80) and a moderate correlation with plasma lymphocyte count (r = 0.55) but had a strong negative correlation with the IIFI (r = -0.76).

3.3.3 PCA: Poor SC

At 14 and 28 dpc, the first two PC of the PCA accounted for 48.9% (PC1 = 30.0%; PC2 = 18.9%) and 55.6% (PC1 = 37.7%; PC2 = 17.9%) of the total variance (Fig. 5). At 14 dpc, light pigs fed S diet and light pigs fed C diet were clustered in two separate groups, while heavy pigs fed S or C diet were clustered in the same group. The PC1 had a strong positive correlation with BL (r = 0.83) but was negatively correlated with BW (r = -0.94), BP (r = -0.52), and serum albumin concentration (r = -0.50). The PC2 had a strong positive correlation with serum urea concentration (r = 0.80) and a moderate positive correlation with triglycerides (r = 0.58) but had a negative correlation with serum albumin concentration (r = -0.57). At 28 days post-challenge, light pigs fed C or S diet were clustered separately from heavy pigs fed C or S diet. The PC1 had a negative strong correlation with BW (r = -0.89), BP (r = -0.79), and serum albumin concentration (r = -0.71) and a negative correlation with plasma lymphocyte count (r = -0.62), but had a moderate positive correlation with serum triglycerides concentration (r = 0.52). The PC2 had a moderate positive correlation with serum urea concentration (r = 0.67) but a negative moderate correlation with BL (r = -0.59) and serum albumin concentration (r = -0.52).

3.4. Feeding behavior

During the first period, an interaction between SC and diet was observed (Table 3) for

total intake (P = 0.04), meal duration (P = 0.02), and the IIFI (P = 0.09). Pigs within good SC fed S had lower total intake (P = 0.05) and shorter meal duration than those fed with C diet (P = 0.05). However, pigs in poor SC fed C or S diet had no differences in total intake and meal duration (P > 0.10). Pigs in good SC fed S had a higher IIFI than those in the C diet (P = 0.05), whereas pigs in poor SC fed C had a higher IIFI than S diet (P = 0.05). Furthermore, BW influenced total intake (P < 0.01), number of visits (P < 0.01), and meal duration (P < 0.01), as heavy pigs had a higher total intake, number of visits, and meal duration than the light pigs (P = 0.05), with no changes in IIFI (P > 0.10).

During period 2, a BW and diet interaction was observed (P = 0.05). Light pigs fed S diet had a lower IIFI than the C diet, whereas an increase in the IIFI was observed for heavy pigs fed the S diet (P = 0.05). The SC impacted the total intake (P < 0.01) and the IIFI (P = 0.09). Pigs within good SC had higher total intake and lower IIFI than pigs within poor SC. Besides, BW influenced total intake (P < 0.01) and meal duration (P < 0.01), as heavy pigs had higher total intake and meal duration compared to light pigs.

4. Discussion

This exploratory study complements a previous publication (Valini et al., 2023). Specifically, we investigated changes in the feeding behavior of heavy and light pigs in good or poor SC. For the first time, the IIFI was used to explore not only how total intake changes but also how the feeding patterns of challenged animals supplemented or not with AA above growth requirements were affected. Notably, observed outcomes indicate that, when exposed to immune challenges, heavy pigs demonstrate heightened total intake, extended meal duration, and improved feed intake regularity compared to their lighter counterparts. Additionally, the study showed the potential benefits of AA supplementation in enhancing feed intake regularity and robustness in light pigs facing immune challenges.

4.1. Rectal temperature, relative FI, and BW changes

We hypothesized that light pigs would have an exacerbated febrile response to the IC compared to heavy pigs. However, no statistical differences were detected in the rectal temperature response of BW groups. Indeed, both BW groups raised the rectal temperature after the challenge in a similar magnitude. This shows that poor housing condition, mainly the ST load, overwhelmed the intestinal immune system and caused inflammation, releasing pro-inflammatory cytokines in the bloodstream. As cytokines can be detected in the plasma up to 5 days post-infection (Lee et al., 2019) and are the mediators of the febrile response, the pyrogenic response of pigs is in agreement with their appearance in the bloodstream. However, there was a different pattern in how the rectal temperature rose between BW groups. Heavy pigs rectal temperature reached its maximum (39.6 ± 0.2 ºC) at 2 dpc, whereas for light pigs, the rectal temperature reached its maximum (39.6 ± 0.3 ºC) at 4 dpc. This different pattern may suggest that heavy pigs have improved intestinal functioning, with higher jejunum expression of genes involved in inflammation and stress responses compared to their lighter littermates (Villagómez-Estrada et al., 2022), and thus, a latter febrile response is expected.

Besides the febrile response, it is well known that enteric challenges, including ST, have a great impact on FI and, as a consequence, BW, especially on the first seven dpc (Wellington et al., 2019; Rodrigues et al., 2021). Indeed, in the present study, changes in relative FI found in challenged pigs are within the range of the 8% to 23% FI reduction observed in other studies (Pastorelli et al., 2012). Even though poor SC pigs fed the S diet had a 9 % greater reduction of FI and a 43 % lower BW increase than the C diet, no statistical differences were detected. This is likely due to the large variation observed within treatments and the small number of animals per group. Surprisingly, pigs in good SC fed C diet showed

changes in relative FI compared to S diet, which was reflected in a greater BW increase for control pigs. It is possible that this may happen due to the rapid absorption and quick appearance in the plasma of free AA (Krehbiel and Matthews, 2003), which may induce anorexigenic hormone release (Westerterp-Plantenga et al., 2012), enhancing satiety.

4.2. Feeding behavior and growth response to treatments

The exploratory PCA analysis suggests that light and heavy pigs of the same genetic background and similar age had different starting characteristics mainly driven by BP, BL, albumin, urea, triglycerides, and lymphocytes. In fact, variations in growth performance have been previously correlated to pigs having different body compositions. For example, pigs with the same BW but greater BL were less sensitive to insulin (Salgado et al., 2022). Meanwhile, insulin resistance has also been linked to lower protein deposition and changes in AA kinetics (Remus and Pomar, 2023). For instance, pigs with low protein deposition had lower BP and showed differences in gene expression for protein metabolism and stress response (Remus et al., 2022). Thus, it is plausible that the difference between light and heavy BW pigs, in terms of blood metabolites at the starting point of the trial, is linked to the differences in BP and BL. As shown by the PCA analysis, at d 0, light pigs had a positive correlation with BL, and on average, light pigs had 7% greater BL and 6% smaller BP than heavy pigs in both SC, and these differences in body composition were maintained throughout the trial. Based on resource allocation theory (van der Waaij, 2004), a lower percentage of lean body mass may impair pigs' capacity to handle immune stimulation. Under an immune system activation, the cytokines produced, such as interleukins 1-β and 6, and the tumor necrosis factor-alpha, are also involved in protein metabolism regulation. These cytokines may induce muscle protein degradation and increase the AAs utilization as a substrate for gluconeogenesis and acute-phase protein synthesis in the liver to boost immune system responses (Zamir et al., 1994;

Obled, 2003). Indeed, heavy pigs had a positive correlation with serum triglyceride (PC2) and a negative correlation with BP (PC1) at 14 dpc in poor SC, which may suggest that heavy pigs may mobilize body reserves to support immune function and cope with an IC. As animals prefer glucose over fatty acids under IC as the energy source, it may impair triglyceride clearance from the blood, related to lower activity of the enzyme lipoprotein lipase (Butcher and Miles, 2002).

Moreover, such differences could thus result in different responses to the SC and increases in dietary AA. Previous studies have shown that light pigs have lower serum triglycerides or other lipid metabolites (Huang et al., 2020; He at al., 2011), which may be associated with intestinal disorders and gut microbiota alterations, leading to long-term abnormalities in lipid metabolism (Yan et al., 2017). Thus, having a negative correlation with BP may contribute to light pigs being less efficient in AA utilization and body reserve mobilization.

Besides the initial differences between BW groups, light and heavy pigs also responded differently to the SC and dietary treatments. For example, in the good SC PCA, at 14 and 28 dpc an association was observed between the IIFI, body composition, and blood variables such as albumin, urea, triglycerides, and lymphocytes. Light pigs supplemented with AA above the requirement, remained clustered apart from heavy pigs overall the 28 d of experimentation. Additionally, AA supplementation increased IIFI at period 1 and enlarged variation between both BW groups supplemented pigs at 14 dpc and supplemented heavy pigs at 28 dpc. Meanwhile, in poor SC, on 14 dpc, light pigs fed the S diet were clustered apart from light, and heavy pigs fed the C diet, and reduced IIFI was observed in supplemented pigs at period 1. The IIFI seems to be one of the driving variables due to its important contribution to explaining the variation observed in the PCAs. A higher IIFI indicates that pigs had a less

constant FI over time, with less frequent meals and longer intervals.

Feeding pigs ad libitum with higher amounts of crystalline AA without a sanitary challenge may impair feeding patterns such as meal duration and FI regularity due to the post-absorptive physiological response to an imbalance of essential AA. However, it is important to consider that the S diet had a slight increase in Lys content (+ 6%) than expected, which may have affected the efficiency of Lys utilization and reduced the amount of FI as well. Although there was a slight difference in Lys content, the relative BW change was similar in both diets, especially at good SC, which may suggest that the C diet met the requirements and did not limit the pigs' performance.

Feeding behavior modifications observed in poor SC may also be triggered by cytokine synthesis, which in the brain induces sickness behavior and decreased appetite. Besides, because of abdominal pain caused by enteric infections, pigs may develop feed aversion from the learned association between feed consumption and post-ingestive effects (Day et al., 1998). Even though pigs in poor SC had a lower total intake and larger IIFI in both periods compared to good SC, heavy pigs in poor SC still had a higher number of visits in period 1, and they also had higher total intake and meal duration than light pigs in poor SC in both periods. Thus, having a lower FI and a more irregular FI may compromise gut integrity and nutrient absorption (Jayaraman and Niachoti, 2017), which may impair nutrients to boost the immune response and, consequently, increase susceptibility to an IC.

Supplementing Trp, Thr, and Met above requirements in poor SC resulted in differences in clustering between light pigs fed S diet and those fed the C diet at 14 dpc. Also, improved FI regularity, TI, and MD were observed in S versus C diet-fed pigs at period 1. These results might indicate that the S diet can boost immune system responses, likely by reducing cytokines and acute phase protein synthesis, weakening the sickness behavior. The S

diet also seemed to shorten the time for coping with an IC. Indeed, PC1 showed a negative correlation with serum lymphocytes at 28 dpc, whereas no correlation between serum lymphocytes and PC1 and 2 occurred in good SC at 28 dpc. Lymphocytes are mainly indicative of the initiation and execution of adaptive immune responses due to their essential and multiple roles in adaptive immunity (Bai et al., 2020). Therefore, having lower lymphocyte concentration in the blood may indicate a resolution or having an effective control of an infection.

An important limitation of this study is the limited number of experimental units per treatment, mainly for the PCA analysis, where removing or adding new animals may alter the clusters' formation. Therefore, the results and conclusions of this study may only apply to these animals submitted to this immune challenge model. Furthermore, our herd cannot be considered representative of a large population because the pigs were not selected at random for this study. Indeed, the light and heavy groups were prefixed at the beginning of the trial after pigs selection and distribution to the treatments. To our knowledge, there is no study in the literature regarding changes in feeding patterns and body composition associated with coping characteristics of pigs with different BW under an IC and supplemented with Trp, Thr, and Met above NRC (2012) requirements. Thus, the present work may serve as an exploratory study to help design future large-scale studies in growing-finishing pigs to better understand the variability within treatments supplemented with different dietary AA levels, especially in poor SC.

5. Conclusion

In conclusion, heavy pigs exposed to immune challenges showed heightened total intake, extended meal duration, and improved feed intake regularity compared to lighter pigs. The study also highlights the potential benefits of amino acid supplementation in enhancing

feed intake regularity and robustness in light pigs facing immune challenges. Supplementing Trp, Thr, and Met + Cys 20% above the NRC (2012) requirements may be a potential precision nutrition strategy with a greater effect on light pigs under a sanitary challenge, improving feed intake regularity and reducing the probability of being susceptible to an immune system challenge.

Conflict of interest statement

The authors declare no conflicts of interest.

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Table 1. Ingredient and nutrient composition of experimental diets[1] (%, as-fed basis).

Item	CN	AA+
Centesimal composition		
Corn	74.32	74.32
Soybean meal	22.08	22.08
Limestone	0.73	0.73
Maltodextrin	0.50	0.50
Dicalcium phosphate	0.53	0.53
Salt	0.45	0.45
Kaolin	0.30	0.05
Soybean oil	0.10	0.10
L-Lysine (60%)[2]	0.480	0.480
DL-Methionine (99 %)[2]	0.090	0.201
L-Threonine (98.5 %)[2]	0.070	0.183
L-Tryptophan (98 %)[2]	0.010	0.045
L-Valine (98 %)[2]	0.0002	0.0002
Vit + min premix[3]	0.150	0.150
Antifungal	0.100	0.100
Choline chloride	0.060	0.060
Phytase[4]	0.005	0.005
Calculated chemical composition[5]		
Net energy, kcal/kg	2,550	2,559
Crude protein, %	16.54	16.73
Total nitrogen, %	2.65	2.68
Lysine:Crude protein	5.92	5.85
SID[6] Lysine, %	0.98	0.98
SID Methionine + Cysteine, %	0.55	0.66
SID Methionine, %	0.32	0.43
SID Threonine, %	0.59	0.70
SID Tryptophan, %	0.17	0.20
SID Valine, %	0.67	0.67
SID Arginine, %	0.95	0.95
SID Isoleucine, %	0.59	0.59
SID Leucine, %	1.28	1.28
SID Histidine, %	0.38	0.38
SID Phenylalanine, %	0.70	0.70
Calcium, %	0.66	0.66
STTD[7] Phosphorus, %	0.31	0.31
Chloride, %	0.34	0.34
Potassium, %	0.64	0.64
Sodium, %	0.19	0.19

[1] Control diet with the basal AA profile; Supplemented diet with AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio.

[2]Amino acids (BioLys, MetAmino, ThreAmino, TrypAmino, ValAmino, respectively) provided by Evonik Nutrition & Care GmbH (Hanau-Wolfgang, Germany).

[3]Mineral premix supplied (per kg of diet): Manganese (40 mg); copper (15 mg); iron (24.93 mg); cobalt (0.168 mg); iodine (1.416 mg); and zinc (74.971 mg). Vitamin premix supplied (per kg of diet): Folic Acid (0.32 mg); D-pantothenic acid (14.8 mg); Biotin (0.04 mg); Niacin (28 mg); Selenium (0.25 mg); Vit. A (6000 IU); Vit. B_1 (1.2 mg); Vit. B_{12} (22 mcg); Vit. B_2 (4.4 mg); Vit. B_6 (1.4 mg); Vit. D_3(1400 IU); Vit. E (26 IU); and Vit. K_3 (2.16 mg).

[4]Phytase provided by Cargill and contained 500 FTU/ton.

[5]Nutrient content of diets based on estimated nutrient contents of ingredients, according to AMINODAT 6.0.

[6]SID, standardized ileal digestible.

[7]STTD, standardized total tract digestible.

Table 2. Analysed crude protein and total amino acid contents of experimental diets[1] (as-fed basis).

Item	CN	AA+
Crude Protein, %	16.00	17.00
Total amino acid[2], %		
Lysine	1.09 (1.07)	1.15 (1.07)
Methionine + Cysteine	0.56 (0.61)	0.71 (0.72)
Methionine	0.32 (0.34)	0.46 (0.45)
Threonine	0.68 (0.67)	0.83 (0.78)
Valine	0.79 (0.75)	0.79 (0.75)
Arginine	1.01 (1.01)	1.04 (1.01)
Isoleucine	0.68 (0.65)	0.69 (0.66)
Leucine	1.50 (1.44)	1.49 (1.43)
Histidine	0.45 (0.43)	0.45 (0.43)
Phenylalanine	0.82 (0.78)	0.82 (0.78)

[1] Control diet with the basal AA profile; Supplemented diet with AA profile containing 20 % above Trp, Thr, and Met + Cys:Lys ratio.

[2]Calculated values are given in parentheses.

Table 3. Index of irregularity of feed intake and feeding behavior variables of heavy and light growing pigs fed control (CN) or supplemented diet with increased Trp, Thr, and Met (AA+) raised under good or poor sanitary condition.

	GOOD				POOR				*P*-value						
	CN		AA+		CN		AA+								
Item[1]	Heavy	Light	Heavy	Light	Heavy	Light	Heavy	Light	SC	D	BW	SC×BW	SC×D	BW×D	SC×D×BW
Period 1 (7 to 14 days post-challenge)															
TI (g)	7426	5677	6810	4919	5209	3541	5574	3998	**<0.01**	0.67	**<0.01**	0.87	**0.04**	0.89	0.44
MD (min)	538	422	476	358	403	292	434	336	**<0.01**	0.45	**<0.01**	0.94	**0.02**	0.98	0.37
Visits (n)	68	50	60	50	66	46	60	46	0.81	0.27	**<0.01**	0.85	0.92	0.73	0.37
IIFI	360.1	365.1	434.6	380.8	576.7	712.9	609.5	538.7	**<0.01**	0.31	0.11	0.24	**0.09**	0.27	0.69
Period 2 (21 to 28 days post-challenge)															
TI (g)	12080	9076	11245	9112	9908	7840	10774	8140	**<0.01**	0.91	**<0.01**	0.85	0.21	0.92	0.42
MD (min)	566	469	531	451	524	455	578	475	0.51	0.73	**<0.01**	0.84	0.13	0.6	0.58
Visits (n)	76	75	62	75	88	75	77	74	0.28	0.11	0.8	0.34	0.68	0.54	0.7
IIFI	193.9	241.5	321.3	173.6	261.6	318.4	216.8	251.8	**0.09**	0.96	0.11	0.13	0.17	**0.05**	0.11

[1]Abbreviations: GOOD = good sanitary condition; POOR = poor sanitary condition; SC = sanitary condition; D = diet effect; BW = body weight group effect; C = control diet (basal AA profile); S = supplemented diet (supplemented AA profile containing 20% above Trp, Thr and Met + Cys: Lys); TI = total intake; MD = meal duration; IIFI = index of irregularity of feed intake.

Figure legend

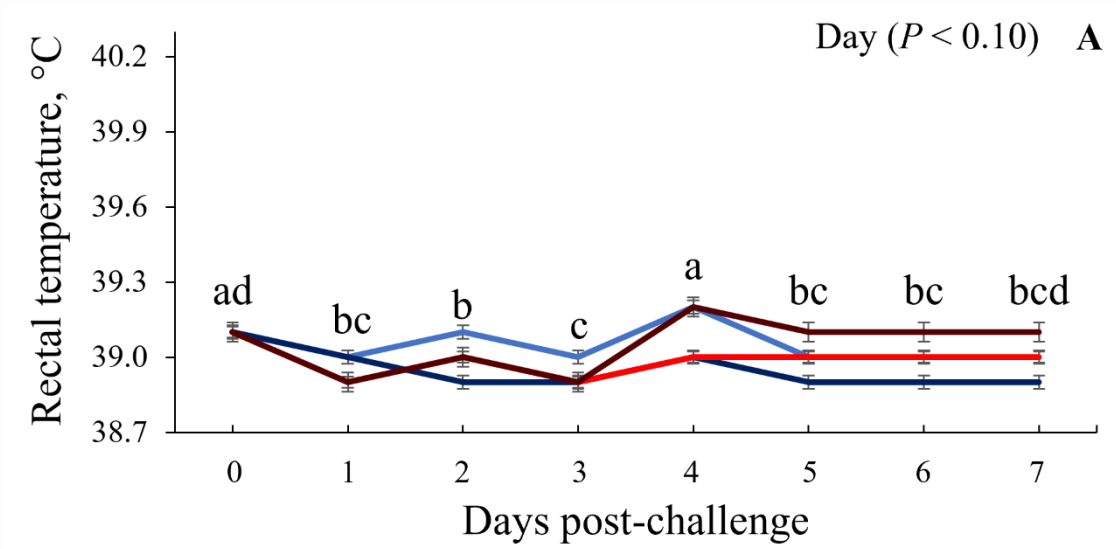
Figure 1. Rectal temperature of heavy and light pigs fed control (C) or supplemented (S) diet and raised in good (A) or poor sanitary conditions (B). Different lowercase letters indicate a difference between days for the same sanitary condition by Tukey test ($P < 0.10$).

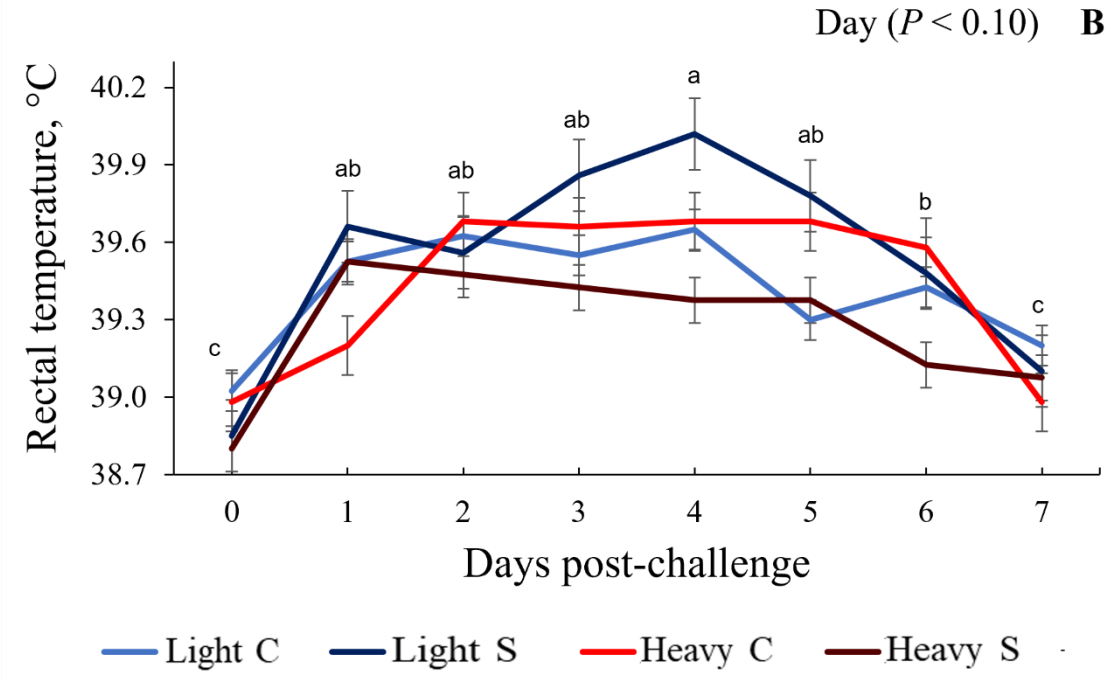
Figure 2. Relative feed intake and body weight changes of heavy and light pigs raised in good (■) or poor (■) sanitary conditions. Different lowercase letters indicate differences between diets in the same sanitary condition by F test ($P < 0.10$).

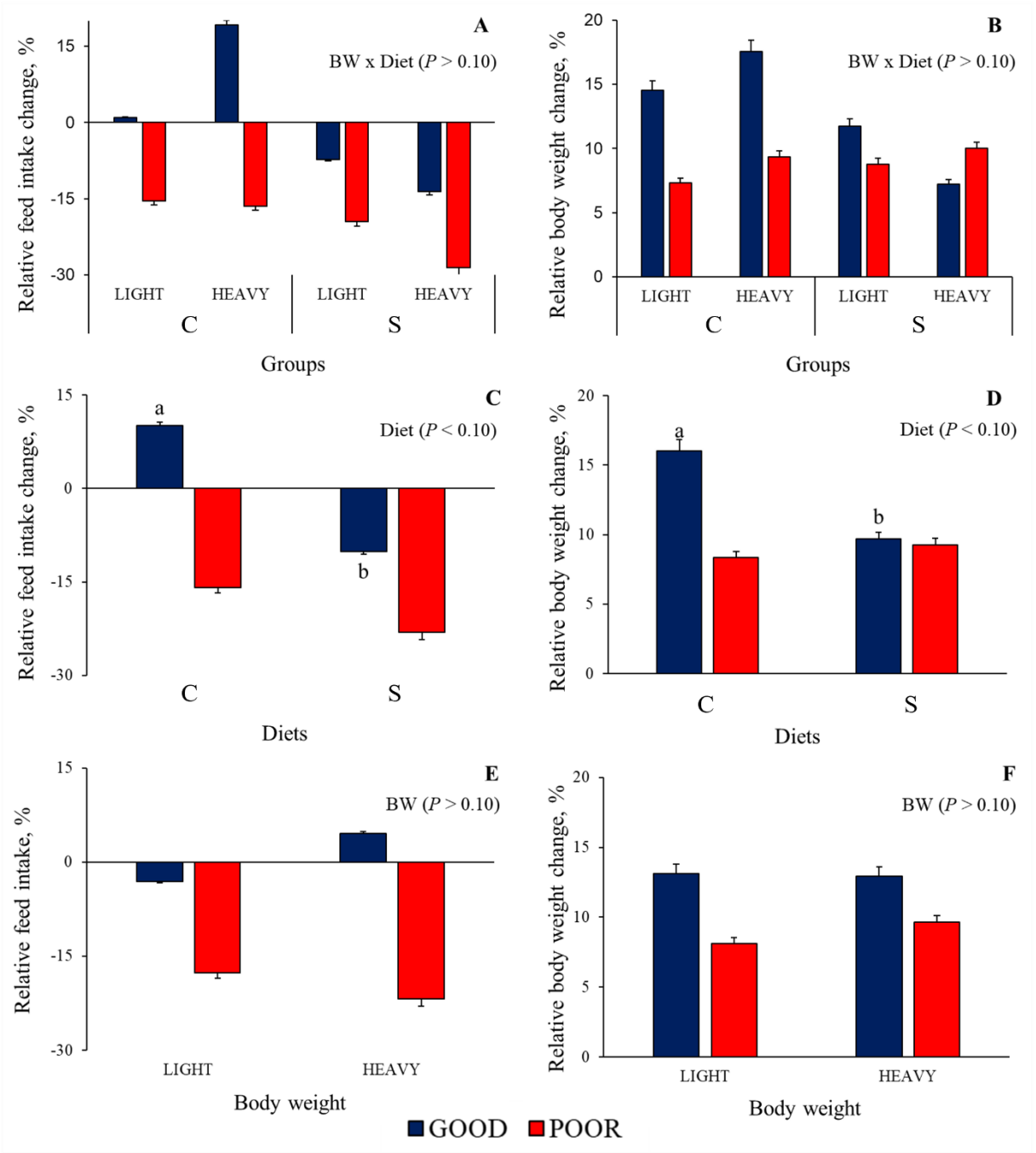
Figure 3. Principal component analysis (PCA) constructed with heavy (H) and light (L) pigs allotted in good (G) or poor (P) sanitary conditions at day 0, previous to the sanitary challenge. PC, principal component; contrib, contribution; bw, body weight; bp, body protein percentage; bl, body lipid percentage.

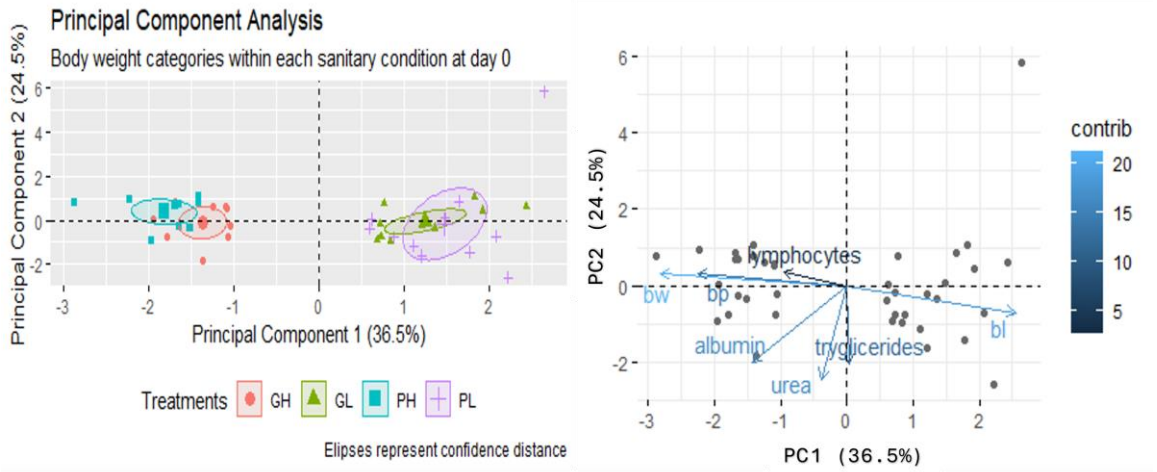
Figure 4. Principal component analysis (PCA) constructed with heavy (H) and light (L) pigs fed control (C) or supplemented diet (S) at 14 and 28 days post-challenge allotted in good sanitary conditions. PC, principal component; contrib, contribution; bw, body weight; bp, body protein percentage; bl, body lipid percentage; index, index of irregularity of feed intake.

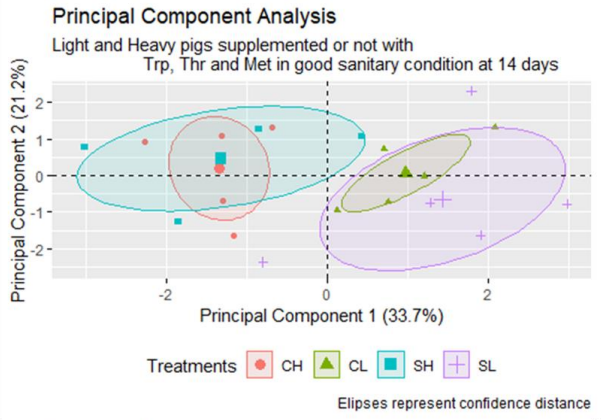
Figure 5. Principal component analysis (PCA) constructed with heavy (H) and light (L) pigs fed control (C) or supplemented diet (S) at 14 and 28 days post-challenge allotted in poor sanitary conditions. PC, principal component; contrib, contribution; bw, body weight; bp, body protein percentage; bl, body lipid percentage; index, index of irregularity of feed intake.

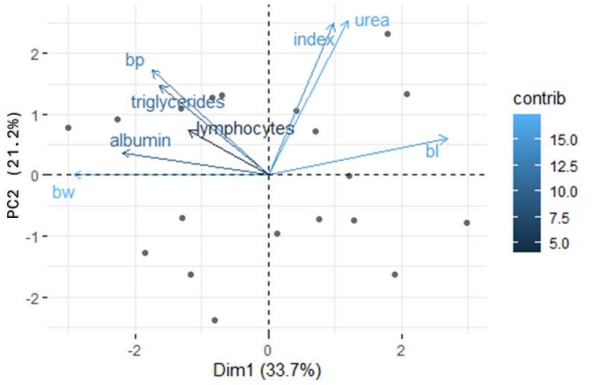

Day ($P < 0.10$) A
Rectal temperature, °C
40.2
39.9
39.6
39.3
39.0
38.7
ad
bc
b
c
a
bc
bc
bcd
0
1
2
3
4
5
6
7
Days post-challenge

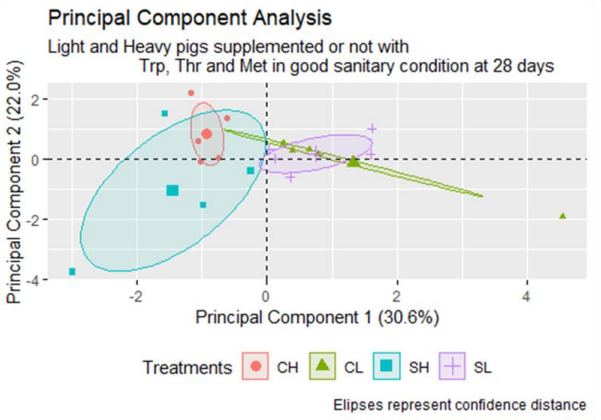

Day ($P < 0.10$) B
Rectal temperature, °C
40.2
39.9
39.6
39.3
39.0
38.7
c
ab
ab
ab
a
ab
b
c
0
1
2
3
4
5
6
7
Days post-challenge
Light C
Light S
Heavy C
Heavy S

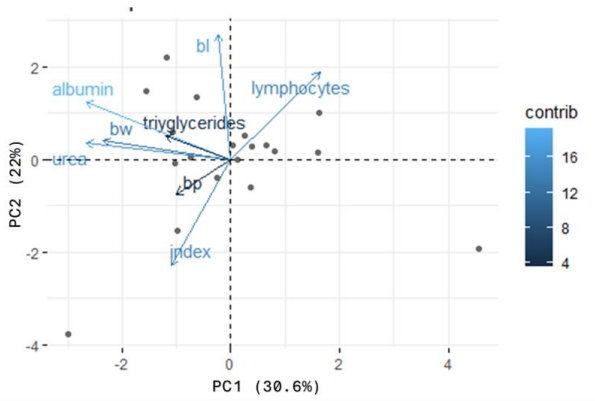

A
BW x Diet (P > 0.10)
Relative feed intake change, %
15
0
-15
-30
LIGHT
HEAVY
LIGHT
HEAVY
C
S
Groups
B
BW x Diet (P > 0.10)
Relative body weight change, %
20
15
10
5
0
LIGHT
HEAVY
LIGHT
HEAVY
C
S
Groups
C
Diet (P < 0.10)
Relative feed intake change, %
15
0
-15
-30
a
b
C
S
Diets
D
Diet (P < 0.10)
Relative body weight change, %
20
15
10
5
0
a
b
C
S
Diets
E
BW (P > 0.10)
Relative feed intake, %
15
0
-15
-30
LIGHT
HEAVY
Body weight
F
BW (P > 0.10)
Relative body weight change, %
20
15
10
5
0
LIGHT
HEAVY
Body weight
GOOD
POOR

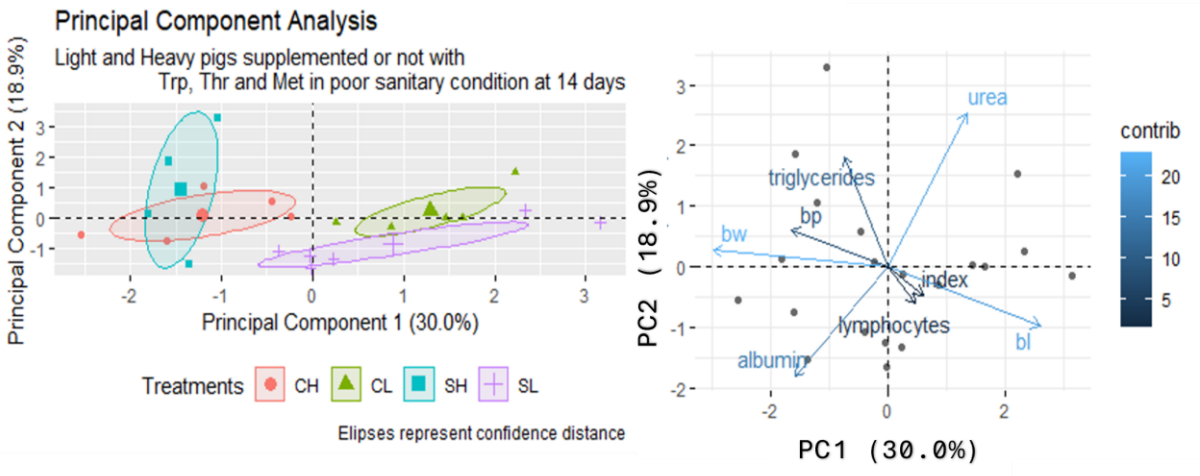

Principal Component Analysis
Body weight categories within each sanitary condition at day 0
Principal Component 2 (24.5%)
Principal Component 1 (36.5%)
Treatments GH GL PH PL
Elipses represent confidence distance
contrib
lymphocytes
bw
bp
albumin
triglicerides
urea
bl
PC2 (24.5%)
PC1 (36.5%)

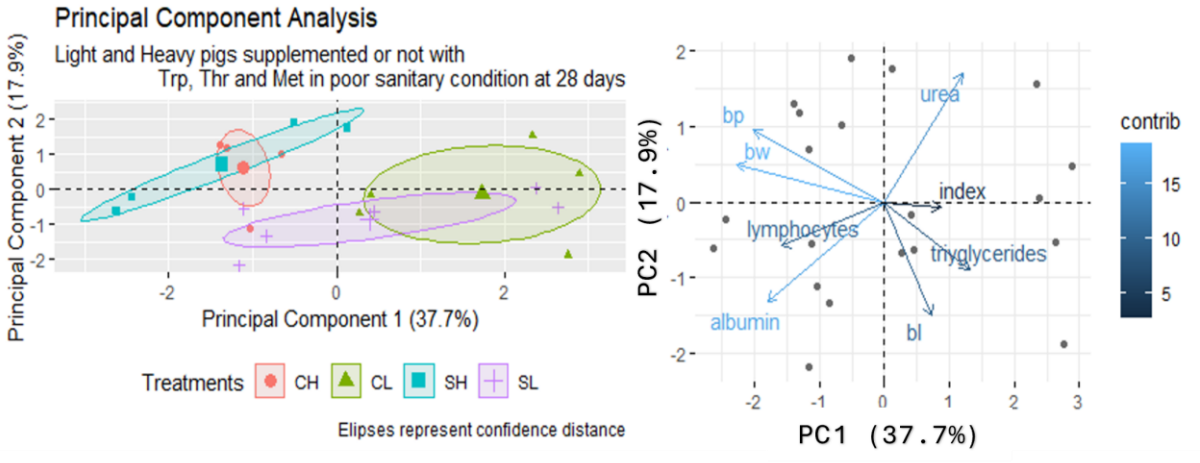

Principal Component Analysis
Light and Heavy pigs supplemented or not with
Trp, Thr and Met in good sanitary condition at 14 days
Principal Component 2 (21.2%)
Principal Component 1 (33.7%)
Treatments CH CL SH SL
Elipses represent confidence distance


urea
index
bp
triglycerides
lymphocytes
albumin
bl
bw
PC2 (21.2%)
Dim1 (33.7%)
contrib
15.0
12.5
10.0
7.5
5.0


Principal Component Analysis
Light and Heavy pigs supplemented or not with
Trp, Thr and Met in good sanitary condition at 28 days
Principal Component 2 (22.0%)
Principal Component 1 (30.6%)
Treatments CH CL SH SL
Elipses represent confidence distance


bl
albumin
lymphocytes
bw
triglycerides
urea
bp
index
PC2 (22%)
PC1 (30.6%)
contrib
16
12
8
4


Principal Component Analysis
Light and Heavy pigs supplemented or not with
Trp, Thr and Met in poor sanitary condition at 14 days
Principal Component 2 (18.9%)
Principal Component 1 (30.0%)
Treatments CH CL SH SL
Elipses represent confidence distance
urea
triglycerides
bp
bw
index
lymphocytes
albumin
bl
contrib
PC2 (18.9%)
PC1 (30.0%)


Principal Component Analysis
Light and Heavy pigs supplemented or not with
Trp, Thr and Met in poor sanitary condition at 28 days
Principal Component 2 (17.9%)
Principal Component 1 (37.7%)
Treatments CH CL SH SL
Elipses represent confidence distance
urea
bp
bw
index
lymphocytes
triglycerides
albumin
bl
contrib
PC2 (17.9%)
PC1 (37.7%)

CHAPTER 6 – Non-target metabolomics profiling indicates the dietary Trp, Thr, and Met supplementation potential to mitigate alterations in protein and energy metabolism in immune-stimulated growing pigs

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Draft to be submitted to Scientific reports

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Abstract: The tryptophan (Trp), threonine (Thr), and methionine (Met) requirements have been shown to play a considerable part in supporting immune system activation in immune-challenged pigs. However, the metabolic pathways affected by dietary Trp, Thr, and Met supplementation on challenged pigs to cope with an immune system stimulation (ISS) have not yet been elucidated. The study aimed to identify blood metabolites and pathway changes in growing pigs under ISS fed with dietary Trp, Thr and Met supplementation. Pigs (10 per treatment) were exposed to oral inoculation of *Salmonella* Typhimurium and poor housing condition (poor SC) or were housed in good sanitary condition (good SC) without being inoculated with *Salmonella* Typhimurium. Two diets were provided within each SC: control (CN) or supplemented (AA+) with Trp, Thr, and Met at 120% of NRC recommended levels. After six hours of fasting, plasma samples were collected via jugular puncture at 0, 14, and 28 days after ISS. Plasma metabolites were analyzed using the GC-MS approach. Partial least squares discriminant analysis (PSL-DA) explored the differential profiling of metabolites among groups. Variable importance for projection (VIP) and volcano plots were used to identify significant features in discriminating groups. Enrichment and pathway analysis were performed to highlight pathway changes between challenged pigs fed with CN or AA+ diet. The PLS-DA showed different clustering for poor SC pigs fed the CN diet and the other treatments throughout the experimental period. On d 14, the VIP showed a decrease in histidine, aminomalonic acid, fumaric acid, tryptophan, ornithine, pseudouridine, cysteine, and threonine - in poor SC pigs fed CN diet compared to other groups. In addition, there was a decrease in plasma α-glucoside and mannose along with an increase in 1,5-anhydro-d-mannitol, indicating insufficient energy supply in poor SC pigs fed CN diet. On d 28, both the cluster and VIP analysis showed a persistent shortage in glucose availability in poor SC pigs fed CN diet compared to good SC pigs fed AA+ diet, as plasma erythronic acid, α-glucoside,

and 2,5-dimethoxymandelic acid decreased. Furthermore, plasma ornithine and Trp were present at lower concentrations in poor SC pigs fed the CN diet than good SC pigs fed the AA+ diet. Finally, the pathway analysis suggested that the poor SC pigs fed the AA+ diet had enhanced gut integrity and antioxidant capacity and lower energy and protein metabolism changes compared to poor SC pigs fed the CN diet. In conclusion, dietary Trp, Thr, and Met supplementation mitigates inflammation through histidine pathway modulation and alleviates oxidative stress and energy consumption through regulation of glutathione metabolism, cysteine and methionine metabolism, and glycine, serine, and threonine metabolism pathways.

Keywords: amino acids supplementation, antioxidant activity, immune system, plasma metabolites.

1. Introduction

Immune system stimulation (ISS) can negatively affect pigs' performance even when no clinical signs of illness are observed[1]. Reduced growth performance is triggered by alterations in pigs' nutrient utilization, leading to nutrients being relocated from growth to immune functions[2]. Under these circumstances, a better understanding of the impact of ISS and innovative strategies on pig physiology and metabolism is essential to attenuate the impact on pig health and farms' profitability.

Dietary supplementation of certain amino acids (AA) such as tryptophan (Trp), threonine (Thr), and methionine (Met) above NRC3 requirements is shown to alleviate the negative effects of ISS on reduced growth rate[4,5,6,7]. These AAs are associated with positive growth and health effects due to improved intestinal mucosa integrity, antioxidant defense, and immune molecule synthesis[8].

The AA supplementation's impact on inflammatory responses has been illustrated by changes in blood parameters, such as immune molecule synthesis, cell proliferation[9], AA plasmatic levels[10], acute phase proteins[5] and antioxidant molecules[11] concentrations. However, recently, great efforts have been directed toward a systematic approach that allows an understanding of global metabolic responses. One of these approaches, which improves the

comprehension of the interactions occurring inside the organisms and their environment, is detecting and quantifying metabolites within a single sample[12]. Thus, plasma samples may be a tool for an accessible metabolic footprint, providing a picture of the metabolic events that occurred and may reveal changes in metabolic pathways under different physiological or nutritional conditions[13,14].

Therefore, this study aimed to explore the metabolite alterations induced by oral Salmonella Typhimurium and poor housing conditions during the growing phase of pigs and determine whether the dietary supplementation of Trp, Thr and Met:Lys above NRC requirements may impact metabolic pathways of ISS growing.

2. Material and Methods

All experimental procedures in this trial followed the Brazilian National Council of the Control of Animal Experimentation (CONCEA) and were reviewed and approved [protocol no. 4784/20] by the Ethical Committee on Animal Use (CEUA) of *Faculdade de Ciências Agrárias e Veterinárias* (FCAV/UNESP – Jaboticabal).

Animals, housing, management, and experimental diets

The dataset used in this study was collected from 40 [25.6 ± 2.3 kg of initial body weight (BW)] high-performance gilts [Pietrain × (Large White × Landrace)] raised at the Swine Research Facility (UNESP, Jaboticabal, SP, Brazil) for a 28 days (d) trial (n = 10 pigs/treatment). A detailed description of the experimental setup (animals, housing, and immune system challenge model protocol) can be found in a previous publication[4].

Shortly, the gilts were housed in two growing-finishing barns, which represented two different sanitary conditions (SC): good (without *Salmonella* Typhimurium (ST) inoculation and with disinfection and cleaning protocol) or poor (challenged with ST and manure spreading on the barn floor). Within each SC, pigs were group-housed on a solid concrete floor pen (0.9 m^2/pig). Humidity and temperature were set at 60% and 24°C (± 2°C), respectively, and a 12 h light period was set from 0700 to 1900. Additionally, the pen was equipped with four electronic feeding stations, and six ball-bite drinkers were placed. Pigs had unrestricted access to feed and water throughout the trial. Pigs allotted in poor SC were orally inoculated with a 5 mL solution containing *Salmonella enterica* serovar Typhimurium (ST, 2 × 10^9 CFU), and fresh manure from a commercial pig farm was spread on the barn floor. No daily cleaning was applied. On the other hand, pigs at the good SC pigs were inoculated with

a saline solution without ST (to have the same handling stress), and no manure was applied on the floor. The good SC was washed twice a day and disinfected weekly using potassium monopersulfate (1:200; Virkon; Lanxess, Colony, Germany). Daily animal monitoring was conducted with the good SC first and then the poor SC to avoid cross-contamination.

Furthermore, two diets (Table 1) were provided in each SC: a control diet (CN), with the AA profile according to NRC (2012), and a supplemented diet (AA+), with the AA profile containing 20 % greater standardized ileal digestible (SID) Trp:Lys, Thr:Lys, and Met + Cys:Lys than the CN. The diets were animal protein-free and corn-soybean meal-based. They were formulated using the reported nutrient and analyzed AA contents of ingredients to provide the nutrient requirements from 25 to 50 kg BW according to NRC[3], except for the Trp, Thr, and Met+Cys in diet AA+. The diets contained 2550 kcal/kg net energy and 16% crude protein. No antibiotics in-feed were used as growth promoters throughout the trial.

Data collection

The electronic feeding stations measured individual daily feed consumption, and the individual BW was obtained before challenge (d -1), at 14, and 28 d post-challenge. After six hours of fasting, blood samples were collected from the jugular vein on 0, 14, and 28 dpc. Each sampling collected one 4 mL EDTA tube (Vacutainer®; BD; Franklin Lakes, NJ, EUA) per animal. After blood sampling, the tube per pig was centrifuged for 10 min at 3,000 × g at 4°C after sampling. The plasma was transferred to storage tubes, frozen in liquid nitrogen, and stored at -80 °C for subsequent metabolomics testing.

Sample preparation for non-target metabolomics profiling

Samples were subjected to metabolite extraction protocol according to Liu et al., 2018). The plasma samples (300 µL) were extracted using a 200 µL ice-cold extraction mix (acetonitrile:methanol, 1:1, v:v). After vortexing for 5 min, the samples were centrifuged at 15,000 × g for 10 min at 4 °C for deproteinization (Eppendorf, Germany). The supernatant fractions were then collected and freeze-dried in a lyophilizer (LS 3000; Terroni, São Carlos, SP, Brazil). The resulting dry residues were re-suspended in 200 µL of methanol: water (4:1), vortexed, and centrifuged at 15,000 × g for 15 min at 4 °C. Lastly, the supernatant fractions were transferred to sampler vials to be analyzed on a GC-MS system.

GC-MS analysis

Aliquots (80 µL) of each sample were freeze-dried in the lyophilizer (LS 3000;

Terroni, São Carlos, SP, Brazil) and subjected to derivatization with 30 μL methoxyamine-HCl (15 mg/mL) in pyridine for 16 h at room temperature. The trimethylsilylation was accomplished with the addition of 1% trimethylchlorosilane in 30 μL of n-methyl-n-(trimethylsilyl)-trifluoroacetamide to the samples, followed by incubation for 1 h at room temperature. After silylation, heptane (30 μL) was added to samples, which were immediately analyzed in a 7890A Agilent Gas Chromatograph coupled to a Pegasus HT TOF Mass Spectrometer (LECO, Saint Joseph, MI, USA).

Samples were injected with a mix of n-alkanes standards (C12 – C40) for the retention times corrections. The derivatized samples (1 μL) were injected in splitless mode using an automatic sampler-CTC Combi Pal Xt Duo (CTC Analytics AG, Zwingen, Switzerland) coupled to the GC–MS system equipped with two silica columns in line. The first was a DB 5 column (20 m long × 0.18 mm internal diameter × 0.18 μm thick) (Agilent J & W Scientific, Folsom, CA, USA), and the second was a Rxi-17 column (0.84 m long × 0.1 mm internal diameter × 0.1 μm thick) (Restek Corporation, Bellefonte, PA, USA). The injector was set at 280 ºC, the septum bleed rate was 20 mL/min, and started 250 s after the data acquisition[15]. The gas flow was 1 mL/min, and the temperature of the first column was maintained at 80 ºC for 2 min and increased at 15 ºC/min to 305 ºC, with a 10 min hold. The temperature of the second column was maintained at 85 ºC for 2 min and then raised to 310 ºC at 15 ºC/min, with a 10 min hold. The column effluent was introduced into the ionization source of the Pegasus HT TOF MS. The transfer line and ionization source temperatures were held at 280 and 250 ºC, respectively. The ions were generated by an electron source (70-eV) at an ionization current of 2.0 mA, and 20 spectra/s were acquired in a mass range of 45 to 800 m/z, with the detector voltage set to 1500 V.

Data processing

The processing of GC-TOF/MS data was performed in two steps. First, the generated chromatograms were exported to the ChromaTOF Software (version 1.2.0.6; LECO, Saint Joseph, MI, USA), in which baseline correction, deconvolution of the spectra, retention rate correction, retention time correction, peak identification, and alignment and identification of metabolites were processed using the NIST library (version 2.4). Only metabolites with three or more characteristic masses and a score of 800 or higher were considered valid. Isomers were manually checked and merged, and feature intensities were normalized by the total ion chromatogram. Before the statistical analysis, the data were log-transformed and Pareto-

scaled. Missing values were imputed by the minimum nonzero value method.

Statistical Analysis

A comparison of the metabolomic profile between groups was performed using the MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca). Partial Least Squares Discriminant Analysis (PLS-DA) 2D score plot and heatmaps were carried out to explore the differential metabolites among groups. Variable Importance for Projection (VIP) score was calculated to determine the influence of every component on the principal scores. Additionally, pairwise analysis was performed using a volcano plot, which combines fold change ($FC \geq |2.0|$) and t-test analysis ($p \leq 0.05$) to identify the features that were potentially significant in discriminating groups. Enrichment and pathway analysis was performed employing the *Homo sapiens* library as a reference since *Sus scrofa* library was not available, using impact value higher than 0.1, −log p-value higher than 2, and false discovery rate (FDR) lower than 0.05 cutoffs values for relevance.

3. Results

The PLS-DA score plot of the metabolites showed no difference among treatments before challenge (Fig. 1).

Meanwhile, the PLS-DA score plot of the metabolites in plasma samples showed differences in clustering for poor SC pigs fed CN diet throughout the experimental period (Fig. 2). This difference was also shown by the Heatmap plot (Fig. 3), where the abundance of several metabolites was different in poor SC pigs fed CN and the other treatments on d 14 and 28.

To further explore the metabolic profile differences among treatments, PLS-DA was performed to compare treatments within days. On d 14, PLS-DA showed differences in clustering for poor SC pigs fed CN diet and the other treatments (Fig. 4a). The VIP score plot showed that the cluster differences were mainly due to downregulation of mannose, histidine, aminomalonic acid, ethyl-alpha-D-glucopyranoside, cysteine, fumaric acid, tryptophan, ornithine, pseudouridine, phenylpropanol and threonine, and upregulation of 1,5-anhydromannitol from poor SC pigs fed CN diet compared to other groups (Fig. 5a). Conversely, on d 28, PLS-DA demonstrated differences in clustering for poor SC pigs fed CN and good SC fed AA+ diet (Fig. 4b). The VIP score plot showed that this difference was mainly due to the downregulation of erythronic acid, ethyl-alpha-d-glucopyranoside,

ornithine, 2,5-dimethoxymandelic acid, and tryptophan between poor SC pigs fed CN diet compared to good SC pigs fed AA+ diet (Fig. 5b).

Since there were pronounced metabolic profile differences in poor SC pigs, especially in pigs fed CN diet, volcano plots and enrichment and pathway analysis were performed to compare poor SC pigs fed CN and AA+ diets within days. On d 14, myo-inositol was downregulated, whereas histidine, pseudouridine, cysteine, fumaric acid, ethyl α-d-glucopyranoside, cysteine, and mannose were upregulated in pigs fed AA+ compared to CN diet (Fig. 6a). Additionally, the most deeply impacted pathway (Fig. 7a) was histidine metabolism (impact = 0.22, −log p value = 3.90, FDR < 0.01). On d 28 d, hydroxyproline was downregulated, while cystine, cysteine, aminomalonic acid, mannose, and fumaric acid were upregulated in pigs fed AA+ compared to CN diet (Fig. 6). The most deeply impacted pathways (Fig. 7b) were glutathione metabolism (impact = 0.12, −log p value = 8.86, FDR < 0.01), arginine and proline metabolism (impact = 0.44, −log p value = 6.53, FDR < 0.01), cysteine and methionine metabolism (impact = 0.20, −log p value = 6.33, FDR < 0.01), glycine, serine and threonine metabolism (impact = 0.24, −log p value = 4.84, FDR < 0.01), histidine metabolism (impact = 0.22, −log p value = 3.15, FDR < 0.01), tyrosine metabolism (impact = 0.15, −log p value = 3.05, FDR < 0.01), alanine, aspartate and glutamate metabolism (impact = 0.53, −log p value = 2.87 FDR < 0.01), arginine biosynthesis (impact = 0.17, −log p value = 2.80, FDR < 0.01), phenylalanine metabolism (impact = 0.35, −log p value = 2.36, FDR < 0.01).

4. Discussion

This study investigated the metabolite changes in growing pigs under ISS by oral Salmonella Typhimurium inoculation and poor housing conditions and explored the plasma metabolites and metabolic pathways affected by AAs supplementation that improves ISS pigs' growth and adaptive responses. To compare the changes between groups, a GC–MS-based metabolomic analysis was conducted on plasma from pigs collected at different time points after ISS.

The current outcomes indicate that poor SC impacted the abundance of intermediate and end products of protein and energy-related pathways throughout the experimental period. These metabolic changes may reflect pigs' adaption to redirected nutrients from different sources to support the immune system activation to cope, mainly when lower feed intake is

observed[4]. Additionally, the study showed the positive effect of AA supplementation above the requirement for growth in regulating inflammation-related pathways, enhancing antioxidant capacity, and lowering changes in carbohydrate metabolism and energy supply.

The PLS-DA plots also showed differences in clustering treatments according to their metabolic profiles within days after ISS, indicating changes in the pigs' metabolic patterns during the challenge period. On d 14 d, there was a separation between poor SC pigs fed the CN diet and the other treatments. The first two weeks after a challenge are considered the acute phase, during which the main inflammatory response and metabolic changes can occur[16], especially for protein-relate metabolites. Indeed, on d 14 d, half of the top downregulated compounds in poor SC pigs fed the CN diet identified in the VIP analysis were related to amino acid metabolism: histidine, aminomalonic acid, cysteine, tryptophan, ornithine, and threonine. As previously reported, AAs may contribute to several metabolic pathways under an ISS, especially cell proliferation, tissue reparation, inflammation, and redox balance to support cellular function[8].

For example, proinflammatory cytokines are produced in the infected cells under ISS. As a result, those molecules lead to the recruitment of inflammatory cells, which secrete histamine, a potent vasodilator, facilitating the migration of immune cells from the blood to inflamed tissues[17]. Histidine is the body's primary AA precursor of histamine production[18]. Therefore, its relative decrease in the plasma in poor SC pigs fed a CN diet may be related to inflammation and histamine production.

Histamine can sway the metabolism of polyamines and amino acids, including arginine and ornithine, to regulate cell proliferation and death[19]. When induced by histamine, immune cells release nitric oxide (NO) in damaged tissue, triggering pathogen neutralization[20]. The NO synthesis is driven by arginine, its main target substrate[21]. In this process, ornithine, a non-proteinogenic amino acid, may be used as a precursor to generate arginine. In fact, the decrease in plasma ornithine concentration was observed in poor SC pigs fed a CN diet, which may be partly related to potential pathogen neutralization. Besides, the alteration in the intestinal lumen environment due to inflammation may also cause a decrease in ornithine concentration. Along with histamine, immune cells and cytokines can lead to gut permeability and cell apoptosis[22]. Consequently, intestinal barrier renovation is stimulated to improve gut barrier integrity. Since ornithine participates in the cycle of polyamine synthases, it may have helped regulate gut epithelial renewal and barrier function, reducing its plasma

concentration.

The relative decrease of mannose, Thr, and Trp may also be linked to gut epithelial renewal and inflammatory response. For example, mannose concentration in the blood has been associated with mannose glycosylation, an important process for glycoprotein synthesis in the gut barrier epithelium[23]. Additionally, its plasma concentration has been reported to negatively correlate with intestinal permeability[24]. Hence, the downregulation of mannose infers that poor SC pigs fed the CN diet were undergoing intense intestinal repair to reduce intestinal permeability and damage.

On the other hand, plasma Thr in poor SC pigs may have been sequestrated by gut cells to reduce pathogen gut adhesion and translocation[25]. During an inflammatory response, histamine is a potent inducer of intestinal mucus, which may increase Thr demand in the gut. Therefore, plasma Thr decrease in poor SC pigs fed CN diet may result from mucus, IgA, and other protein synthesis to maintain intestinal functionality. Meanwhile, lower plasma Trp concentration may be linked to cytokine activating the indoleamine 2,3-dioxygenase enzyme. This enzyme is responsible for the catabolism of Trp into kynurenine[26] and regulates T-cell proliferation[27]. As a result, the decreased Trp in poor SC pigs fed CN diet may emerge from immune response regulation.

The relative decrease in pseudouridine concentration may also be related to the oxidative stress caused by the inflammatory response, along with Cys, fumaric acid, and aminomalonic acid. The reactive oxygen species generated under inflammatory response can cause oxidative damage to macromolecules such as DNA/RNA and cell membranes. Pseudouridine is RNA's most abundant modified nucleoside[28], forming stable pairs with all nucleotides[29]. Thus, a decrease in pseudouridine relative concentration in poor SC pigs fed CN diet may occur to support RNA synthesis. At the same time, oxidative stress may increase the demand for antioxidant compounds, such as glutathione, which needs three AAs for its synthesis: glycine, cysteine, and glutamic acid. Aminomalonic acid is a metabolite resulting from glycine oxidation[30], and its relative decrease in plasma of poor SC pigs fed CN diet may indicate a metabolic shift towards prioritizing glutathione synthesis. Concomitantly, the Cys' plasma reduction in poor SC pigs fed a CN diet may be linked to glutathione production, which reduces oxidative damage. Along with glutathione, plasma fumaric acid may have been reduced in poor SC pigs fed CN diet to mitigate inflammation and oxidative stress. Under inflammation, fumaric acid can be converted into itaconic acid, which possesses anti-

inflammatory properties that inhibit the production of pro-inflammatory cytokines[31], regulating the immune response and avoiding overstimulation. Additionally, esters of fumaric acid, mainly dimethyl fumarate, exhibit anti-oxidative effects, contributing to cell protection against damage caused by oxidative stress[32].

During inflammation, alterations in energy metabolism are frequently observed. Poor SC pigs fed CN diet had downregulation of α-glucoside, a glucose analog form, and upregulation of 1,5-anhydro-mannitol, a glucose-related metabolite associated with glycemic control[33]. When challenged pigs have reduced feed intake, a short-term caloric restriction may occur due to a lower amount of carbohydrates available for digestion. At the same time, pigs might have impaired digestion caused by inflammation[34], affecting blood glucose levels and tissue availability. Indeed, poor SC pigs fed CN diet showed reduced α-glucoside, corroborating the decrease in carbohydrates for energy production. Concomitantly, the energy demand of immune cells increases under inflammatory response[35]. To improve glucose concentration in the bloodstream, degradation of α-1,4-glucans, including glycogen, occurs. Some intermediate metabolites of glycogen metabolism also increase, such as 1,5-Anhydro-D-fructose (AF), which has antioxidative and antibacterial activities[36]. In addition, the NADPH-dependent reductases can reduce AF to 1,5-anhydro-D-mannitol (AM), increasing its concentration in the bloodstream. High amounts of AM and its isomers may induce glucose tubular reabsorption to control glycemia[33], and they are also effective against NO, regulating cytokine production and inflammation[37]. Therefore, the relative increase in AM in poor SC pigs fed the CN diet may be correlated to glycemia control and anti-inflammatory response.

Meanwhile, the dietary AA supplementation of Trp, Thr, and Met may have attenuated the effects of inflammation in pigs' AA and energy-related metabolites, which may have reduced the differences with the good SC pigs, as observed in PLS analysis on d 14. The volcano plot showed that myo-inositol was downregulated, whereas histidine, pseudouridine, cysteine, fumaric acid, α-glucoside, and mannose were upregulated in pigs fed AA+ compared to the CN diet. In addition, the enrichment and pathway analysis demonstrated that the pathway that had the most impact was histidine metabolism in poor SC pigs fed CN compared to the AA+ diet.

The higher apport of Trp, Thr, and Met may have mitigated gut inflammation, immune cell proliferation, and oxidative stress to neutralize pathogen adhesion and gut translocation. For example, the supplementation of Trp and Thr might have enhanced gut integrity, while

Met might have improved the blood antioxidant capacity[38]. Indeed, the histidine pathway modulation in poor SC-fed AA+ diet indicates enhanced antioxidant capacity and lower systemic inflammation in those pigs. In addition, the increase in plasma pseudouridine, cysteine, fumaric acid, and mannose also corroborates the reduction of inflammation and oxidative stress. Lower oxidative stress and ROS might have triggered a decrease in cell reparation, proliferation, and gut permeability, which may have increased the relative concentration of pseudouridine and mannose in plasma. Additionally, lower anti-inflammatory molecules may have been demanded, increasing the relative concentration of cysteine, fumaric acid, and aminomalonic acid in the plasma of poor SC pigs fed an AA+ diet. Lastly, as inflammation was reduced in poor SC pigs fed an AA+ diet, mitigating alterations in glucose metabolism may have occurred to support immune system activation. For example, myo-inositol is a glucose metabolite utilized to generate second messengers and regulate macrophages' cell volume[39]. Higher plasma myo-inositol concentration has been associated with reduced insulin secretion, lipolysis rate, and cell proliferation[40,41]. Accordingly, the downregulation of myo-inositol and the upregulation of α-glucoside in poor SC pigs fed the AA+ diet may indicate that blood glucose was redirected from immune cells to other tissues, supporting growth and protein accretion.

On d 28, some metabolic differences were still observed between groups, especially between poor SC pigs fed the CN diet and good SC pigs fed the AA+ diet. The VIP scores showed that the differences were mainly driven by the downregulation in erythronic acid, α-glucoside, ornithine, 2,5-dimethoxymandelic acid, and tryptophan relative plasma concentrations. The ornithine decrease might indicate that poor SC pigs fed the CN diet were undergoing protein and urea cycle disorders, which might be caused by gut inflammation and reparation. Besides, reduced plasma Trp may be linked to T cell proliferation to neutralize pathogens compared to good SC pigs fed an AA+ diet.

In the meantime, the downregulation of α-glucoside and erythronic acid might indicate a metabolic change in glucose formation and energy utilization in poor SC pigs fed CN diet. The reduced α-glucoside might suggest there was still a shortage in glucose availability in poor SC pigs fed the CN diet compared to good SC pigs fed the AA+ diet. However, erythronic acid reduction may demonstrate that glucose generation from other substrates, such as Thr[42], and the pentose-phosphate pathway dysfunction may have decreased[43]. Consequently, a reduction in catecholamine release may have followed to regulate

carbohydrate metabolism in the liver[44]. Mandelic acid and its derivatives are produced during the metabolism of epinephrine and norepinephrine[45]. Therefore, a relative decrease in plasma might indicate an improvement in the energy metabolism of poor SC pigs fed the CN diet compared to good SC pigs fed the AA+ diet.

Even though on d 28 no cluster differences were observed between poor SC-fed CN or AA+ diet, some metabolites and pathway topology analysis identified differences between groups, regarding muscle catabolism, gut inflammation, antioxidant molecules synthesis, and energy generation. During inflammation, the decrease in protein anabolism and increase in muscle catabolism are commonly observed, enhancing AA release by the muscle into the blood. For example, hydroxyproline, a non-proteinogenic amino acid, has been used as a marker of muscle protein catabolism, as hydroxyproline and proline represent approximately one-third of AA in muscle collagen. In this way, the hydroxyproline relative decrease in the plasma of pigs fed the AA+ diet may indicate a reduction in muscle collagen catabolism[46]. The higher collagen catabolism in poor SC pigs fed the CN diet may be linked to the glycine and proline release in the bloodstream. Proline is the major substrate for the synthesis of arginine, which is required to produce NO and polyamines. The pathway topology analysis showed that arginine and proline metabolism, arginine biosynthesis, and histidine metabolic pathway were enriched in poor SC pigs fed CN compared to the AA+ diet. This may indicate higher gut immune system activation and inflammation. The higher gut inflammation is corroborated by the mannose relative decrease in the plasma of poor SC pigs fed the CN diet compared to the AA+ diet, as its plasma concentration negatively correlates with intestinal permeability and damage. Additionally, the enrichment of alanine, aspartate, and glutamate metabolic pathways suggests the need for energy substrates for rapidly dividing cells, especially enterocytes[47].

Along with gut inflammation and epithelial damage, some metabolites, such as cysteine and aminomalonic acid, indicate that poor SC pigs fed the CN diet had a higher demand for antioxidant compounds than pigs fed the AA+ diet. The higher relative concentration of aminomalonic acid in the plasma of poor SC pigs fed an AA+ diet may indicate a higher glycine utilization in transamination reactions rather than its incorporation in other proteins, such as glutathione. Additionally, the enrichment of the glycine, serine, and threonine pathway might indicate the higher demand for glycine to produce glutathione. Conversely, pigs fed the AA+ diet may increase plasma cysteine due to the higher apport of

Met + Cys. However, the mannose relative downregulation and the enrichment of the glutathione and cysteine and methionine metabolic pathways may infer the deviation of cysteine to antioxidant compounds synthesis and cell proliferation. Despite cysteine's utilization in glutathione synthesis, Met and Cys are involved in the biosynthesis of immune system proteins[48], mucosal integrity, and methylation reactions, such as methylation of DNA and other proteins[49].

Finally, the pathway topology analysis also indicated that the poor SC may have induced phenylalanine, tyrosine, alanine, glutamate, and aspartate metabolic pathways to synthesize more glucose for cellular physiological regulation in pigs fed CN diet compared to the AA+ diet. Tyrosine is a potent ketogenic amino acid[50], whereas alanine, glutamate, and aspartate are glucogenic amino acids when lower glucose is available. Through hydroxylation reaction, phenylalanine can generate tyrosine, which can undergo various metabolic pathways, such as neurotransmitters like norepinephrine and epinephrine, to regulate carbohydrate metabolism in the liver[44]. Moreover, all these AAs can be metabolized to produce intermediates that enter the Krebs cycle as energy substrates. As some intermediates from the Krebs cycle had a relative plasmatic decrease in poor SC pigs fed CN diet due to its anti-inflammatory properties, such as fumaric acid, those pathways may have been enriched to maintain energy availability.

5. Conclusions

Immune system stimulation decreases plasma AAs such as histidine, tryptophan, ornithine, cysteine, and threonine to neutralize pathogen gut mucosa adhesion and systemic translocation. Additionally, the ISS impairs energy-related plasma metabolites such as α-glucoside and 1,5-anhydro-d-mannitol, indicating a metabolic change in glucose formation and energy utilization in challenged pigs. However, Trp, Thr, and Met + Cys supplementation, 20 % above NRC requirements for growth, mitigates inflammation through the downregulation of the histidine pathway, increases the antioxidant capacity, and lowers changes in energy supply through regulation of glutathione metabolism, cysteine and methionine metabolism, and glycine, serine, and threonine metabolism pathways.

6. Conflict of interest statement

The authors declare no conflicts of interest.

7. Data Availability

Due to our institutional policy, data will be available upon request only.

8. Author Contributions

Conceptualization, G.A.V.C., A.R. and L.H.; methodology, G.A.C.V., A.R. software, G.A.V.C.; validation, G.A.V.C.; formal analysis, G.A.V.C., and A.R.; investigation, G.A.C.V.; resources, L. H.; data curation, G.A.C.V. and D.A.M.; writing—original draft preparation, G.A.C.V.; writing—review and editing, A.R., and L.H.; visualization G.A.C.V.; supervision, A. R., and L.H.; project administration, L.H.; funding acquisition, L.H. All authors have read and agreed to the published version of the manuscript.

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Figure legend

Fig. 1. Partial Least-Squares Discriminant Analysis (PLS-DA) plot in plasma samples before challenge. The PLS-DA plot showed no difference among clusters between treatments at day 0. Shaded areas in different colors represent the 95% confidence interval.

Fig. 2. Partial Least-Squares Discriminant Analysis (PLS-DA) plot in plasma samples after challenge (days 14 and 28 post-challenge). The PLS-DA plot of the metabolites in plasma samples showed differences in clustering for pigs housed in poor sanitary conditions fed a control (CN) diet at 14- and 28-days post-challenge and the other treatments. Shaded areas in different colors represent the 95% confidence interval.

Fig. 3. Heatmap of metabolites of treatments at 14- and 28-days post-challenge. The heatmap showed a distinct pattern of abundance of metabolites between pigs housed in poor sanitary conditions fed a control (CN) diet at 14- and 28-days post-challenge and the other treatments.

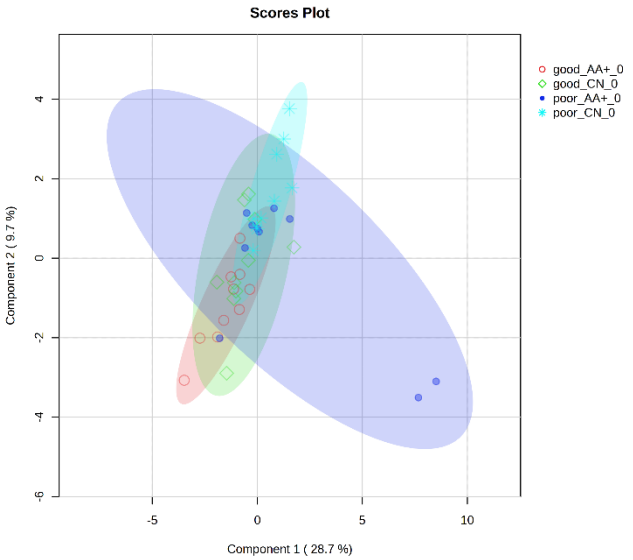
Fig. 4. Partial Least-Squares Discriminant Analysis (PLS-DA) plot in plasma samples within 14- and 28-days post-challenge. **a**. The PLS-DA plot of the metabolites in plasma samples showed differences in clustering for pigs housed in poor sanitary conditions fed the control (CN) diet at 14 days post-challenge from the other treatments. Shaded areas in different colors represent the 95% confidence interval. **b**. The PLS-DA plot of the metabolites in plasma samples showed differences in clustering for pigs housed in poor sanitary conditions fed the control (CN) diet and pigs housed in good sanitary conditions fed the supplemented (AA+) diet at 28 days post-challenge. Shaded areas in different colors represent the 95% confidence interval.

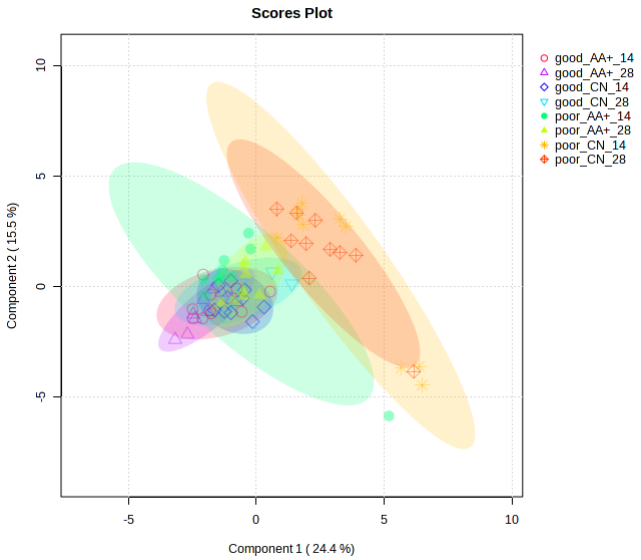
Fig. 5. Variable Importance for Projection (VIP) score of plasma metabolites of pigs housed in good or poor sanitary conditions fed control (CN) or supplemented (AA+) diet at 14- (**a**) or 28- days post-challenge (**b**). The colored boxes indicate the relative intensities of the corresponding compounds in each treatment. Red represents higher relative abundance, while blue represents lower abundance in the VIP score plot.

Fig. 6. Volcano plot of plasma metabolites of pig housed in poor sanitary condition at 14- (**a**) or 28- days post-challenge (**b**). X-axis values correspond to log 2 (fold change), and y-axis values represent -log 10 (false discovery rate). Blue points represent a significant decrease of a plasma metabolite (DOWN), grey points represent no difference (Non-SIG), and red points represent a significant increase of a plasma metabolite (UP).

Fig. 7. Topology analysis of metabolic pathways identified of pigs housed in poor sanitary conditions fed a control diet in relation to a supplemented diet at 14- or 28-days post-challenge. **a**. At 14 days post-challenge, the pathway identified was histidine metabolism (I). **b.** At 28 days post-challenge, the pathways identified were glutathione metabolism (I), arginine and proline metabolism (II), cysteine and methionine metabolism (III), glycine, serine, and threonine metabolism (IV), histidine metabolism (V), tyrosine metabolism (VI), arginine biosynthesis (VII), phenylalanine metabolism (VIII), alanine, aspartate and glutamate metabolism (IX). The X-axes represent the pathway impact, and the Y-axes represent the pathway enrichment. Larger sizes and darker colors represent greater pathway enrichment and

higher pathway impact values. The cut-off values were pathway impact higher than 0.1 and −log P-value higher than 2. The pathways identified showed a false discovery rate lower than 0.05


Scores Plot
good_AA+_0
good_CN_0
poor_AA+_0
poor_CN_0
Component 2 (9.7 %)
Component 1 (28.7 %)
-6
-4
-2
0
2
4
-5
0
5
10


Scores Plot
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good_AA+_28
good_CN_14
good_CN_28
poor_AA+_14
poor_AA+_28
poor_CN_14
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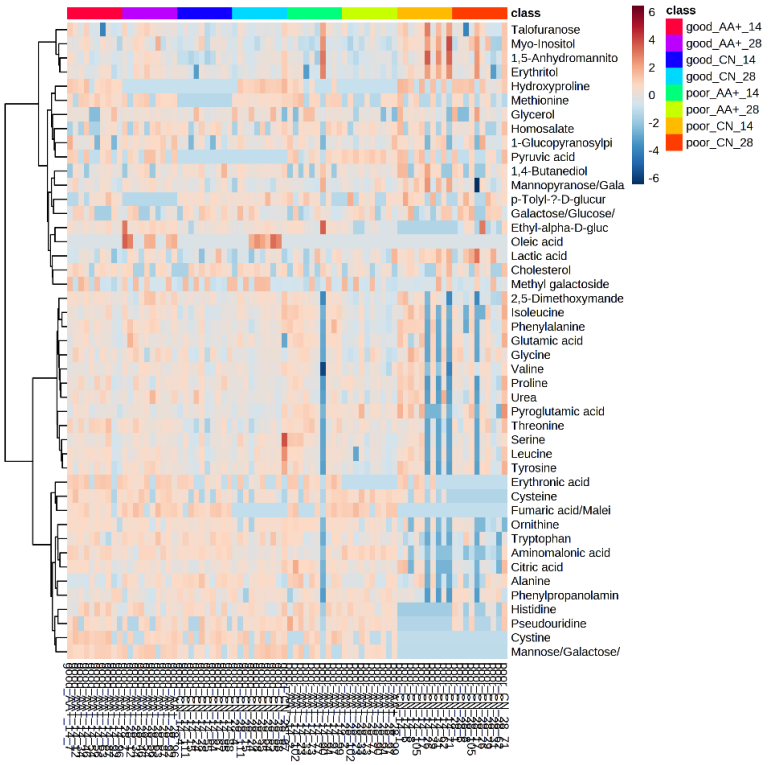
Component 1 (24.4 %)
10
5
0
-5
-5
0
5
10


class
Talofuranose
Myo-Inositol
1,5-Anhydromannito
Erythritol
Hydroxyproline
Methionine
Glycerol
Homosalate
1-Glucopyranosylpi
Pyruvic acid
1,4-Butanediol
Mannopyranose/Gala
p-Tolyl-?-D-glucur
Galactose/Glucose/
Ethyl-alpha-D-gluc
Oleic acid
Lactic acid
Cholesterol
Methyl galactoside
2,5-Dimethoxymande
Isoleucine
Phenylalanine
Glutamic acid
Glycine
Valine
Proline
Urea
Pyroglutamic acid
Threonine
Serine
Leucine
Tyrosine
Erythronic acid
Cysteine
Fumaric acid/Malei
Ornithine
Tryptophan
Aminomalonic acid
Citric acid
Alanine
Phenylpropanolamin
Histidine
Pseudouridine
Cystine
Mannose/Galactose/
6
4
2
0
-2
-4
-6
class
good_AA+_14
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good_CN_14
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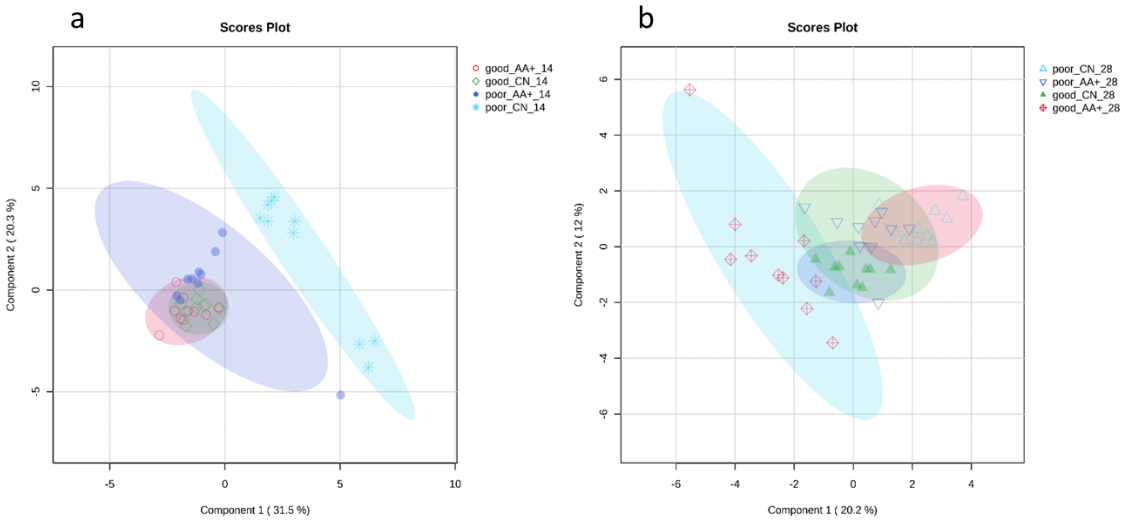
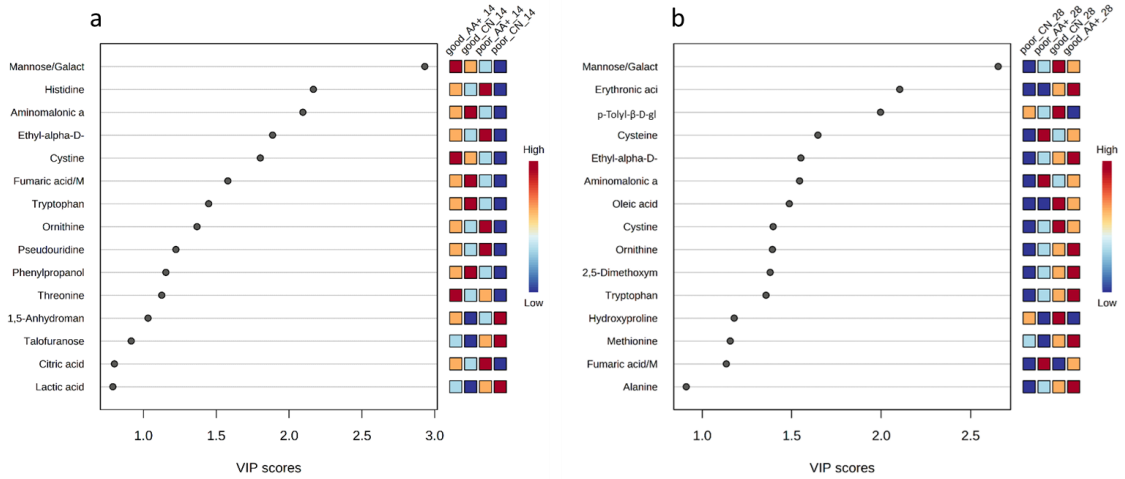
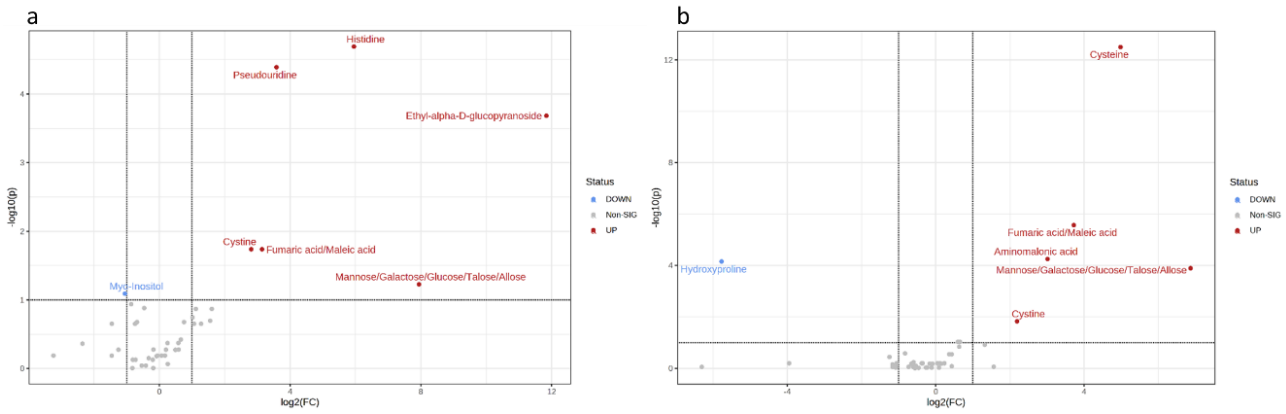
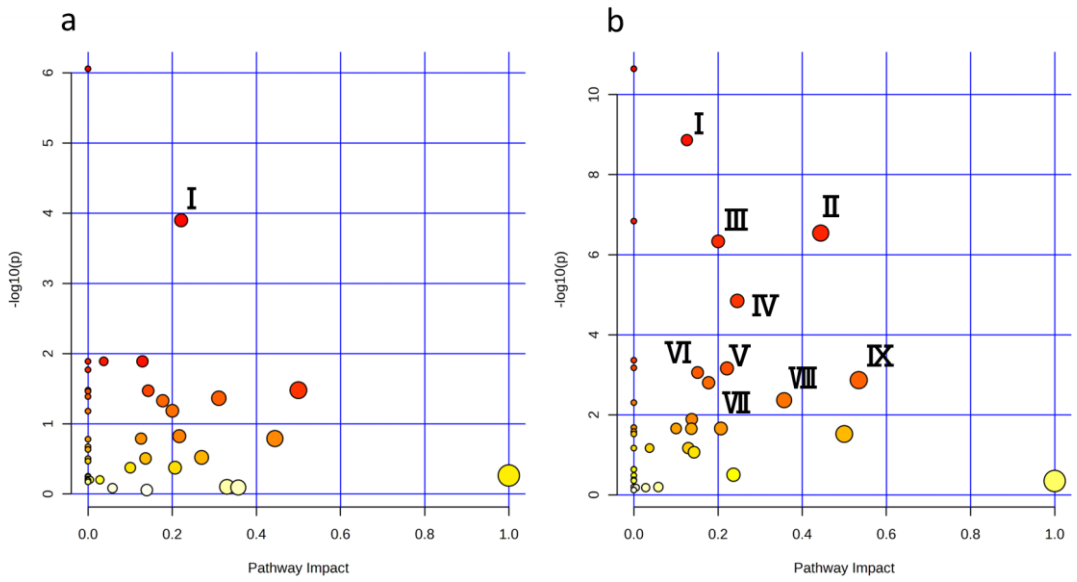
poor_AA+_28
poor_CN_14
poor_CN_28


a
Scores Plot
good_AA+_14
good_CN_14
poor_AA+_14
poor_CN_14
Component 2 (20.3 %)
Component 1 (31.5 %)
b
Scores Plot
poor_CN_28
poor_AA+_28
good_CN_28
good_AA+_28
Component 2 (12 %)
Component 1 (20.2 %)


a
Mannose/Galact
Histidine
Aminomalonic a
Ethyl-alpha-D-
Cystine
Fumaric acid/M
Tryptophan
Ornithine
Pseudouridine
Phenylpropanol
Threonine
1,5-Anhydroman
Talofuranose
Citric acid
Lactic acid
1.0
1.5
2.0
2.5
3.0
VIP scores
High
Low
b
Mannose/Galact
Erythronic aci
p-Tolyl-β-D-gl
Cysteine
Ethyl-alpha-D-
Aminomalonic a
Oleic acid
Cystine
Ornithine
2,5-Dimethoxym
Tryptophan
Hydroxyproline
Methionine
Fumaric acid/M
Alanine
1.0
1.5
2.0
2.5
VIP scores
High
Low


a
Histidine
Pseudouridine
Ethyl-alpha-D-glucopyranoside
Cystine
Fumaric acid/Maleic acid
Mannose/Galactose/Glucose/Talose/Allose
Myo-Inositol
-log10(p)
log2(FC)
Status
DOWN
Non-SIG
UP
b
Cysteine
Fumaric acid/Maleic acid
Aminomalonic acid
Mannose/Galactose/Glucose/Talose/Allose
Hydroxyproline
Cystine
-log10(p)
log2(FC)
Status
DOWN
Non-SIG
UP


a
b
-log10(p)
Pathway Impact
I
II
III
IV
V
VI
VII
VIII
IX
0.0
0.2
0.4
0.6
0.8
1.0

CHAPTER 7 – GENERAL DISCUSSION

One of the goals of the pig production sector is to maintain pigs' performance and low production costs while reducing antibiotic usage due to societal, environmental, and health perspectives. This may be achieved through nutritional strategies, as feed costs are responsible for at least 70% of production costs (Pomar et al., 2019). Therefore, the original objective of this thesis research was to understand the mechanisms of how a dietary amino acid supplementation above the estimated requirements for growth improves and regulates growing pigs' ability to cope with a sanitary challenge to increase the leanness/protein deposition in group-housed challenged growing pigs.

To achieve this objective, it was required a preliminary study to determine the SC model to induce a constant stimulation of the immune system and limit growth performance as observed in commercial production facilities (Le Floc'h et al., 2006). Indeed, there are several SC models available in the literature; however, there was no consensual conclusion about the best model for growing pigs. In addition, most of the SC models were developed and evaluated in weaned piglets for a short period of time (maximum of three weeks of trial).

Thus, when comparing the SC models available based on the mechanisms to induce an immune system activation, the impairment in metabolism and growth performance, and their limitations, we decided to use ST inoculation and no cleaning routine as the SC model. The choice was driven by the production phase of interest (growing pigs, in which pigs are susceptible/predisposed to ST infection), the type of response to be evaluated (chronic response/long-term response), and mimic a commercial condition where pigs are exposed to a higher environmental pathogenic pressure. To this end, we tested two oral gavage dosage inoculations with ST required to assess immunological, physiological, and growth performance alterations in pigs.

Our findings showed that oral inoculation of ST could impair pigs' growth performance and health status. Indeed, in both ST-challenged groups, pigs had diarrhea with semiliquid to liquid feces. Additionally, in both challenged groups, there was a decrease in ADG and worsened G:F in the first week post-challenged, followed by alterations in blood parameters, such as higher hematocrit and hemoglobin concentrations at 7 and 14 days post-challenge. However, the 1.5×10^8

CFU pigs had a more pronounced reduction in performance (ADG: 21% and G:F: 19%) compared to the Basal group (no challenge), which can be related to the higher ST concentration.

The presence of diarrhea was the direct impact of ST in the gut mucosa, where inflammation modifies the chloride channel function and disrupts tight junctions, leading to gut leakage and diarrhea (Campbell et al., 2013; Wang et al., 2015). In the meantime, the blood parameters alteration and the reduction in growth performance results from the indirect effect of the ST challenge model. The higher hematocrit and hemoglobin concentrations detected may be caused by dehydration but also by the pro-inflammatory cytokines' interference on erythropoietin activity and reduced erythrocyte survival during inflammation (Weiss et al., 2019). These effects together may negatively affect growth performance. Besides, the decreased performance may be a consequence of the reduced gut nutrient digestion and absorption (Adedokun et al., 2018), lowering nutrient availability for growth, as well as from the redirection of dietary and body nutrient reserves to support the immune system (Campos et al., 2014; 2019).

Even though the oral dosage of 1.5×10^8 CFU of *Salmonella* Typhimurium may have achieved our goal, which was to induce pigs' immune system activation and impair growth performance, there was a lack of effect on growth parameters after 14 days of trial, and the small alterations in blood parameters. The choice of the oral dosage of 10^8 was made based on case reports and papers (Rostagno et al., 2011; Walsh et al., 2012; Moura et al., 2021), which indicated that when sick pigs are detected in the field, the ST load that caused the infection generally is between 10^5 and 10^7, and the ST presence in the heard with low performance and sick behavior would be detected up to 28 days post-infection (Nielsen et al., 1995; Rostagno et al., 2011). As we know, the outcomes in commercial units may differ from those observed in experimental conditions (up to 28% lower/worse performance; Campbell and Taverner, 1985). Thus, we decided to increase the dosage concentration to ensure a reliable assessment of immunological and growth performance alterations in those ST challenged pigs. However, those field/previous results were not fully observed in the first trial.

The first hypothesis we raise is the housing condition itself. In the trial, pigs

were housed individually in pens with fully slatted floors. As it was a pilot test, with few experimental units, we did not have another facility available that could house the pigs collectively, as we did in the second experiment. Thus, this housing system may have prevented challenged pigs from being reinfected by the fecal-oral cycle or nose-to-nose contact, especially from long-term carrier pigs that can, continuously or intermittently, shed ST in feces and in oropharyngeal secretions (Oliveira et al., 2010). The second hypothesis is that those pigs' sows have been vaccinated against *Salmonella sp.* at farrowing, which increased their resistance to ST infection. As the pigs were acquired from a commercial farm with high health status and a strict vaccination program, they may have a low immune system activation and a faster recovery to the ST challenge applied.

Therefore, given the results obtained from the pilot study, we decided to increase the ST dose inoculated (from 10^8 to 10^9) and include manure spreading (from a commercial pig herd) in the pen floor as part of the SC model in the third chapter of this thesis. These modifications aimed to enhance and recycle the pathogenic pressure and prolong the immune system activation of pigs SC throughout the trial (28 days).

With this new SC model, in the second study, we investigated the effect of dietary AA supplementation above the estimated requirements for growth to improve pigs' ability to cope with a sanitary challenge and keep the leanness/protein deposition in a group-housed environment. Our major and original findings were that increasing dietary Trp, Thr, and Met + Cys to Lys ratio mitigated fever in the first week of challenge, reduced the probability of diarrhea, reduced the number of positive pigs and the fecal ST shedding, improved protein deposition and nitrogen efficiency of group-housed growing pigs under a SC. These results suggest that SC-supplemented pigs had a greater capacity to use those AA to support immune responses compared to SC non-supplemented ones, which may explain their better ability to cope with an immune challenge as previously reported by other studies (e Floc'h et al., 2018; Wellington et al., 2019; Rodrigues et al., 2021; Fraga et al., 2023). Furthermore, supplemented pigs had a trend for reduction in urea serum concentration, which may be probably a consequence of better efficiency of nitrogen utilization for protein retention. It is important to highlight that with these results,

some gaps are filled about immune system overstimulation on AA requirements and protein metabolism of growing pigs, especially for pigs housed in large groups and exposed to different challenges, as limited information is available in the literature.

Although we demonstrated important AA supplementation effects on the performance of SC pigs, in this study, we assumed that the response of all pigs in a population would be the same as the response of the average individual to the AA supplementation and to the SC-applied. However, not all animals in the population have equal growth potential, and all react in the same way to encounter stressors. Thus, it is expected that variation in the ability to cope exists within individuals and a large variation in growth (Wellock et al., 2004; van der Peet-Schwering et al., 2019). Indeed, pigs in good SC had lower variation coefficients for BW, daily FI, and protein deposition (10.9, 20.7, and 13.7%, respectively) compared to poor SC pigs (13.5, 26.7, and 23.4%, respectively) (Figures 2 and 3).

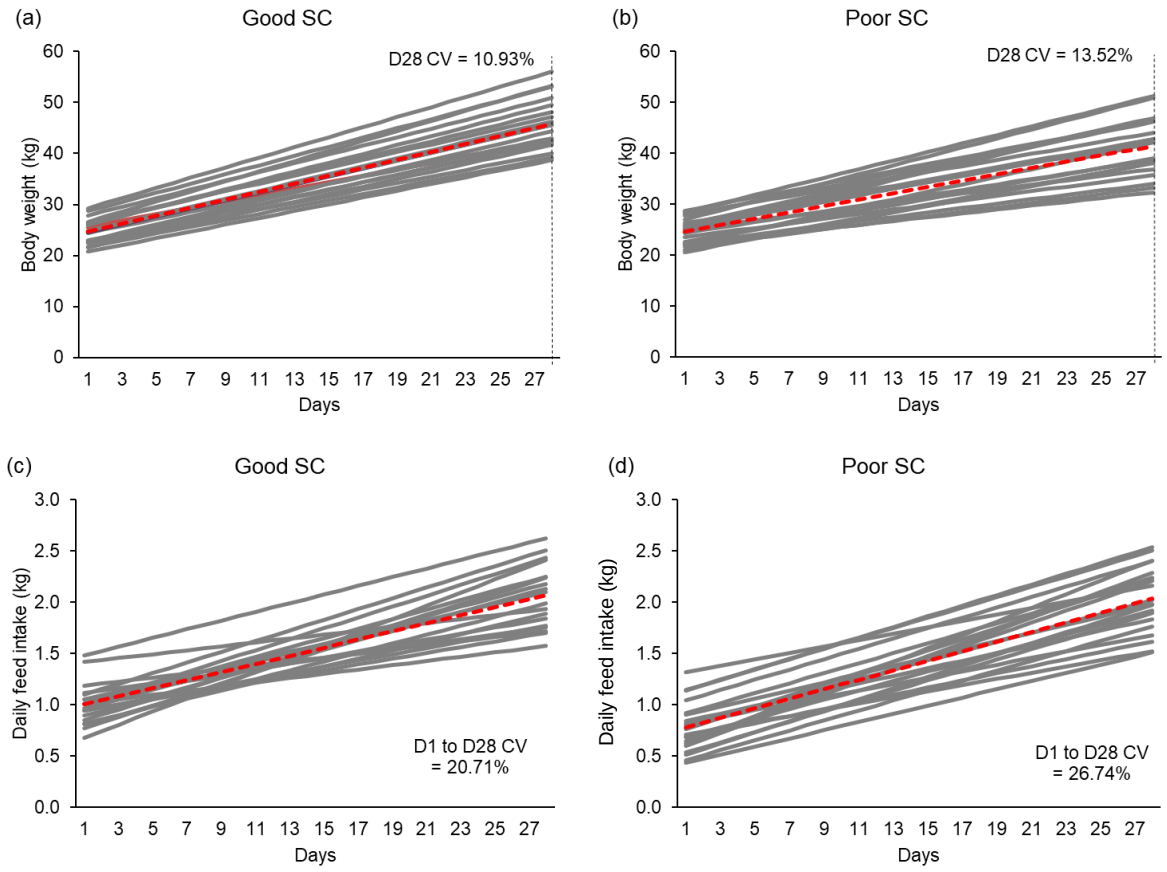


Figure 2: Individual body weight (kg) of growing pigs after 28 experimental days under good (a) or poor (b) sanitary conditions. Individual daily feed intake (DFI, kg) of

growing pigs after 28 experimental days under good (c) or poor (d) sanitary conditions.

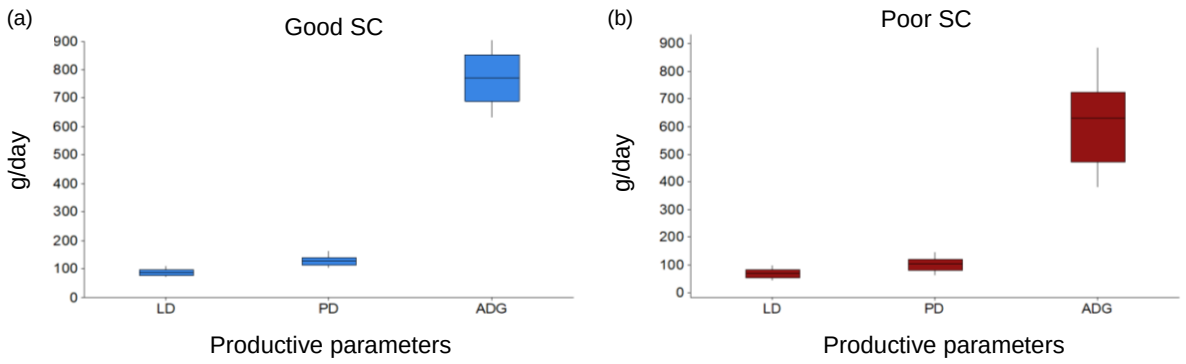


Figure 3: Productive parameters dispersion (lipid deposition (LD), protein deposition (PD), and average daily gain (ADG)) of growing pigs after 28 experimental days under good (a) or poor (b) sanitary conditions.

Additionally, there is likely to be variation in the initial state (described by initial BW) between pigs at the start of a SC (Wellock et al., 2004), even under experimental conditions, which may also affect the ability of an individual to cope in a given environment. Therefore, a nutritional strategy may have a higher effect on some pigs compared to others within the same group. For that reason, we investigated the association between nutritional and immune response and the variation in the initial BW of pigs in Chapter 5. Data from the 30 light and 30 heavy gilts from the second trial were used. Half of each BW group was fed the control diet, and the other was supplemented with 20% higher Trp:Lys, Thr:Lys, and Met + Cys:Lys concentrations. Our main objective was to evaluate the effect of BW (light vs. heavy) and Trp, Thr, and Met supplementation on feeding behavior and the coping capacity of growing pigs under a SC.

Our findings showed that light and heavy pigs had different responses to the same environment. Light and heavy pigs were clustered separately from the beginning until the end of the trial, with light pigs having a positive correlation with body lipid percentage and heavy pigs having a positive correlation with body protein percentage. Additionally, different feeding behaviors were observed, as light pigs had lower total feed intake and shorter meals than heavy pigs in both SCs throughout the

trial. These initial and throughout the trial differences between groups might help us understand why some pigs in a population have greater coping abilities when housed in poor SC. For example, even though no statistical differences were observed, the rectal temperature (RT) for heavy pigs in poor SC reached its maximum at day 2 (39.6 ± 0.2 ºC) whereas, for light pigs, the RT only reached its maximum at day 4 (39.6 ± 0.3 ºC). At day 7, it was still numerically higher than heavy pigs. This faster immune response/coping ability of heavy pigs may be related to a higher body lean mass percentage (van der Waaij, 2004) and a higher FI, which, together, might help individuals to handle immune stimulation. However, the dietary AA supplementation might have influenced more light pigs than heavy pigs. At poor SC, supplemented light pigs were clustered separately from non-supplemented light pigs on day 14, whereas this effect was not observed for poor SC heavy pigs. Furthermore, supplemented light pigs had a more regular feed intake pattern than non-supplemented ones, while a more irregular pattern was observed for supplemented heavy pigs than the non-supplemented individuals at period 2.

To our knowledge, there are no other publications with pigs exploring the individual response under a SC and quantifying the effect of dietary supplementation on that in the growing phase. However, unfortunately, we had a limited number of animals (5 animals/experimental treatment) in some analyses of this Chapter, where removing or adding new animals may modify the results obtained. Additionally, in both SC, there was a large difference in BW between groups (mean of 6 kg) since the beginning of the trial, which might have made it difficult to detect differences between groups, especially when classical statistical analysis is applied.

Even with those limitations, we explored as an exploratory analysis the individual variation through slopes comparison between light and heavy BW pigs within SC and diets. This analysis was performed because different clusters between BW groups were observed in PCA analysis. Therefore, the objective was to understand the metabolic effect that explained differences among clusters, and whether differences among clusters were due to the imposed dietary treatments. To do so, linear multilevel regression models were performed with the lme4 (v1.1-26; Bates et al., 2015) of the R software (version 4.2.3) considering repeated measures in time to compare the slopes of the BW groups, day, and their interaction within diets

and for each SC. Thus, the model used to evaluate the individual response was:

$$Y = \alpha_1\, BW\ heavy + \alpha_2\ BW\ light + \beta_1\, day + \beta_2\, day \times BW\ \text{group}$$

Where Y is the response variable, α_1 represents the starting value for a given variable in the BW heavy group, α_2 is the change in α_1 value for BW light in relation to heavy, thus characterizing the different intercepts for each BW group. Whereas β_1 is the slope change for the experimental days (0, 14, and 28), and β_2 represents the interaction between BW group and the day. The last allows a comparison of whether the response differed between BW groups over time within each diet. The R-square was calculated (Agresti, 2018) considering the random effects (RSQ package of R), and the Performance package (Lüdecke et al., 2021) was used to evaluate the normality of the distribution of the residuals. The individual pig was the experimental unit, and the significance level adopted was 10% ($P \leq 0.10$). Within this analysis, here is what we observed:

The BW and BP increased, whereas BL decreased with time ($P < 0.10$; Table 1) within both SC. However, light pigs had, on average, 7% greater body lipid percentage (BL) and 6% smaller body protein percentage (BP) than heavy pigs ($P < 0.10$) in both SC. Diets influenced BW, and BL changes differently between BW groups over time within each SC. In good SC, light pigs had a faster decrease in BL and a slower increase in BW over time than heavy pigs, regardless of the diet ($P < 0.10$). However, in poor SC, light pigs fed CN diet had a faster decrease in BL and a slower increase in BW over time than heavy pigs (observed by the interaction in the model; β_2 = -0.06; $P < 0.10$), which was not significant in model for pigs fed AA+ diet (β_2 = -0.05; $P > 0.10$). For blood parameters, dietary treatments affected serum albumin differently over time between BW groups within each SC. In good SC, light pigs fed CN diet had a decrease in serum albumin over time compared to heavy pigs (β_2= -0.01; $P < 0.10$), with no differences in these parameters in the model fit for pigs fed AA+ diet ($P > 0.10$). However, no changes in serum albumin were observed between BW groups in poor SC over time (β_1 = -0.008; $P > 0.10$). No serum urea changes were observed between BW groups within diets (β_1 = -0.09 CN, and -0.004 AA+; $P > 0.10$), in good SC, whereas in poor SC, light pigs fed CN diet (α_2 = 4.78) had greater serum urea than heavy pigs (α_1 = 19.48; $P < 0.10$), with no differences for pigs fed AA+ diet ($P > 0.10$). No differences in serum triglycerides were observed

between BW groups within diets and SC over time (P > 0.10). Plasma lymphocytes were influenced by diets, regardless of the BW group. In good SC, pigs fed AA+ diet had increased plasma lymphocytes over time (β_1= 113; P < 0.10), which was not observed in the model fit for pigs fed CN diet (P > 0.10). Conversely, in poor SC, pigs fed CN diet had increased plasma lymphocytes over time (β_1= 84; P < 0.10), which was not observed in pigs fed AA+ diet (P > 0.10).

Table 1. Effect of initial body weight group (heavy or light) in the time (days) on body composition and blood metabolites of growing pigs fed control or supplemented diet with increased Trp, Thr, and Met raised under good or poor sanitary condition.

Item	Parameter				R^2	RMSE
	Heavy (α_1)	Light (α_2)	Day (β_1)	Interaction (β_2)		
Good sanitary condition						
Control diet						
Body weight, kg	27.50*	-5.97*	0.96*	-0.24*	0.96	2.00
Body lipid, %BW	18.54*	1.59*	-0.08*	-0.06*	0.76	0.76
Body protein, %BW	14.07*	-0.97*	0.06*	0.02	0.46	0.92
Albumin, g/L	2.55*	-0.23*	0.005	-0.01*	0.62	0.15
Urea, g/L	20.57*	2.57	-0.09	-0.13	0.39	2.99
Triglycerides, g/L	40.65*	-4.22	-0.28	0.33	0.16	13.2
Lymphocytes, mm^3	7,530*	2,468	108	-122	0.10	2,784
Supplemented diet						
Body weight, kg	27.27*	-5.91*	0.81*	-0.13*	0.95	1.89
Body lipid, %BW	18.52*	2.26*	-0.10*	-0.08*	0.81	0.77
Body protein, %BW	13.9*	-1.1*	0.07*	0.02	0.53	0.91
Albumin, g/L	2.69*	-0.37	0.0004	0.007	0.83	0.12
Urea, g/L	20.19*	-0.37	-0.004	0.17	0.15	4.60
Triglycerides, g/L	36.02*	-12.59	-0.32	0.3	0.15	12.7
Lymphocytes, mm^3	6,677*	-150	113*	30	0.27	1,840
Poor sanitary condition						
Control diet						
Body weight, kg	27.52*	-5.93*	0.68*	-0.13*	0.92	1.91
Body lipid, %BW	18.28*	3.02*	-0.11*	-0.06*	0.87	0.69
Body protein, %BW	14.72*	-1.09*	0.06*	0.02	0.49	0.83
Albumin, g/L	2.44*	-0.13	-0.008	-0.001	0.54	0.17
Urea, g/L	19.48*	4.78*	0.03	-0.08	0.30	3.69
Triglycerides, g/L	35.47*	18.25	0.23	-0.23	0.13	20.1
Lymphocytes, $10^3/mm^3$	7,311*	-498	84*	-46	0.19	1,860
Supplemented diet						
Body weight, kg	27.67*	-6.07*	0.68*	-0.05	0.90	2.34
Body lipid, %BW	18.87*	1.87*	-0.11*	-0.05	0.80	0.75
Body protein, %BW	14.82*	-1.40*	0.07*	0.02	0.57	0.88
Albumin, g/L	2.48*	-0.26	-0.006	0.01	0.74	0.17
Urea, g/L	21.25*	-1.46	0.000	-0.09	0.42	3.67
Triglycerides, g/L	49.75*	-7.37	0.39	-0.46	0.10	20.5
Lymphocytes, $10^3/mm^3$	8,009*	1,790	2.43	-68	0.05	2,857

RSME: root mean square error;
R^2 = R-square for mixed models considering the random effects;
*Stands for $P < 0.10$;

This analysis also corroborates with the previous results of the feeding behavior, showing that the additional dietary Trp, Thr, and Met might influence pigs' responses differently within each SC. In good SC, the AA supplementation did not mitigate differences between BW groups, as demonstrated by the different slopes between light and heavy pigs for BL and BW in both diets over time. These results provide us with two ideas. First, feeding pigs with additional dietary AA in an ideal environment attempting to improve the growth of light pigs/slow growers is not a good strategy. The second one is that light pigs may have some metabolic differences compared to heavy pigs. As an example, light pigs had higher BL over time compared to heavy pigs, regardless of the diet. This difference may be related to lower insulin sensitivity and increased de novo lipogenesis (Salgado et al., 2022), which may impair light pigs' response to nutrient intake. On the other hand, in poor SC, additional dietary Trp, Thr, and Met improved light pigs' ability to cope with a SC. This was demonstrated by the similar slopes for BW and BL between supplemented light and heavy pigs. Increasing Trp, Thr, and Met concentration in the diet may partially overcome low Trp, Thr, and Met intake for light pigs and boost the immune system responses, maintaining growth over time. Besides, the absence of differences in serum urea concentration between supplemented BW groups indicates that Trp, Thr, and Met may have increased pigs' AA utilization efficiency of light pigs for growth. As serum urea concentration was measured with pigs in the fasted state, it also suggests that the additional Trp, Thr, and Met may reduce muscle protein breakdown (Eugenio et al., 2023). Therefore, supplementing Trp, Thr, and Met may have attenuated systemic inflammation, muscle protein breakdown, and, consequently, reduced variation between BW groups.

As mentioned before, there is a limited number of pigs/experimental units per treatment, and, therefore, the results and conclusions may only apply to these animals submitted to this SC model. Furthermore, our herd may not be considered representative of a huge population because the pigs were not selected at random: the groups light and heavy were already prefixed in the previous study (Chapter 4). Thus, this approach may serve as an exploratory analysis, providing insights into

individual differences in coping with environmental challenges, but further studies are still needed to understand better, identify, and quantify the sources of variation in the response among challenged individuals.

Finally, in the last chapter of this thesis (Chapter 6), we investigated the changes in metabolites and metabolic pathways caused by the dietary acid supplementation strategy and the sanitary challenge in growing pigs. Our major outcomes indicated that SC impacted the abundance of intermediate and end products of protein and energy-related metabolites. Additionally, the pathway analysis showed the effect of AA supplementation above the requirement for growth in regulating inflammation pathways and enhancing the antioxidant capacity of supplemented challenged pigs.

Even though the SC impact on some blood biomarkers related to protein and carbohydrate metabolism has been previously reported (Le Floc'h et al., 2006; 2009; Merlot et al., 2016; van der Meer et al., 2016; Fraga et al., 2023), our results demonstrated how dynamic and connected those pathways are. Additionally, the outcomes pointed out how an immune system activation may impact energy production efficiency and nitrogen metabolism. For example, some intermediates in the citric acid cycle have antioxidant and anti-inflammatory properties, such as fumaric acid. During the inflammatory response, it may have been used to reduce the oxidative stress in challenged pigs, which may have impaired the efficiency of the citric acid cycle and aerobic respiration. Furthermore, some intermediates in the urea cycle, such as ornithine, may have been redirected to improve gut barrier integrity through polyamine synthesis, impairing nitrogen metabolism.

Our findings also pointed out that during a SC, especially in the acute phase, some AAs, such as His, Trp, Thr, and Cys, are more likely to be used by the immune system than others, confirming the need for higher ratios in the diet, especially those AA supplemented 20% in the diet. This finding brings new knowledge about the immune system stimulation effect on AA requirements of group-housed growing pigs, as only a few studies have reported a reduction in plasma AA concentration in challenged pigs (Melchior et al., 2004; Le Floc'h et al., 2009; Wirthgen et al., 2014).

Additionally, the results demonstrated that challenged pigs supplemented with Trp, Thr, and Met had changes in plasma metabolites compared with non-challenged

pigs throughout the trial. Besides, in this chapter, through pathway analysis, it was indicated by which pathways the AA supplementation may have improved challenged pigs' adaptive responses. To our knowledge, there are no other publications with group-housed growing pigs exploring the pathways modulated by this nutritional strategy. From our results, it was indicated that dietary Trp, Thr, and Met supplementation modulated the histidine pathway modulation, mitigating inflammation at 14 days of challenge. At the end of the trial, this nutritional strategy also regulated glutathione metabolism, cysteine and methionine metabolism, and glycine, serine, and threonine pathways, alleviating oxidative stress. Consequently, those AAs may have had lower muscle catabolism, indicated by the downregulation of hydroxyproline, and reduced energy metabolism perturbations as demonstrated by the differences in phenylalanine, tyrosine, alanine, glutamate, and aspartate metabolic pathways between AA+ and CN diet in challenged pigs. Therefore, this chapter elicited how those AAs interacted with the immune system, and, consequently, improved protein deposition and weight gain compared to non-supplemented challenged pigs (results observed in chapter 4).

7.1 Experimental strengths, limitations, and perspectives

This thesis investigated the effects of an environmental challenge model (caused by *Salmonella* Typhimurium and poor housing conditions) and a mixture of three AA supplemented above estimated requirements for growth on performance and the coping abilities of growing pigs. Previous studies have shown positive effects of dietary Trp, Thr, and Met supplementation in pigs under SC. However, most of those studies have evaluated the AA effect when supplemented individually or in a combination of two of those AA (Trp + Met (Capozzalo et al., 2016); Trp + Thr (Xu et al., 2015)) at the postweaning phase, and not for growing-finishing period. Therefore, the studies in this thesis bring novelty about the immune system response and nutritional requirements of challenged-growing pigs.

It is also important to highlight that oral inoculation of *Salmonella* Typhimurium + poor housing conditions is a reliable SC model. It successfully induced an immune response (higher serum levels of haptoglobin) through constant exposure of pigs to several environmental agents, as seen in practical conditions

when there is a disease outbreak. Thus, this model could be used in other studies to validate several aspects of the immunomodulating effects of nutrients for challenged-growing pigs.

Furthermore, the dietary supplementation with a mixture of Trp, Thr, and Met above (120%) estimated requirements for growth improved growing pigs' performance, nitrogen efficiency, and robustness to cope with SC. The reliability of the results relies on the experimental design utilized, with a non-challenged sanitary condition. Even though having a good sanitary condition increased the experimental costs, it was crucial to validate the SC model efficiency and also confirm the functional effects of those AAs to support the immune system only under SC, as minimal effects of dietary supplementation on blood parameters and growth performance was observed in non-challenged pigs. Additionally, this thesis also evidenced that heavy pigs responded differently to SC and AA supplementation than light pigs, especially under SC, which is probably due to their greater feed intake capacity and fast body reserve mobilization. Nutritionists may use this knowledge to adjust optimal dietary nutrient levels for pigs in commercial systems to improve the animal's capacity to cope with challenges.

Another experimental strength was the use of electronic feeders (AIPFs) and body composition scanning. Those feeders allowed the continuous monitoring of each individual in real-time. In Chapter 5, it has been shown that their use may contribute to pig production by allowing the monitoring of pigs' feed intake and the deviations from their typical feeding patterns for early disease detection. Research to explore the use of this technique for growing-finishing pigs under commercial conditions is encouraged. The dual-energy X-ray absorptiometry (DXA) assessment allows the evaluation of fat mass, lean mass, and bone mineral content with low cost, rapidity, and low bias compared to body chemical composition analysis. Additionally, it is a non-invasive technique, allowing the same animal to be evaluated over time. Thus, its use in further studies is also encouraged.

One limitation of the thesis studies is the lack of long-term data on performance and immunological parameters, as the experimental pigs were monitored for 28 d post-inoculation only. It was shown that pigs had slight recovery from the SC by d 28, as demonstrated by the reduced serum haptoglobin level,

improved fecal score, low fecal ST shedding, and low percentage of pigs shedding ST. However, it remains unknown whether the AA benefits reported in this thesis will maintain any long-term impact (e.g., slaughter).

Another limitation was the same supplementation ratio used for all AAs. Although the results presented positive effects when supplementing a fixed ratio (20% of each AA), comparing those findings with different supplementation ratios of each AA may be relevant to understanding the role of each AA in supporting the immune system, especially when designing cost-effective nutritional strategies. As a result, it will allow a more comprehensive approach to gut challenges and AA uptake.

As observed in Chapter 5, pigs within a population may respond differently to a SC. Thus, an additional major development in AA supplementation in SC studies would be accounting for variability in animal response on the adjustment of AA requirements. Precision feeding concepts, for example, could integrate knowledge of individual requirements and help maximize nutrient utilization. Adjusting AA requirements individually would allow pigs to express individual potential and redirect the correct amount of nutrients to improve robustness to cope with environmental challenges and bring economic benefits to the pig production sector. Finally, the PCA results reinforced the need for a better understanding of the modulatory effects of AA supplementation and its association with animal health and metabolism indicators. Therefore, advanced statistical analysis, including multivariate approaches and pathway analysis, should be used to bring additional robustness to that analysis.

Indeed, in Chapter 6, with the metabolomic aid, it was possible to have a deep understanding of the changes in metabolites and metabolic pathways caused by the dietary acid supplementation strategy and the sanitary challenge in growing pigs. The outcomes indicate that SC impacted the abundance of intermediate and end products, not only of protein but also of energy-related metabolites, showing how dynamic and connected those pathways are, which can be considered a strength of this study. Those results also point out the need for a better understanding of the SC effect on the energy requirement of challenged growing pigs. Another advantage was that with a single blood sample per animal, a robust

comparative pathway analysis could be performed, in which the effect of AA supplementation above the requirement for growth in regulating inflammation-related pathways was shown. However, one limitation of this approach is that metabolites only represent a snapshot of current bodily or cellular activity. As it is a dynamic flux, sampling time is an important factor in this analysis. Another limitation is the pathway analysis, in which, only two treatments can be compared at a time. Even with those cons, metabolomics should keep being applied in further studies to potentially capture patterns and identify biomarkers that trigger adaptative responses in challenged pigs. Additionally, the incorporation of metabolite data in disease occurrence prediction and performance prediction is encouraged.

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CHAPTER 8 – CONCLUSIONS

This thesis evaluated the effect of environmental challenges (chronic inflammation) on performance and AA metabolism in growing pigs. The results provide a scientific basis for developing targeted nutritional strategies for pigs under sanitary challenges. In brief, the following conclusions were drawn:

- Using oral inoculation of *Salmonella* Typhimurium and poor housing conditions to create a contrast in chronic immune system stimulation leads to:
    - Fever, diarrhea, increased serum haptoglobin, 9% reduced body weight, 19% reduced weight gain, 10% reduced feed intake, 10% reduced feed efficiency, and 25% increase in lipid deposition in poor SC compared with good SC pigs.
    - Affected feeding behavior patterns – more irregular meals in poor SC pigs than good SC pigs.
    - Decreased plasma AAs such as histidine, tryptophan, ornithine, cysteine, and threonine to neutralize pathogen gut mucosa adhesion and systemic translocation. Additionally, the challenge model impaired energy-related plasma metabolites such as myo-inositol, α-glucoside, and 1,5-anhydro-d-mannitol, indicating a metabolic change in glucose formation and energy utilization in challenged pigs.
- Extra dietary supplementation of Trp, Thr, Met + Cys was beneficial for growth performance in poor SC pigs: 7% increased body weight, 17% increased weight gain, 14% increased feed efficiency, and 16% increase in protein deposition in challenged pigs fed the supplemented diet compared with challenged pigs fed control diet.

The observed variation in pig performance and immune responses among pigs in the current thesis indicates the importance of accounting for variability in animal response in adjusting nutritional requirements.

- Extra dietary supplementation of Trp, Thr, Met + Cys had a pronounced effect on light pigs compared with heavy pigs under a poor SC.
- Supplementing Trp, Thr, and Met + Cys mitigated inflammation through the downregulation of the histidine pathway. This nutritional strategy also increased the antioxidant capacity and lowered changes in energy supply through regulation of glutathione metabolism, cysteine and methionine metabolism, and glycine, serine, and threonine metabolism pathways.

Overall, the results of this thesis indicate that AA requirements are greater in challenged pigs compared with non-challenged pigs. The present thesis demonstrates that feeding behavior assessment can be used to detect sanitary problems within a herd, for early SC detection. Additionally, this thesis shows that the adjustment of AA requirements for categories when there is a sanitary challenge may improve immune responses and reduce variation in pig performance.