UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

Faculdade de Ciências Agrárias e Veterinárias - Campus de Jaboticabal

RELATÓRIO CIENTÍFICO FINAL APRESENTADO À FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE SÃO PAULO

Bolsa no País – BCO – Pós-Doutorado

Processo 2019/19588-4

Referente ao período: 01/10/2019 a 05/03/2024

Avaliação da resposta fisiológica e metabólica e desempenho de suínos submetidos a diferentes condições de manejo e ambiente nas fases de crescimento

Relatório PD FAPESP: Antonio Diego Brandão Melo

Supervisor: Prof. Luciano Hauschild

Professor do Departamento de Zootecnia da Universidade Estadual Paulista

Campus de Jaboticabal

Bolsista PD: Dr. Antonio Diego Brandão Melo

Supervisor: Prof. Dr. Luciano Hauschild

Jaboticabal
Agosto/2025

1. Resumo das atividades realizadas no período

Este relatório descreve as atividades desenvolvidas pelo bolsista de pós-doutorado pelo período de 53 meses (01/10/2019 a 05/03/2024). Este período compreende atividades desenvolvidas na FCAV/Unesp e na University of Nebraska-Lincoln (processo Fapesp BEPE n. 2022/04322-1). Este relatório antecede em 2 meses o fim das atividades do bolsista BCO PD, haja vista ao fim do pojeto JP2 ao qual sua bolsa é vinculada em 30/04/2024. O bolsista optou por encerrar suas atividades como PD e dar início a um novo vínculo empragatício no setor privado. As principais atividades desenvolvidas no período estão resumidas a seguir:

1. Discutir, revisar e auxiliar, como coorientador, os alunos de iniciação científica, mestrado e doutorado na elaboração dos protocolos experimentais, resumos, manuscritos, dissertações, teses e outras atividades de pesquisa.
2. Auxiliar o supervisor Prof. Dr. Luciano Hauschild na coordenação das atividades, na redação dos relatórios científicos, na prestação de contas e na coordenação dos experimentos do JP2 no Laboratório de Estudos em Suinocultura da Unesp (LabSui).
3. Participar de reuniões técnicas com pessoal de empresas parceiras para desenvolvimento e elaboração dos protocolos experimentais, experimentos, manuscritos e relatórios.
4. Participar com a equipe de pesquisa, membros externos e o supervisor Prof. Dr. Luciano Hauschild nas discussões e análises de dados experimentais.
5. Auxiliar na gestão de infraestrutura do Laboratório de Estudos em Suinocultura da Unesp para adequada condução dos experimentos.
6. Ministrar parte da disciplina de Zootecnia IV (Suínos) durante o período 2020.1 no curso de Graduação em Medicina Veterinária da Unesp.
7. Ministrar parte da disciplina de Zootecnia IV (Suínos) durante o período 2021.1 no curso de Graduação em Medicina Veterinária da Unesp.
8. Ministrar parte da disciplina de Zootecnia IV (Suínos) durante o período 2022.1 no curso de Graduação em Medicina Veterinária da Unesp.
9. Revisão e submissão de manuscritos de autoria ou coautoria do bolsista relacionados a experimentos do plano de atividades do bolsista submetido à Fapesp e ao JP2.
10. Coordenação da condução da tese do doutorando Pedro Righetti Arnaut sob

coorientação do pós-doutorando.

11. Coordenação da condução da tese do doutorando direto Ismael França sob coorientação do pós-doutorando.
12. Conclusão da condução da tese do doutorando Marllon José Karpeggiane de Oliveira sob coorientação do pós-doutorando.
13. Conclusão da condução da tese da doutoranda Graziela Alves da Cunha Valini sob coorientação do pós-doutorando.
14. Conclusão da condução da dissertação da mestranda Joseane Penteado Rosa sob coorientação do pós-doutorando.
15. Submissão de resumos a eventos científicos para apresentação de resultados experimentais.
16. Execução de BEPE por periodo de 10/10/2022 a 05/10/2023.
17. Um manuscrito publicado referente ao período de BEPE no periódico *Frontiers in Veterinary Science* com o título "*Temporal changes in fecal swine microbiome primarily reflect Salmonella Typhimurium challenge and poor sanitary housing conditions, even with functional amino acid supplementation*"
18. Um manuscrito publicado referente ao experimento de Fase 2 do plano de atividade do bolsista PD no periódico *Animals* com o título *"Increased Dietary Trp, Thr, and Met Supplementation Improves Performance, Health, and Protein Metabolism of Weaned Piglets under Mixed Management and Poor Housing Conditions"*.
19. Finalização do projeto JP2 e das atividades do bolsista PD.

2. Resumo da Proposta Inicial do Projeto

As granjas comerciais apresentam grande variação no desempenho de suínos na fase de crescimento e terminação. Essa variação tem sido identificada mesmo quando utilizando a mesma genética e planos nutricionais similares. O status sanitário dos animais na fase inicial do crescimento (quando recebidos da creche) pode ser considerado um dos principais fatores que contribuem para essa variação. Diferentes status sanitários observados nas granjas podem ser determinados pelas diferentes práticas de manejo (ex: higiene das instalações) e ambiente (ex: temperatura, altas densidades) adotadas na fase creche. Quando submetidos a más condições de manejo, o sistema imune dos animais é

ativado com consequente redução do consumo e do desempenho. Portanto, o objetivo desse projeto é avaliar a resposta fisiológica, metabólicas e o desempenho de leitões submetidos a diferentes condições de manejo e ambiente na fase de creche. Esse projeto está dividido em duas fases. Na Fase 1 será realizado um levantamento das práticas de manejo adotadas na creche de acordo com o desempenho das granjas. Nessa fase serão realizadas reuniões com técnicos de campo e pesquisadores para diagnóstico das práticas de manejo adotadas na fase creche (higiene, alimentação, programa de vacina, ambiência etc.) de acordo com o nível de desempenho das granjas em relação à média do Brasil (nível de desempenho alto, médio e baixo). Inicialmente, uma planilha eletrônica será sistematizada com todas as informações levantadas. Posteriormente, dentro de cada categoria de desempenho, será descrito em detalhes as práticas de manejo normalmente realizadas em cada sistema de produção. Na Fase 2, um experimento será conduzido com o objetivo de avaliar o impacto das práticas de manejo realizadas na fase de creche (manejo e ambiente) sobre as respostas fisiológicas e metabólicas e, consequentemente, no status sanitário dos animais. Serão utilizados 216 suínos fêmeas (6 kg de peso inicial) de linhagem com alto potencial genético. Os animais serão alojados em dois galpões de creche. Para simulação de manejo de uma condição de granja de baixo e alto desempenho, serão utilizados os dados a serem levantados na Fase 1. O período experimental será de 42 dias dividido em três fases alimentares. O delineamento experimental para disposição dos tratamentos será inteiramente casualizado com dois tratamentos (condições de manejo) utilizando-se 36 repetições (3 leitões/repetição). As dietas serão similares nas duas condições e formuladas de acordo com as exigências nutricionais recomendadas pelo NCR (2012). As variáveis a serem estudadas serão de fisiologia e metabolismo, composição corporal e desempenho. As respostas fisiológicas (morfologia intestinal e digestibilidade ileal) e metabólicas (metabolismo proteico e de AA, e resposta imune) serão avaliadas por meio de coleta de sangue (início, meio e fim) e de tecidos. A composição corporal dos animais será avaliada com uso do equipamento de absorciometria por duplo feixe de raio X (DXA, GE Lunar Prodigy Advance, Madison, EUA).

Resumo da proposta de renovação da BCO PD

A disbiose intestinal observada em leitões no período pós-desmame reduz a ação protetiva exercida pela microbiota comensal à mucosa intestinal. A mistura de lotes de diferentes origens nas creches pode representar um desafio sanitário para leitões de bom

estado de saúde quando alojados com animais com estado de saúde precário. Em um cenário de disbiose intestinal pós-desmame e mistura de lotes de leitões com diferentes condições sanitárias, a proliferação e disseminação de patógenos são potencializadas, resultando em considerável variabilidade de respostas produtivas dos animais. Além disso, quando práticas de biosseguridade das instalações são negligenciadas, a entrada de patógenos entéricos como *Salmonella* podem resultar em prejuízos ainda maiores. Nesse sentido, estratégias nutricionais como uso de aminoácidos funcionais nas dietas têm sido propostas para amenizar o impacto de condições adversas as quais os leitões têm sido expostos. Dessa forma, além de continuar as atividades desenvolvidas até o momento, esta proposta de renovação tem como objetivo também avaliar o impacto da mistura de lotes, de um inoculo contendo *Salmonella* Typhimurium e da suplementação de aminoácidos funcionais sobre a microbiota intestinal de leitões na fase de creche. Durante o período experimental de 42 dias, os animais serão alimentados com uma dieta controle ou suplementada, a fim de atingir 100 % ou exceder em 20% as exigências dos aminoácidos funcionais Trip, Tre e Met, de acordo com as recomendações do NRC. Em geral, os resultados do estudo irão permitir avançar no conhecimento das respostas dos suínos (microbiota) à suplementação de aminoácidos funcionais quando expostos a condição de desafio sanitário.

3. Atividades realizadas no projeto

3.1 *Efeito do sistema de alimentação de precisão e nível de aminoácidos funcionais na dieta de suínos sob desafio sanitário (condições de higiene das instalações e inoculação com Salmonella Typhimurium) (Projeto de doutorado: Pedro Righetti Arnaut).* Tese em andamento sob orientação do Prof. Dr. Luciano Hauschild. Um experimento parte da tese do discente foi conduzido de abril a julho de 2022. O manuscrito intitulado "*Precision feeding system and functional amino acids supplementation for growing pigs raised under challenging sanitary conditions*" está submetido a um periódico de interesse. A tese foi concluída em 2024.

3.2 *Protein and amino acids dietary levels in growing-finishing pigs reared under cyclic heat stress (Projeto de doutorado: Marllon J. K. Oliveira)*. Tese concluída sob coorientação do pós-doutorando Antonio Diego Brandão Melo e orientação do Prof. Dr. Luciano Hauschild. No período deste relatório foram publicados 3 artigos oriundos da tese.

3.3 *Efeito da suplementação dietética de aminoácidos e condição sanitária de alojamento sobre a fisiologia e metabolismo de suínos em crescimento (Projeto de doutorado: Graziela A. C. Valini)*. Tese concluída sob coorientação do pós-doutorando Antonio Diego Brandão Melo e orientação do Prof. Dr. Luciano Hauschild. Foram publicados 2 artigos oriundos da tese.

3.4 *Efeito da suplementação dietética com aminoácidos funcionais (Tre, Met e Trip) para suínos em crescimento sob desafio sanitário (mistura de lotes, sala suja e inoculados com Salmonella Typhimurium) (Projeto doutorado: Ismael França).* Nesse período foram concluídas as análises laboratoriais (sangue) e estatisticas do experimento conduzido neste projeto. Em adição, um manuscrito foi publicado no periodico *Animal Feed Science and Technology* com o Título "*Dietary supplementation with functional amino acids improves the capacity of growing pigs to cope with a health challenge*".

3.5 *Avaliação da resposta fisiológica, metabólica e desempenho de leitões submetidos a diferentes condições de manejo e ambiente na fase de creche (Projeto de Pós-Doutorado: Antônio Diego Brandão).* Projeto concluído com as atividades do bolsista PD descritas no presente relatório. Os resultados das atividades de BEPE Fapesp execultadas pelo bolsista PD no período junto à *University of Nebraska-Lincoln* foram apresentados nos artigos anexados a este relatório. No período, 2 manuscritos oriundos das atividades de PD do bolsista foram publicados. Um manuscrito publicado referente ao período de BEPE no periódico *Frontiers in Veterinary Science* com o título "*Temporal changes in fecal swine microbiome primarily reflect Salmonella Typhimurium challenge and poor sanitary housing conditions, even with functional amino acid supplementation*". Um manuscrito publicado referente ao experimento de Fase 2 do plano de atividade do bolsista PD no periódico *Animals* com o título *"Increased Dietary Trp, Thr, and Met Supplementation Improves Performance, Health, and Protein Metabolism of Weaned Piglets under Mixed Management and Poor Housing Conditions".*

4. Lista das publicações resultantes do auxílio

4.1 Artigos publicados em revistas científicas indexadas

4.1.1 BRANDÃO MELO, ANTONIO DIEGO; ALVES DA CUNHA VALINI, GRAZIELA; YANG, QINNAN; KARPEGGIANE DE OLIVEIRA, MARLLON JOSÉ;

ALVES MARÇAL, DANILO; RIGHETTI ARNAUT, PEDRO; FRANÇA, ISMAEL; ALISSON SILVA, CLESLEI; KORTH, NATE; PAVLOVIKJ, NATASHA; REIS FURTADO CAMPOS, PAULO HENRIQUE; GASTMANN BRAND, HENRIQUE; HTOO, JOHN KYAW; BENSON, ANDREW K.; HAUSCHILD, LUCIANO; GOMES-NETO, JOAO CARLOS. Temporal changes in fecal swine microbiome primarily reflect Salmonella Typhimurium challenge and poor sanitary housing conditions, even with functional amino acid supplementation. FRONTIERS IN VETERINARY SCIENCE, v. 12, p. 12:1597857-16, 2025.

4.1.2. DE OLIVEIRA, MARLLON JOSÉ KARPEGGIANE; YANG, QINNAN; **BRANDÃO MELO, ANTÔNIO DIEGO**; MARÇAL, DANILO ALVES; KORTH, NATE; PAVLOVIKJ, NATASHA; BENSON, ANDREW K.; HTOO, JOHN KHUN KYAW; BRAND, HENRIQUE GASTMANN; HAUSCHILD, LUCIANO; GOMES-NETO, JOAO CARLOS. Fecal microbiome of pigs fed diets differing in protein and amino acid content raised in thermoneutral or cyclical heat stress conditions. Frontiers in Microbiology, v. 16, p. 16:1585374-18, 2025.

4.1.3 DE OLIVEIRA, M.J.K.; POLYCARPO, G.V.; ANDRETTA, I.; **MELO, A.D.B.**; MARÇAL, D.A.; LÉTOURNEAU-MONTMINY, M.P.; HAUSCHILD, L. Effect of constant and cyclic heat stress on growth performance, water intake, and physiological responses in pigs: A meta-analysis. Animal Feed Science and Technology, v. 309, p. 115904, 2024.

4.1.4 GONÇALVES, JOSEANE PENTEADO ROSA; **MELO, ANTONIO DIEGO BRANDÃO**; YANG, QINNAN; DE OLIVEIRA, MARLLON JOSÉ KARPEGGIANE; MARÇAL, DANILO ALVES; ORTIZ, MANOELA TREVISAN; RIGHETTI ARNAUT, PEDRO; FRANÇA, ISMAEL; ALVES DA CUNHA VALINI, GRAZIELA; SILVA, CLESLEI ALISSON; KORTH, NATE; PAVLOVIKJ, NATASHA; CAMPOS, PAULO HENRIQUE REIS FURTADO; BRAND, HENRIQUE GASTMANN; HTOO, JOHN KYAW; GOMES-NETO, JOÃO CARLOS; BENSON, ANDREW K.; HAUSCHILD, LUCIANO. Increased Dietary Trp, Thr, and Met Supplementation Improves Performance, Health, and Protein Metabolism of Weaned Piglets under Mixed Management and Poor Housing Conditions. ANIMALS, v. 14, p. 1143, 2024.

4.1.5 FRANÇA, I.; VALINI, G.A.C.; ARNAUT, P.R.; ORTIZ, M.T.; SILVA, C.A.; OLIVEIRA, M.J.K. DE; PAULINO, G.S.C.; MARÇAL, D.A.; **MELO, A.D.B.**; HTOO, J.K.; BRAND, H.G.; ANDRETTA, I.; HAUSCHILD, L. Dietary supplementation with functional amino acids improves the capacity of growing pigs to cope with a health challenge. Animal Feed Science and Technology, v. 318, p. 116148, 2024.

4.1.6 ORTIZ, M.T.; ARNAUT, P.R.; VALINI, G.A.C.; FRANÇA, I.; SILVA, C.A.; DE OLIVEIRA, M.J.K.; MARÇAL, D.A.; **MELO, A.D.B.**; HAUSCHILD, L. A salmonella-challenge impacts the variability of performance, body composition and lysine requirements of growing pigs under poor housing conditions. Livestock Science, v. 283, p. 105462, 2024.

4.1.7 JOSÉ KARPEGGIANE DE OLIVEIRA, MARLLON; **DIEGO BRANDÃO MELO, ANTONIO**; ALVES MARÇAL, DANILO; ALVES DA CUNHA VALINI, GRAZIELA; ALISSON SILVA, CLESLEI; MARI VEIRA, ALINI; ZEM FRAGA, ALÍCIA; RIGHETTI ARNAUT, PEDRO; HENRIQUE REIS FURTADO CAMPOS, PAULO; SOUSA DOS SANTOS, LUAN; KHUN KYAW HTOO, JOHN; GASTMANN BRAND, HENRIQUE; HAUSCHILD, LUCIANO. 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, 2023.

4.1.8 ALVES DA CUNHA VALINI, GRAZIELA; ARNAUT, PEDRO RIGHETTI; BARBOSA, LARISSA GONÇALVES; DE AZEVEDO, PAULO HENRIQUE AMADEU; **MELO, ANTONIO DIEGO BRANDÃO**; MARÇAL, DANILO ALVES; CAMPOS, PAULO HENRIQUE REIS FURTADO; HAUSCHILD, LUCIANO. A Simple Assay to Assess Salmonella Typhimurium Impact on Performance and Immune Status of Growing Pigs after Different Inoculation Doses. Microorganisms, v. 11, p. 446, 2023.

4.1.9 DE OLIVEIRA, MARLLON JOSÉ KARPEGGIANE; VALK, MARCIO ; **MELO, ANTÔNIO DIEGO BRANDÃO**; MARÇAL, DANILO ALVES; SILVA, CLESLEI ALISSON; VALINI, GRAZIELA ALVES DA CUNHA; ARNAUT, PEDRO RIGHETTI; GONÇALVES, JOSEANE PENTEADO ROSA; ANDRETTA, INES; HAUSCHILD, LUCIANO. Feeding Behavior of Finishing Pigs under Diurnal Cyclic Heat Stress. Animals, v. 13, p. 908, 2023.

4.1.10 ALVES DA CUNHA VALINI, GRAZIELA; RIGHETTI ARNAUT, PEDRO; FRANÇA, ISMAEL; TREVISAN ORTIZ, MANOELA; KARPEGGIANE DE OLIVEIRA, MARLLON JOSÉ; **BRANDÃO MELO, ANTONIO DIEGO**; ALVES MARÇAL, DANILO; REIS FURTADO CAMPOS, PAULO HENRIQUE; KHUN HTOO, JOHN; GASTMANN BRAND, HENRIQUE; HAUSCHILD, LUCIANO. 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, 2023.

4.2 **Resumos publicados e aceitos em anais de conferências nacionais e internacionais**

4.2.1 MELO ADB, ROSA JP, FRANÇA I, VALINI GAC, DE OLIVEIRA MJK, SILVA CA, ARNAUT PR, ORTIZ M, MORAES WH, MARÇAL DA, HTOO JK, BRAND HG, LANFERDINI E, NAGAE RY, CAMPOS PHRF, HAUSCHILD L. Impacto da suplementação dietética de aminoácidos funcionais e condições de manejo no desempenho de leitões. 14º Simpósio Internacional de Suinocultura (SINSUI) – Produção, Reprodução e Sanidade Suína. Porto Alegre – RS, Centro de Eventos da PUCRS, 17 a 19 de maio de 2022.

4.2.2 VALINI GAC, FRANÇA I, ARNAUT PR, ORTIZ MT, BARBOSA LG, SILVA CA, ROSA JP, OLIVEIRA MJK, HTOO JK, BRAND HG, LANFERDINI E, **MELO ADB**, MARÇAL DA, CAMPOS PHRF, HAUSCHILD L. Dietary Trp, Thr, and Met supplementation attenuates changes in pig metabolism caused by sanitary challenge. 14º Simpósio Internacional de Suinocultura (SINSUI) – Produção, Reprodução e Sanidade Suína. Porto Alegre – RS, Centro de Eventos da PUCRS, 17 a 19 de maio de 2022.

4.2.3 FRANÇA I, VALINI GAC, MILLA IC, ORTIZ TM, SILVA CA, ARNAUT PR, ROSA JP, DE OLIVEIRA MJK, **MELO ADB**, MARÇAL DA, HTOO JKK, BRAND HG, NAGAE RY, LANFERDINI E, CAMPOS PHRF, ANDRETTA I, HAUSCHILD L. Suplementação dietética com aminoácidos funcionais durante o desafio sanitário atenua o baixo desempenho de suínos. 14º Simpósio Internacional de Suinocultura (SINSUI) – Produção, Reprodução e Sanidade Suína. Porto Alegre – RS, Centro de Eventos da PUCRS, 17 a 19 de maio de 2022.

4.2.4 ROSA JP, **MELO ADB**, FRANÇA I, VALINI GAC, OLIVEIRA MJK, SILVA CA, ARNAUT PR, ORTIZ MT, SILVA JA, MARÇAL DA, LANFERDINI E, CAMPOS PHRF, HAUSCHILD L. Efeito da suplementação de aminoácidos funcionais no desempenho pós-desmame de leitões de baixo padrão sanitário. 14º Simpósio Internacional de Suinocultura (SINSUI) – Produção, Reprodução e Sanidade Suína. Porto Alegre – RS, Centro de Eventos da PUCRS, 17 a 19 de maio de 2022.

4.2.5 ORTIZ MT, VALINI GAC, FRANÇA I, DE OLIVEIRA MJK, SILVA CA, ROSA JP, ARNAUT PR, **MELO ADB**, MARÇAL DA, HAUSCHILD L. Modelo de desafio com *Salmonella* Typhimurium e condições precárias de alojamento para suínos em crescimento. 14º Simpósio Internacional de Suinocultura (SINSUI) – Produção, Reprodução e Sanidade Suína. Porto Alegre – RS, Centro de Eventos da PUCRS, 17 a 19 de maio de 2022.

4.2.6 ARNAUT PA, VALINI GAC, ORTIZ MT, FRANÇA I, ROSA JP, SILVA CA, OLIVEIRA MJK, HTOO JK, BRAND HG, **MELO ADB**, MARÇAL DA, ANDRETTA I, HAUSCHILD L. Aminoácidos funcionais atenuam o impacto do desafio sanitário na exigência de mantença e melhoram a resiliência de suínos. 14º Simpósio Internacional de Suinocultura (SINSUI) – Produção, Reprodução e Sanidade Suína. Porto Alegre – RS, Centro de Eventos da PUCRS, 17 a 19 de maio de 2022.

4.2.7 DE OLIVEIRA MJK, AZEVEDO PHA, VALINI GAC, SILVA CA, ROSA JP, ARNAUT PR, FRANÇA I, **MELO ADB**, MARÇAL DA, CAMPOS IBS, HTOO JK, BRAND HG, CAMPOS PHRF, HAUSCHILD L. Effect of protein and amino acids diet level on performance and body composition of finishing-pigs under daily cyclic heat

stress. 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP), Granada, Espanha, de 12 a 15 de setembro de 2022.

4.2.8 SILVA CA, MARÇAL DA, **MELO ADB**, VALINI GAC, VEIRA AM, DE OLIVEIRA MJK, ROSA JP, FRAGA AZ, LIMA GFR, REMUS A, HAUSCHILD L. Protein deposition affects lysine efficiency of utilization on growing pigs. **7th EAAP** International Symposium on Energy and Protein Metabolism and Nutrition (ISEP), Granada, Espanha, de 12 a 15 de setembro de 2022.

4.2.9 FRANÇA I, VALINI GAC, MILLA IC, BLANCO MBCR, ORTIZ M, SILVA CA, ARNAUT PR, ROSA JP, DE OLIVEIRA MJK, **MELO ADB**, MARÇAL DA, HTOO JK, BRAND HG, LANFERDINI E, CAMPOS PHRF, HAUSCHILD L. Effect of functional amino acid supplementation from nursery to growing diets on performance and body composition of growing pigs under sanitary challenge. 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP), Granada, Espanha, de 12 a 15 de setembro de 2022.

4.2.10 MELO ADB, ROSA JP, FRANÇA I, VALINI GAC, DE OLIVEIRA MJK, SILVA CA, ARNAUT PR, ORTIZ M, MARÇAL DA, HTOO JK, BRAND HG, LANFERDINI E, CAMPOS PHRF, HAUSCHILD L. Effects of dietary supplementation of functional amino acid and weaning conditions on growth performance of piglets. 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP), Granada, Espanha, de 12 a 15 de setembro de 2022.

4.2.11 VALINI GAC, FRANÇA I, DE OLIVEIRA MJK, BARBOSA LG, SILVA CA, ROSA JP, ARNAUT PR, ORTIZ MT, DE AZEVEDO PHA, **MELO ADB**, MARÇAL DA, HTOO JK, BRAND HG, LANFERDINI E, CAMPOS PHRF, HAUSCHILD L. Dietary supplementation with L-Trp, L-Thr and DL-Met improves the immune status and protein deposition of growing pigs under sanitary challenge. 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP), Granada, Espanha, de 12 a 15 de setembro de 2022

4.2.12 SILVA CA, MARÇAL DANILO, HAUSCHILD L, **MELO ADB**, OLIVEIRA MJK, ROSA JP, FRAGA AZ, VEIRA AM, VALINI GAC, LIMA GFR. Compensatory

growth response and body composition after a period of lysine restriction in entire male growing pigs. PORK EXPO 2022 & X Congresso Internacional de Suinocultura, realizado nos dia 26 e 27 de outubro de 2022 em Foz do Iguaçu, Paraná, Brasil.

4.2.13 DE MORAES WH, GONÇALVES JPR, **MELO ADB**, MARÇAL DA, HAUSCHILD L. Efeito da suplementação de aminoácidos funcionais no desempenho de leitões desmamados alojados sob condições sanitárias precárias. XXXIV Congresso de Iniciação Científica da Unesp: Reflexos da Pandemia no Letramento e na Cultura Científica, realizado entre os dias 29/11/2022 e 01/12/2022 no Hotel Fazenda Hípica Atibaia, na cidade de Atibaia/SP.

4.2.14 FRANÇA I, VALINI GAC, ORTIZ MT, SILVA CA, ARNAUT PR, DE OLIVEIRA MJK, GONÇALVES JPR, MELO ADB, MARÇAL DA, **HAUSCHILD L**. Suplementação com aminoácidos funcionais modula o metabolismo e melhora a utilização de nitrogênio em suínos sob desafio sanitário. 15º Simpósio Internacional de Suinocultura - SINSUI 2023, realizado entre os dias 09 e 11 de maio de 2023, no Centro de Eventos da PUCRS, em Porto Alegre/RS.

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4.2.16 MARÇAL DA, **MELO ADB**, GONÇALVES JPR, DE OLIVEIRA MJK, ORTIZ MT, SILVA CA, HTOO JK, GOMES-NETO JC, BENSON A, HAUSCHILD L. Suplementação extra de Tre, Trp e Met atenua a redução de desempenho sem alterar a composição microbiana central de leitões sob desafio sanitário. XX Congresso Nacional da ABRAVES, promovido pela ABRAVES - Regional Rio Grande do Sul, realizado entre 16 e 19 de outubro de 2023, no Centro de Eventos da PUCRS, em Porto Alegre/RS.

4.2.17 VEIRA AM, SANTOS LS, SILVA CA, MARÇAL DA, **MELO ADB**, DE OLIVEIRA MJK, LOPES BT, CAMPOS LD, BARBOSA LG, HAUSCHILD L.

Comportamento alimentar de suínos alimentados com níveis de aminoácidos na dieta ajustados de acordo com ritmo circadiano. XX Congresso Nacional da ABRAVES, promovido pela ABRAVES - Regional Rio Grande do Sul, realizado entre 16 e 19 de outubro de 2023, no Centro de Eventos da PUCRS, em Porto Alegre/RS. **(Anexo 3_437)**

4.2.18 MELO ADB, MARÇAL DA, GONÇALVES JPR, ARNAUT PR, VALINI GAC, FRANÇA I, SILVA CA, ORTIZ MT, GASTMANN H, HAUSCHILD L. Suplementação de Thr, Met e Trp para leitões na creche criados em condições sanitárias precárias: estresse oxidativo. XX Congresso Nacional da ABRAVES, promovido pela ABRAVES - Regional Rio Grande do Sul, realizado entre 16 e 19 de outubro de 2023, no Centro de Eventos da PUCRS, em Porto Alegre/RS.

4.2.19 ORTIZ MT, PAULINO GSC, MARÇAL DA, **MELO ADB**, VALINI GAC, ARNAUT PR, FRANÇA I, DE OLIVEIRA AF, VEIRA AM, HAUSCHILD L. Variabilidade das exigências em lisina de suínos em crescimento sob desafio sanitário. XX Congresso Nacional da ABRAVES, promovido pela ABRAVES - Regional Rio Grande do Sul, realizado entre 16 e 19 de outubro de 2023, no Centro de Eventos da PUCRS, em Porto Alegre/RS.

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4.2.22 MARÇAL DA, **MELO ADB**, GONÇALVES JPR, YANG Q, DE OLIVEIRA MJK, ARNAUT PR, ORTIZ MT, FRANÇA I, VALINI GAC, SILVA CA, KORTH N, PAVLOVIKJ N, CAMPOS PHRF, BRAND HG, HTOO JK, GOMES-NETO JC, BENSON A, HAUSCHILD L. Fecal microbiome composition of weaned piglets under mixed management and poor housing conditions fed diets increased in tryptophan, threonine, and methionine supplementation. Aceito para ser apresentado no 2024 ASAS Midwest Annual Meeting, a ser realizado entre 10 e 13 de março de 2024 em Madison, Wisconsin, EUA.

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4.3 Dissertações defendidas sob coorientação do bolsista PD

4.3.1 GONÇALVES, JOSEANE PENTEADO ROSA. Suplementação de aminoácidos funcionais (tre, met e trp) para leitões criados sob condições sanitárias precárias. Dissertação (Mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho" Faculdade de Ciências Agrárias e Veterinárias.

4.3.2 ORTIZ, MANOELA TREVIZAN. Impacto do desafio sanitário na variabilidade do desempenho, da composição corporal e das exigências de lisina de suínos em crescimento. Dissertação (Mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho" Faculdade de Ciências Agrárias e Veterinárias.

4.4 Teses defendidas sob coorientação do bolsista PD

4.4.1 DE OLIVEIRA, MARLLON JOSÉ KARPEGGIANE. Impacto do estresse por calor e do nível dietético de proteína bruta e aminoácidos nas respostas de suínos em terminação. Tese (Doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho" Faculdade de Ciências Agrárias e Veterinárias.

4.4.2 ARNAUT, PEDRO RIGHETTI. Efeito do sistema de alimentação de precisão e nível de aminoácidos funcionais na dieta de suínos sob desafio sanitário (condições de higiene das instalações e inoculação com Salmonella Typhimurium). Tese (Doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho" Faculdade de Ciências Agrárias e Veterinárias.

4.4.2 VALINI, GRAZIELA A. C. Efeito da suplementação dietética de aminoácidos e condição sanitária de alojamento sobre a fisiologia e metabolismo de suínos em crescimento. Tese (Doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho" Faculdade de Ciências Agrárias e Veterinárias.

animals

MDPI

Article

Increased Dietary Trp, Thr, and Met Supplementation Improves Performance, Health, and Protein Metabolism of Weaned Piglets under Mixed Management and Poor Housing Conditions

Joseane Penteado Rosa Gonçalves [1,†], Antonio Diego Brandão Melo [1,2,†], Qinnan Yang [2,3], Marllon José Karpeggiane de Oliveira [1], Danilo Alves Marçal [1], Manoela Trevisan Ortiz [1], Pedro Righetti Arnaut [1], Ismael França [1], Graziela Alves da Cunha Valini [1], Cleslei Alisson Silva [1], Nate Korth [2,3], Natasha Pavlovikj [4], Paulo Henrique Reis Furtado Campos [5], Henrique Gastmann Brand [6], John Kyaw Htoo [7], João Carlos Gomes-Neto [2,3,8], Andrew K. Benson [2,3] and Luciano Hauschild [1,*]

1 Department of Animal Science, School of Agricultural and Veterinarian Sciences, São Paulo State University (UNESP), Campus Jaboticabal, São Paulo 14884-900, Brazil; jp.rosa@unesp.br (J.P.R.G.); diego.brandao@unesp.br (A.D.B.M.); marllon.oliveira@unesp.br (M.J.K.d.O.); danilo.a.marcal@unesp.br (D.A.M.); manoela.ortiz@unesp.br (M.T.O.); pedro.arnaut@unesp.br (P.R.A.); ismael.franca@unesp.br (I.F.); graziela.valini@unesp.br (G.A.d.C.V.); cleslei.silva@unesp.br (C.A.S.)
2 Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; qinnan@umich.edu (Q.Y.); nkorth2@huskers.unl.edu (N.K.); gomesneto.2@osu.edu (J.C.G.-N.); abenson1@unl.edu (A.K.B.)
3 Nebraska Food for Health Center, University of Nebraska-Lincoln, Lincoln, NE 68588, USA
4 Holland Computing Center, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; npavlovikj@unl.edu
5 Department of Animal Science, Universidade Federal de Viçosa, Viçosa 36570-900, Brazil; paulo.campos@ufv.br
6 Evonik Brazil Ltd.a, São Paulo 04711-904, Brazil; henrique.brand@evonik.com
7 Evonik Operations GmbH, 63457 Hanau, Germany; john.htoo@evonik.com
8 Department of Animal Science, Center for Food Animal Health, College of Food, Agricultural, and Environmental Sciences, The Ohio State University, Columbus, OH 43210, USA
* Correspondence: luciano.hauschild@unesp.br
† These authors contributed equally to this work.

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Simple Summary: Poor sanitary conditions (SC) in modern swine production can be associated with a variety of factors, including but not limited to: poor biosecurity protocol and management, erratic production logistics/workflow, ineffective cleaning and disinfection protocols, endemic respiratory and enteric diseases, and nutritional program inadequacy for the genotype × environmental interactions present in any given production system. Since nutrition is a large contributor to pig production cost and profitability, and it directly alters pig health individually and at the herd level, it has the potential to alter pig performance under poor sanitary conditions. Threonine, methionine, and tryptophan surplus supplementation did not affect performance nor immune or fecal microbiome parameters on high-sanitary-status pigs housed under good SC. However, our study supports that hypothesis by demonstrating that functional amino acids surplus supplementation improved the performance of piglets housed in a nursery mimicking a poor sanitary condition. Overall, an increased supply of key amino acids can be a viable nutritional strategy for commercial swine production, which is often under immune challenge conditions.

Abstract: A sanitary challenge was carried out to induce suboptimal herd health while investigating the effect of amino acids supplementation on piglet responses. Weaned piglets of high sanitary status (6.33 ± 0.91 kg of BW) were distributed in a 2 × 2 factorial arrangement into two similar facilities with contrasting sanitary conditions and two different diets. Our results suggest that increased Trp, Thr, and Met dietary supplementation could support the immune systems of piglets under a sanitary challenge. In this manner, AA+ supplementation improved the performance and metabolism of piglets under mixed management and poor sanitary conditions. No major temporal microbiome changes were associated with differences in performance regardless of sanitary conditions or diets. Since piglets often become mixed in multiple-site production systems and facility hygiene is also

often neglected, this study suggests that increased Trp, Thr, and Met (AA+) dietary supplementation could contribute to mitigating the side effects of these harmful risk factors in modern pig farms.

Keywords: amino acids; haptoglobin; immune challenge; microbiome; multiple origins

1. Introduction

Poor sanitary conditions (SC) affecting herd health may be associated with improper biosecurity practices adopted in modern swine farms [1]. Poor or suboptimal herd health is often a target of concern due to a diversity of factors that may contribute to a high prevalence of infections, including a variety of pathogen sources and transmission routes [2,3]. In this way, mixing management of pigs from different origins, as occurs in large-scale pig operations using multisite systems, could be a potential source of infectious disease contamination across farms [4]. Since sanitary conditions can largely vary between farms, this practice may trigger the introduction of endemic and epidemic viral and bacterial pathogens, which in turn may lead to illnesses, reducing growth performance and feed efficiency [5,6]. Furthermore, variability in herd health status could be a determinant factor increasing heterogeneity in pig performance within and between modern production farms [7,8]. Considering the improved financial return associated with good sanitary practices [9], mixing management and farm sanitary conditions should be frequent targets of improvement to maintain the herd's healthy status.

Poor management conditions impair health and growth by generating a cascade of physiological and metabolic changes in pigs [10], including reduced feed intake and immune system activation, which can alter nutrient utilization [11,12]. For instance, immune activation and inflammatory processes can alter feed intake patterns and protein deposition while increasing degradation of body muscle protein to provide amino acids (AAs) for acute phase protein synthesis in the liver [13]. Furthermore, circulating AAs can be repartitioned for the synthesis of immune cells and immune metabolites at the expense of growth performance [14]. The overactivation of the immune system may also increase the release of free radicals, which can overwhelm the steady-state antioxidant capacity [15]. Consequently, the average growth rate of pigs under poor sanitary conditions can be reduced by as much as 30% compared with that of healthy pigs [16,17].

As AAs, such as Thr, Met, and Trp, play essential roles in modulating immune responses and oxidative stress homeostasis [14,18–23], their dietary requirements may be increased for weaned piglets raised under poor sanitary conditions. In fact, there is supportive evidence that Thr, Met, and Trp supplementation above dietary requirements may attenuate the negative impact of sanitary challenges on growth performance while promoting the immune status of pigs [11,24,25]. Furthermore, there is the potential for AA to modulate the gut microbiome's composition, which in turn may be beneficial for host health in terms of nutrient absorption, overall metabolism, and immune functions [26]. Therefore, this study was performed to evaluate the effects of increased dietary Thr, Met, and Trp supplementation on the performance, metabolism, immunological and oxidative status, and fecal microbiota composition of weaned piglets raised under good sanitary conditions or under mixed management and poor housing conditions.

2. Materials and Methods

2.1. Housing, Animals, and Experimental Design

A total of 144 entire male piglets (Agroceres Pig Improvement Co. [AGPIC Camborough], Rio Claro, SP, Brazil), weaned at 21 days of age, with an initial body weight (BW) of 6.33 $\pm$ 0.91 kg, were used in the experiment. The piglets originated from a high-sanitary-status (HSS) multiplier farm. Piglets were allocated in the experiment using a randomized block design in a 2 $\times$ 2 factorial arrangement, composed of two SC (GOOD or MM + POOR) and two diets (control (CON), formulated to meet the nutritional requirements according

to [27]; or surplus supplementation with Thr:Lys, Met+Cys:Lys, and Trp:Lys ratios adjusted to 20% above the CON diet (AA+)). In total, 12 replicates per treatment with 3 piglets per replicate were used, considering each pen as an experimental unit, except for the fecal microbiome analysis, where each piglet was considered an experimental unit. Figure 1 illustrates the experimental design and shows the distribution of experimental piglets across the two facilities under contrasting SC (GOOD or MM + POOR). The serological results of the health-monitoring program of the high and low sanitary status farms are presented in Supplementary Table S1. In summary, the sanitary challenge model featured the mixing of piglets from farms with contrasting sanitary statuses, resulting in housing an additional 48 piglets from a farm with a low sanitary status, following a poor cleaning routine for floor manure removal.

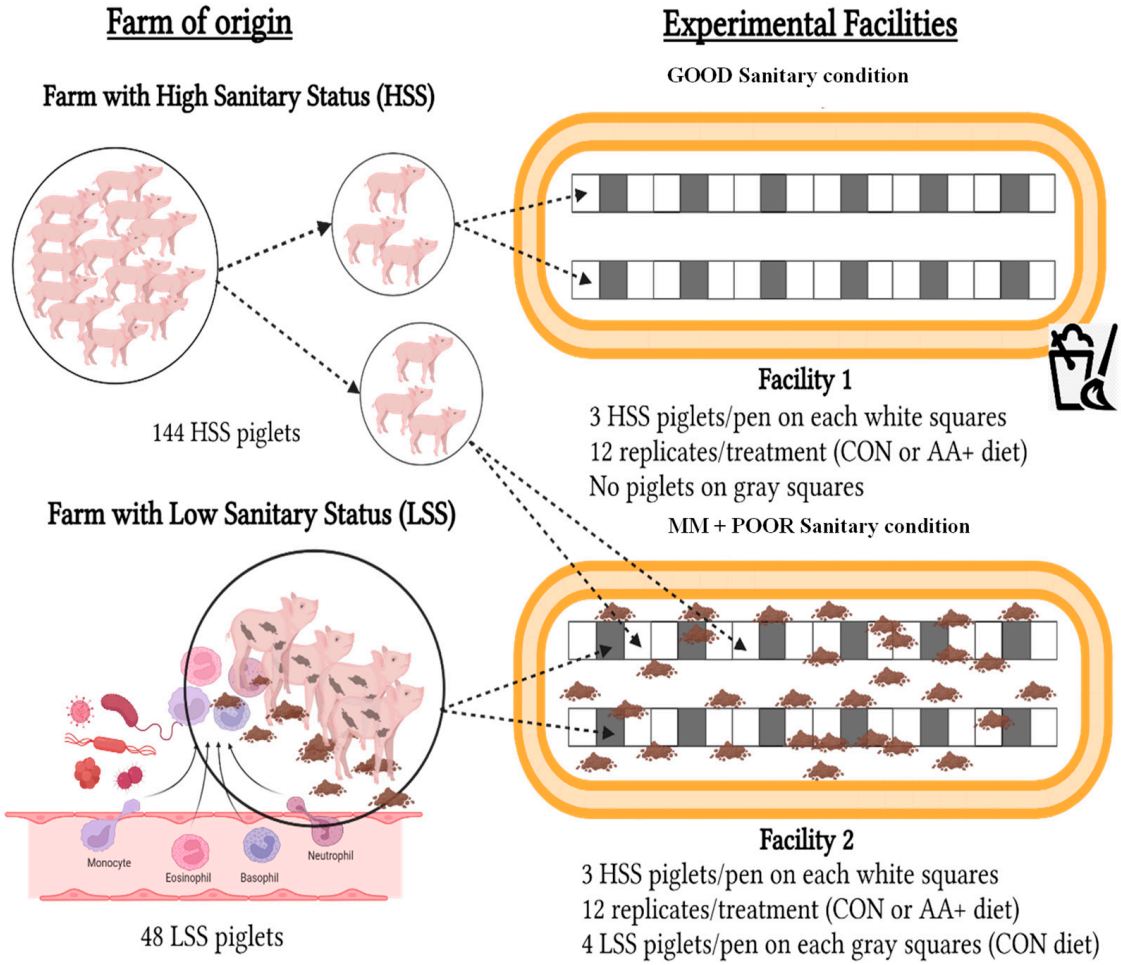

Figure 1. Experimental design presenting pen-based allocation of piglets across treatments. A total of 144 entire male piglets from a high-sanitary-status (HSS) multiplier farm were randomly distributed in a 2 × 2 factorial arrangement, composed of two sanitary conditions (Good or MM + Poor) and two diets (control (CON) or supplemented (AA+)). Each pen with HSS piglets was considered an experimental unit, except for the fecal microbiome analysis, where each piglet was considered an experimental unit. Good sanitary conditions were maintained by a daily cleaning protocol used to remove all manure from and under the slatted plastic floor. On the other hand, mixed management and poor sanitary conditions were mimicked by weekly cleaning of the facility while removing manure only from under the slatted plastic floor. Additionally, piglets from high- and low-sanitary-status (HSS and LSS, respectively) origins were co-housed to model poor sanitary conditions, where LSS piglets were non-experimental animals and were used only to compose the challenge model.

The experiment was carried out during the nursery phase in two similar facilities, each with 36 fully slatted plastic floor pens, for a total period of 42 days. Each pen (2.7 m^2) had slatted laterals and was equipped with a semiautomatic feeder and a nipple drinker. Both facilities underwent the same cleaning, disinfection, and sanitary interval protocol before piglet housing. As shown in Figure 1, 144 HSS piglets were allocated to one of two facilities under contrasting SC (GOOD or MM+POOR). In this way, 72 HSS piglets (3 piglets per pen; 24 pens in total) were distributed in each facility under GOOD or MM+POOR sanitary conditions. The 12 remaining pens were located between the HSS pens and were kept empty (without piglets) during the experiment in the facility with GOOD SC. In contrast, mimicking mixed management (MM) in the facility with POOR SC, a total of 48 non-experimental entire male piglets (AGPIC 337 × female Camborough), weaned at 21 days of age with an initial BW of 5.43 ± 0.24 kg, obtained from a commercial farm with low sanitary status (LSS), were randomly allocated at 4 piglets/pen into the 12 remaining pens located between pens with HSS piglets. In this way, all HSS pens were paralleled with LSS pens, allowing for physical contact between the piglets through the slatted laterals. In summary, 72 HSS piglets were allocated to the facility with GOOD SC, while 120 piglets were allocated into the facility with MM + POOR SC, of which 72 piglets were HSS and 48 piglets were LSS.

The MM+POOR SC facility had a weekly cleaning routine only to remove the manure located under the slatted plastic floor, which thus represented poor housing conditions. However, in the GOOD SC facility where only HSS animals were allocated, a daily cleaning routine was adopted to remove all manure on and under the slatted plastic floor. Antibiotics were not administered to piglets, regardless of the biosecurity pattern or facility in which they were housed. In addition, each management team was unique to each facility. The teams used clean and disinfected clothing and footwear and protective clothing to enter the facilities in order to avoid cross-contamination among both facilities. A day after ending the experimental period of 42 days, the POOR SC facility was cleaned daily, and experimental and non-experimental pigs were submitted to antibiotic therapy based on Enrofloxacin and Tulathromycin (single dosage). After a week, all pigs were transferred to a commercial farm for the growing and finishing phases.

2.2. Experimental Diets

The experimental diets (Table 1) were provided in three feeding phases lasting 14 days each (Phase 1: Days 0 to 14; Phase 2: Days 15 to 28; and Phase 3: Days 29 to 42). All experimental diets were corn- and soybean meal-based. In each phase, HSS piglets were fed the CON diet, formulated to meet the nutritional requirements according to [27], or the AA+ diet, with Thr:Lys, Met+Cys:Lys, and Trp:Lys ratios increased to 20% above the ratios used in the CON diet. LSS piglets were fed the same control diet provided for HSS piglets in each feeding phase during the entire experiment. The diets were formulated using the reported nutrient content, the analyzed AA content of ingredients (NIRS), and the standardized ileal digestibility coefficients (SID) of the AAs, according to [28]. All piglets were given ad libitum access to water and feed throughout the study in both SCs.

Table 1. Ingredients and nutrient compositions of the experimental diets.

Ingredients, %	Phase 1 (d 0 to 14)		Phase 2 (d 15 to 28)		Phase 3 (d 29 to 42)	
	CON [1]	AA+ [1]	CON	AA+	CON	AA+
Corn	49.41	49.41	58.85	58.85	69.64	69.64
Soybean meal	15.00	15.00	20.00	20.00	26.00	26.00
Whey protein	21.42	21.42	12.14	12.14	-	-
Soy protein concentrated (60% CP)	5.23	5.23	2.55	2.55	-	-
Spray-dried blood plasma	5.00	5.00	2.50	2.50	-	-
Dicalcium phosphate	0.870	0.870	0.655	0.655	0.512	0.512

Table 1. *Cont.*

Ingredients, %	Phase 1 (d 0 to 14)		Phase 2 (d 15 to 28)		Phase 3 (d 29 to 42)	
	CON [1]	AA+ [1]	CON	AA+	CON	AA+
Limestone	0.795	0.795	0.884	0.884	0.819	0.819
Soybean oil	-	-	0.100	0.100	0.125	0.125
Salt	0.330	0.330	0.445	0.445	0.450	0.450
Mineral premix [2]	0.110	0.110	0.100	0.100	0.090	0.090
Vitamin premix [3]	0.060	0.060	0.050	0.050	0.050	0.050
Choline chloride	0.060	0.060	0.060	0.060	0.060	0.060
Corn starch	0.500	0.105	0.500	0.146	1.000	0.665
Sodium bicarbonate	-	-	-	-	0.053	0.053
Enzae® (phytase) [4]	0.005	0.005	0.005	0.005	0.005	0.005
Biolys (54.6%) [5]	0.66	0.66	0.68	0.68	0.77	0.77
DL-Met (99%) [5]	0.23	0.39	0.19	0.34	0.16	0.30
L-Thr (98.5%) [5]	0.14	0.32	0.13	0.30	0.15	0.30
L-Trp (98%) [5]	0.001	0.05	0.008	0.04	0.008	0.04
L-Val (98%) [5]	0.11	0.11	0.11	0.11	0.09	0.09
L-Ile	0.04	0.04	0.01	0.01	-	-
Total	100	100	100	100	100	100
Nutrients, calculated values						
ME, kcal/kg	3386	3391	3354	3358	3331	3335
NE, kcal/kg	2521	2524	2516	2518	2470	2473
CP, %	21.2	21.5	19.5	19.9	18.8	18.98
SID Lys, %	1.50	1.50	1.35	1.35	1.23	1.23
SID Met + Cys, %	0.82	0.98	0.74	0.89	0.68	0.82
SID Met, %	0.48	0.64	0.44	0.59	0.41	0.55
SID Thr, %	0.88	1.06	0.79	0.95	0.73	0.88
SID Trp, %	0.25	0.30	0.22	0.26	0.20	0.24
SID Val, %	1.02	1.02	0.92	0.92	0.84	0.84
SID Arg, %	1.11	1.11	1.05	1.05	1.08	1.08
SID Ile, %	0.80	0.80	0.72	0.72	0.69	0.69
SID Leu, %	1.58	1.58	1.46	1.46	1.42	1.42
SID His, %	0.52	0.52	0.46	0.46	0.44	0.44
SID Phe + Tyr, %	1.61	1.61	1.45	1.45	1.38	1.38
SID Phe, %	0.94	0.94	0.85	0.85	0.81	0.81
STTD P, %	0.45	0.45	0.40	0.40	0.33	0.33
Ca, %	0.85	0.85	0.80	0.80	0.70	0.70
Na, %	0.40	0.40	0.35	0.35	0.20	0.20
Lactose, %	15.00	15.00	8.50	8.50	-	-
Nutrients, total analyzed values						
CP, %	21.6	22.0	20.7	21.0	19.2	19.3
Lys, %	1.58	1.59	1.50	1.58	1.36	1.38
Met + Cys, %	0.89	0.94	0.85	0.99	0.74	0.81
Met, %	0.48	0.53	0.48	0.63	0.43	0.51
Thr, %	1.02	1.08	0.91	1.05	0.84	0.90
Val, %	1.16	1.20	1.10	1.09	0.96	0.94
Arg, %	1.23	1.25	1.20	1.22	1.15	1.13
Ile, %	0.84	0.85	0.80	0.82	0.77	0.76
Leu, %	1.79	1.81	1.72	1.73	1.66	1.70
His, %	0.56	0.56	0.53	0.51	0.50	0.50
Phe, %	1.01	1.03	0.95	0.96	0.89	0.90

[1] CON, AA profile diet according to the NRC (2012); AA+, diet with Thr:Lys, Met+Cys:Lys, and Trp:Lys ratios increased by 20% above the CON diet. [2] Mineral supplement (per kg of feed): manganese 0.0522 g; zinc 0.1375 g; iron 0.1045 g; copper 0.016 g; iodine 1.2 mg; selenium 0.121 mg. [3] Vitamin supplement (per kg of feed): vitamin A 20600000 IU; 25-Hydroxy Vitamin D3 4600000 IU; vitamin E 130000 IU; vitamin K3 0.054 g; vitamin B1 0.0036 g; vitamin B2 0.012 g; vitamin B6 0.036 g; folic acid 0.00204 g, biotin 0.000192 g; niacin 0.054 g; vitamin B5 0.03 g. [4] Phytase was provided by Cargill and contained 500 FTU/ton. [5] Amino acids (BioLys, MetAmino, ThreAmino, TrypAmino, ValAmino, respectively) were provided by Evonik Nutrition & Care GmbH (Hanau-Wolfgang, Germany).

2.3. Data Collection

2.3.1. Performance

All piglets were weighed individually at the beginning of the experiment and at the end of each feeding phase without fasting. All feed provided and associated leftovers from each pen were weighed weekly. The BW and feed intake of 144 HSS piglets were recorded from Day 0. Thus, BW, average daily gain (ADG), average daily feed intake (ADFI), and gain:feed ratio (G:F) were evaluated for each feeding phase and for the overall experimental period (Days 0 to 42).

2.3.2. Blood Sampling

On Day 0, after fasting for 8 h, blood samples were nonrandomly collected from one piglet per pen (n = 8 for HSS piglets and n = 8 for LSS piglets) between 06:00 and 08:00 h. On Days 21 and 42, after fasting for 8 h, blood samples were collected only from HSS piglets allocated into the GOOD and MM+POOR SC groups. Blood samples were always collected from the piglet with a BW closest to the average BW of the pen on Day 0. The four pens not selected for blood sampling consisted of two pens with the heaviest piglets and two pens with the lightest piglets, totaling n = 8 for HSS piglets for each treatment in both facilities.

A volume of 8 mL of blood was drawn by puncture of the jugular vein into sterile tubes containing EDTA (BD Vacutainer, São Paulo, SP, Brazil). On Day 0, shortly after blood drawing, a fraction of the blood aliquot was used for total and differential leukocyte counting (eosinophils, neutrophils, lymphocytes, and monocytes) by an automated Microcell counter (Horiba Micros-60; Horiba ABX SAS, Montpellier, France). Blood samples collected on Days 0, 21, and 42 were kept refrigerated on ice and centrifuged at 1500× *g* for 10 min at 4 °C (Novatecnica®, NT 835, Piracicaba, SP, Brazil) to obtain serum and plasma. Subsequently, both serum and plasma aliquots were stored at −80 °C for subsequent analyses of the serum concentration of acute phase proteins, metabolites, and plasma oxidative parameters.

2.3.3. Analysis of Acute Phase Proteins and Biochemical and Redox Parameters

Serum acute phase proteins (immunoglobulin A (IgA), immunoglobulin G (IgG), ceruloplasmin, transferrin, albumin, haptoglobin, and α1 acid glycoprotein) were analyzed by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels [29], and the concentrations were normalized by the total protein serum concentration (Labmax Plenno; Labtest Diagnostics SA, Lagoa Santa, Brazil). Serum concentrations of total protein, urea, creatinine, lactate, and lactate dehydrogenase (LDH) were analyzed in a high-performance semiautomatic spectrophotometer using the biuret method for biochemical tests (Labmax Plenno; Labtest Diagnostics SA, Lagoa Santa, Brazil), with the support of Labtest commercial reagents®. Blood plasma on Day 21 was used to evaluate the piglets' oxidative status according to the following parameters: reactive oxygen species (ROS), lipid peroxidation (LPO), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione S-transferase (GST), reduced glutathione (GSH), glutathione disulfide (GSSH), and GSH:GSSH ratio. T-AOC was evaluated by the ferric-reducing ability of plasma (FRAP) according to the method of [30]. SOD activity was determined by a spectrophotometric method [31]. The ferrous oxidation in xylenol orange (FOX) assay was used for the detection of plasma LPO [32]. A fluorometric method was used to determine plasma GSH and GSSH concentrations [33]. The Nernst equation ($Eh = -264 - (61.5/2) \times \log GSH2/GSSG$) was applied to determine the redox potential of the GSH:GSSG ratio. In addition, a fluorometric method was also used for the analysis of plasma ROS determination [34]. GST was determined with the conjugation of GSH with 1-chloro-2,4-dinitrobenzene catalyzed by GST [35].

2.3.4. Fecal Sampling and DNA Extraction

Fecal samples were always collected from the HSS and LSS piglet with the BW closest to the average BW of the pen on Day 0 (n = 11). All fecal DNA extractions were conducted using the Qiagen DNeasy® PowerSoil® Pro kit according to the manufacturer's guidelines.

All samples were eluted in 60 µL of elution buffer and frozen at −80 °C prior to quality assessment and sequencing. All samples were quality checked for DNA concentration using a Nanodrop One spectrophotometer (Thermo Fisher Scientific c Inc., Middletown, VA, USA).

2.3.5. 16S rRNA Amplicon Sequencing and Bioinformatic Processing

The V4 region of the bacterial 16S rRNA gene was amplified from each sample using Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific, Waltham, MA, USA), following the modified dual-indexing sequencing strategy [36,37]. Paired-end sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) program (version 2, 2021.2) [38]. Sequences were truncated (220 bases for forward reads and 160 bases for reverse reads) and denoised into amplicon sequence variants (ASVs) using DADA2 [39], and then rarefied to 5000 reads per sample. A total of 3,384,304 sequences were reads, with an average of 25,638 reads per sample for the bacterial 16S rRNA analysis (Table S1). All ASVs were assigned taxonomic information using the prefitted sklearn-based taxonomy classifier SILVA database (release 138) [40,41]. All sequences can be found on NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA985664, accessed on 20 June 2023). Prior to statistical analysis, only bacterial taxa (those containing the domain "Bacteria") containing genus-level information in the name (g from QIIME2 output) were filtered for both diversity and taxonomic analyses.

2.4. Network Analysis of the Fecal Microbiome Data

Network construction and visualization were performed using the NetCoMi package [42] within the R (v4.2.2) statistical framework. Overall, the samples were normalized to 5000 reads and grouped by sampling time point, SC, and diet. Each network was constructed for each group or identified treatment by employing a centered log-ratio transformation and Spearman-based correlations between the 50 most abundant microbial taxa. Data interpretation was summarized based on each node representing a bacterial taxon, and the size of each node was scaled according to its centrality. Edges represent associations between taxa, with positive associations colored green and negative associations colored red; the thickness of each edge corresponds to the strength of the association. Edges representing a value less than 0.5 are not shown. Taxa were colored based on clusters calculated in the network construction. The three taxa with the highest degree of centrality were selected to highlight cluster-specific central taxa. When the sample size was limited, the data were excluded from the analysis.

2.5. Statistical Analyses

For pig performance and metabolic, immunological, and oxidative parameters, data normality was verified using the Shapiro–Wilk test, and studentized residues were used to verify outliers. Subsequently, data were analyzed using the GLIMMIX procedure of SAS [43] and presented as least squares and means. The mixed model used for data analysis from HSS piglets following the factorial design included the fixed effects of SC, diets (Ds), and their interactions (SC × D). The effect of the diet was also analyzed separately within each SC. For mixed models, the blocks of BW were included as a random effect. The results of the analysis of acute phase proteins and biochemical parameters on Day 0 were used as covariates for Days 21 and 42. The model used for data analysis of leucocyte counts included a fixed effect for sanitary status (HSS vs. LSS piglets) on Day 0. No other responsive parameter was evaluated for LSS piglets during the experiment, except for comparing HSS and LSS fecal microbiome alpha- and beta-diversity (D21 and 42). LSS piglets are non-experimental animals that were allocated into the poor SC facility only to compose the mixed management as part of the challenge model. The effects were considered significant, with values of $p \leq 0.05$ and trends with values of $p \leq 0.10$.

All bacterial taxonomic outputs from quality control to statistical modeling were processed using R version 4.0.5. Only bacterial genus-level annotation was used in all statistical

analyses. In brief, the tidyverse library (version 1.3.1) was used for data exploration, analysis, and plotting. All analyses were conducted by separating the two facilities differing in SC: "GOOD" vs. "POOR". For alpha-diversity (Shannon's and Simpson's D indices), Welch's two-sample T test (two treatments only—diet or SC effect) was used to assess significance ($p \leq 0.05$). Both the Shannon's and Simpson's D indices of alpha-diversity were calculated with the diversity function in R from the vegan library (version 2.6.2). The Welch two-sample t test function used was from the stats library (version 4.0.5).

Beta-diversity was calculated using the vegdist function from the vegan library in R (version 2.6.2) while using a Bray–Curtis distance matrix and removing missing values of the analysis. For the principal component analysis (PCA), a classical multidimensional scaling model was used to reduce the data to two dimensions (2 principal components—PCs) using the cmdscale function (k = 2) in R from the stats library (version 4.0.5). PERMANOVA was used to calculate the effect of treatment on beta-diversity (Bray–Curtis distances) using the adonis2 function in R from the vegan library (version 2.6.2).

Additionally, for two-group differences in taxon relative abundances, a Welch two-sample T test function was used. However, both the false discovery rate (FDR) and Bonferroni correction (BC) were applied to reduce false discoveries. Only the taxa that passed the significance level for both the FDR and BC at the end points (Day 21 and Day 42) were considered significantly different between diet groups for the HSS animals ($p \leq 0.05$). Ultimately, differences between the mean relative abundance for each family/genus/species (taxon) across groups were depicted as a heatmap using the $\log_2$-transformed relative abundance of the most dominant taxa based on a 2% cutoff and network analysis of clusters of co-occurrences of the most relevant taxa. For taxa with zero mean counts for a given treatment, the value of 1 was added prior to $\log_2$ transformation being applied. For all R functions used, when not stated, the default parameters were used for calculations. Due to the individuality of the gastrointestinal microbiome composition in animals, each piglet was considered an experimental unit for the analysis [44].

3. Results

3.1. Characterization of the Health and GI Microbiome Status

On Day 0 (at arrival), piglets from the LSS farm had, on average, higher counts of total leukocytes ($p < 0.01$), granulocytes ($p < 0.01$), and monocytes ($p < 0.01$) than those in piglets from the HSS farm (Table 2). However, there was no significant difference in the lymphocyte count between HSS and LSS piglets ($p > 0.05$). GI microbiome analysis further revealed no significant differences in alpha-diversity (Shannon's and Simpson's D indices) between HSS and LSS piglets on Day 0 (Figure 2A,B, respectively). Notably, LSS piglets appeared to have started the trial with lower alpha-diversity, although the *p* value was not significant (Figure 2A,B). However, beta-diversity analysis captured different clustering on Day 0, separating HSS vs. LSS piglets ($p < 0.005$, R-squared = 0.12) (Figure 2C). Multiple taxa showed significant differences between HSS and LSS on Day 0, but only *Oribacterium* passed the family-wise *p* value correction thresholds after accounting for the number of comparisons made between groups ($p = 0.00014$, FDR = 0.0333, Bonferroni = 0.0333). A posteriori (Days 21 and 42), no taxon was found to be significantly different between HSS and LSS piglets allocated to the MM + POOR SC. In addition, the serological results of the health-monitoring program conducted for high- and low-sanitary-status farms are presented in Table S2.

Table 2. Serum white blood cell (WBC) counts in piglets from farms with high or low sanitary status (HSS or LSS).

Item (no. × 10^9/L)	HSS Piglets	LSS Piglets	RSD [1]	*p*-Value
Leukocytes	17.2	20.6	0.349	<0.01
Granulocytes [2]	7.7	9.9	0.749	<0.01
Lymphocytes	8.5	9.4	0.422	0.19
Monocytes	1.0	1.3	0.053	<0.01

[1] Residual standard deviation; [2] granulocytes correspond to the sum of neutrophils, eosinophils, and basophils. Each pen was considered an experimental unit (HSS piglets n = 12; LSS piglets n = 12).

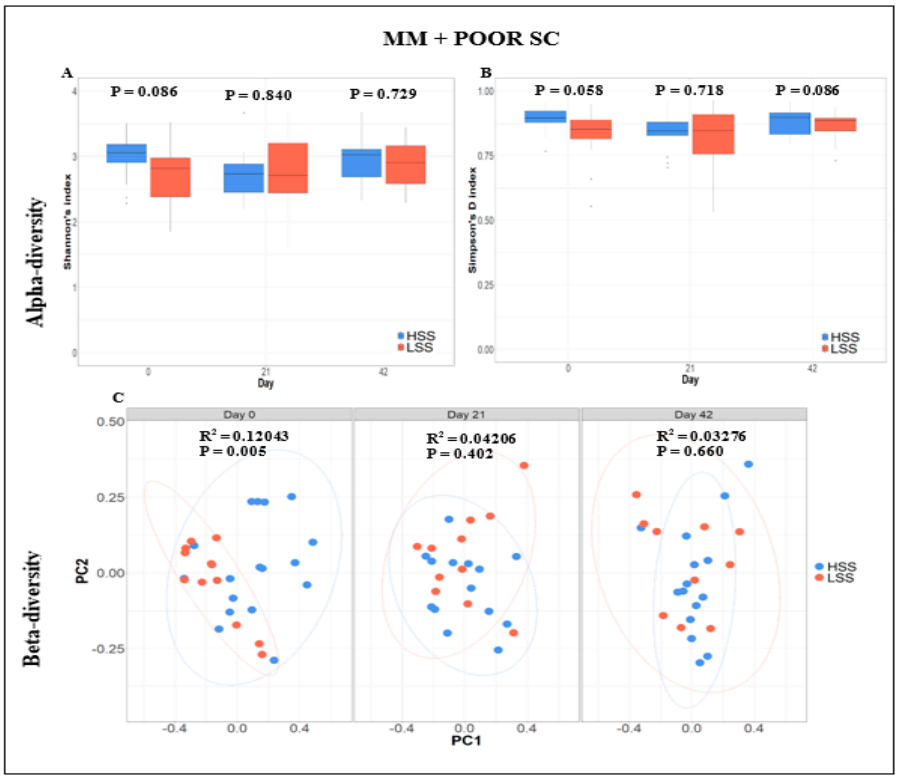

Figure 2. Alpha- and beta-diversity analysis of fecal microbiome samples across piglets of either high or low sanitary status (HSS vs. LSS), housed under mixed management and poor sanitary conditions (MM + Poor SC) regardless of the diet provided. (**A**) shows no significant difference in alpha-diversity (Shannon's index) across HSS and LSS piglets over time under mixed management and poor sanitary conditions. (**B**) shows no significant difference in alpha-diversity (Simpson's D Index) across HSS piglets over time under poor sanitary conditions. A Welch two-sample T-test was used to assess the difference between HSS and LSS piglets for alpha-diversity (Shannon's and Simpson's D indexes). (**C**) shows a significant difference in Bray–Curtis distance matrix across HSS and LSS piglets. Two principal components (PC1 and PC2) are shown for fecal samples across 0, 21, and 42 days of experiment. A PERMANOVA model was used to assess significant differences ($p < 0.05$), which were only found across HSS and LSS piglets at day 0 ($p = 0.005$, $R^2 = 0.12043$). Beta-diversity differences across HSS and LSS piglets at arrival (d0) represent that farm sanitary conditions affect gastrointestinal microbiome composition. The sample sizes for the fecal samples were: MM + Poor SC (**A**–**C**) at day 0, HSS (n = 17) and LSS (n = 12); at day 21, HSS (n = 14) and LSS (n = 11); and day 42, HSS (n = 15) and LSS (n = 11). Each animal was considered an experimental unit throughout the analysis.

3.2. Growth Performance

There was no interaction ($p > 0.05$) between SC and D on growth performance variables in any of the experimental phases evaluated (Table 3). However, HSS piglets under GOOD

SC had higher final BW, ADFI, and ADG than HSS piglets under MM + POOR SC in all experimental phases ($p < 0.05$). Furthermore, throughout the experiment (Days 0 to 42), piglets under GOOD SC had higher final BW, ADFI, ADG, and G:F than piglets under MM + POOR SC did ($p < 0.05$). Within each SC, piglets under MM + POOR SC fed AA+ showed a trend ($p < 0.10$) of higher final BW, ADFI, and ADG compared to those of piglets fed the CON diet during Phase 2. During Phase 3, and for the entire nursery phase, piglets under MM + POOR SC fed the AA+ diet had greater ($p < 0.05$) final BW, ADFI, and ADG compared to those of CON diet piglets.

Table 3. Performance of piglets fed control or amino-acid-supplemented diet above the requirement, raised under good or mixed management and poor sanitary conditions (SC) during the nursery phase (42 days).

	GOOD SC [1]			MM + POOR SC [1]				*p*-Value
Item	**CON [1]**	**AA+ [1]**	***p* Value**	**CON**	**AA+**	***p* Value**	**RSD [2]**	**SC**
Initial BW, kg	6.35	6.26	0.64	6.26	6.16	0.57	0.215	0.51
Phase 1 (d 0 to 14)								
Final BW, kg	8.91	8.85	0.83	8.24	8.61	0.17	0.313	0.03
ADFI, g	282	256	0.19	245	223	0.45	32	0.03
ADG, g	182	185	0.89	123	154	0.32	37	0.02
G:F	0.67	0.72	0.70	0.49	0.65	0.19	0.138	0.15
Phase 2 (d 15 to 28)								
Final BW, kg	15.60	16.12	0.45	12.76	13.86	0.09	0.723	<0.01
ADFI, g	595	646	0.35	434	523	0.06	55	<0.01
ADG, g	482	524	0.22	325	373	0.10	46	<0.01
G:F	0.84	0.83	0.91	0.77	0.77	1.00	0.078	0.16
Phase 3 (d 29 to 42)								
Final BW, kg	22.71	23.85	0.26	18.20	20.95	<0.01	1.053	<0.01
ADFI, g	834	953	0.02	652	824	<0.01	64	<0.01
ADG, g	503	555	0.24	395	512	<0.01	46	<0.01
G:F	0.61	0.58	0.34	0.61	0.61	0.87	0.031	0.68
Overall (d 0 to 42)								
ADFI, g	573	614	0.17	441	523	0.01	38	<0.01
ADG, g	394	425	0.32	282	345	0.01	37	<0.01
G:F	0.69	0.69	0.86	0.63	0.65	0.34	0.034	0.01

[1] CON, AA profile diet according to the NRC (2012) [27]; AA+, diet with Thr:Lys, Met+Cys:Lys, and Trp:Lys ratios increased by 20% above the CON diet; GOOD SC, facility without mixing piglets from different origins and daily cleaning; MM + POOR SC, facility with a mixed management of piglets from different origins and poor sanitary conditions. [2] Residual standard deviation. Each pen was considered an experimental unit (n = 12).

3.3. Acute-Phase Proteins, Biochemical and Redox Parameters

There was no interaction ($p > 0.05$) between SC and D for any of the acute phase proteins analyzed on Days 21 and 42 (Table 4). On Day 21, the average serum concentration of haptoglobin, a positive acute phase protein, in piglets under MM + POOR SC was approximately 44% higher (0.35 vs. 0.80 mg/mL, $p < 0.05$) than that in piglets under GOOD SC. On Day 42, haptoglobin concentrations were similar between SC ($p > 0.05$), whereas the average serum concentration of albumin, a negative acute-phase protein, in piglets under MM + POOR SC was lower (33.8 vs. 31.2 g/L, $p < 0.05$) than that in piglets under GOOD SC.

There was no interaction ($p > 0.05$) between SC and D and non-SC effects for any of the biochemical variables analyzed on Days 21 and 42 (Table 5). However, on Day 21, piglets under MM + POOR SC fed the AA+ diet had lower ($p < 0.05$) serum urea and creatinine concentrations than those in CON diet piglets (16.35 vs. 27.13 mg/dL and 1.04 vs. 1.19 mg/dL, respectively). On Day 42, piglets under MM + POOR SC fed the AA+ diet had lower ($p < 0.05$) serum urea and lactate concentrations than those in CON diet piglets (19.21 vs. 26.06 mg/dL and 85.75 vs. 48.71 mg/dL, respectively). On this same day, AA supplementation tended to increase LDH serum concentration in GOOD SC piglets

(p = 0.09), with concentrations, on average, being 1256 vs. 1403 U/L in piglets fed the AA+ diet compared to the CON diet, respectively.

Table 4. Serum concentration of acute-phase proteins of piglets fed control or amino-acid-supplemented diet above the requirement raised under good or mixed management and poor sanitary conditions (SCs) during the nursery phase (42 days).

Item	GOOD SC [1]			MM + POOR SC [1]				*p*-Value
	CON [1]	AA+ [1]	*p*-Value	CON	AA+	*p*-Value	RSD [2]	SC
Day 0								
IgA, mg/mL	1.50	1.40	0.51	1.30	1.50	0.36	0.024	0.68
IgG, mg/mL	5.90	6.10	0.76	5.10	6.40	0.13	0.096	0.62
Ceruloplasmin, mg/mL	0.90	0.90	0.95	0.80	1.00	0.15	0.013	0.45
Transferrin, mg/mL	3.60	3.90	0.29	3.50	3.50	0.99	0.031	0.15
Albumin, g/L	35.3	35.1	0.94	34.9	34.5	0.85	0.238	0.75
Haptoglobin, mg/mL	0.50	0.60	0.73	0.60	0.70	0.22	0.014	0.19
α-1 acid glycoprotein, µg/mL	38.6	48.6	0.84	45.7	51.4	0.69	0.013	0.53
Day 21								
IgA, mg/mL	1.30	1.20	0.29	1.40	1.20	0.17	0.025	0.73
IgG, mg/mL	6.90	6.60	0.82	8.40	7.20	0.24	0.106	0.16
Ceruloplasmin, mg/mL	0.60	0.60	0.54	0.70	0.70	0.77	0.014	0.31
Transferrin, mg/mL	3.80	3.90	0.84	3.70	4.10	0.26	0.044	0.91
Albumin, g/L	28.8	28.8	0.99	28.4	29.5	0.53	0.197	0.92
Haptoglobin, mg/mL	0.40	0.30	0.52	1.00	0.60	0.07	0.025	0.002
α-1 acid glycoprotein, µg/mL	45.0	38.3	0.44	48.6	51.4	0.29	0.019	0.13
Day 42								
IgA, mg/mL	1.50	1.30	0.41	1.40	1.40	0.80	0.025	0.89
IgG, mg/mL	9.50	9.80	0.87	10.40	12.30	0.29	0.185	0.18
Ceruloplasmin, mg/mL	1.10	0.90	0.20	0.80	1.00	0.18	0.014	0.13
Transferrin, mg/mL	4.30	4.60	0.21	4.20	4.30	0.82	0.026	0.44
Albumin, g/L	33.5	34.1	0.78	31.3	31.1	0.91	0.187	0.05
Haptoglobin, mg/mL	1.00	0.70	0.16	0.60	0.80	0.35	0.022	0.43
α-1 acid glycoprotein, µg/mL	48.3	48.6	0.94	50.0	34.3	0.14	0.014	0.38

[1] CON, AA profile diet according to the NRC (2012); AA+, diet with Thr:Lys, Met+Cys:Lys, and Trp:Lys ratios increased by 20% above the CON diet; GOOD SCs, facility without mixing piglets from different origins and daily cleaning; MM + POOR SCs, facility with a mixed management of piglets from different origins and poor sanitary conditions. [2] Residual standard deviation. Each pen was considered an experimental unit (n = 12).

Table 5. Serum biochemical profile of piglets fed control or amino-acid-supplemented diet above the requirement and raised under good or mixed management and poor sanitary conditions (SC) during the nursery phase (42 days).

Item	GOOD SC [1]			MM + POOR SC [1]				*p*-Value
	CON [1]	AA+ [1]	*p*-Value	CON	AA+	*p*-Value	RSD [2]	SC
Day 0								
Total protein, g/dL	5.22	5.21	0.95	5.01	5.09	0.76	0.277	0.39
Urea, mg/dL	17.96	19.44	0.66	24.77	18.60	0.10	3.520	0.24
Creatinine, mg/dL	1.59	1.44	0.10	1.65	1.46	0.05	0.090	0.51
Lactate, mg/dL	50.57	59.38	0.26	43.07	31.13	0.13	7.710	0.003
Lactate dehydrogenase, U/L	1415	1345	0.33	1485	1303	0.01	69.980	0.78
Day 21								
Total protein, g/dL	4.68	4.65	0.91	4.78	4.84	0.824	0.272	0.45
Urea, mg/dL	19.35	19.18	0.96	27.13	16.35	0.003	3.420	0.30
Creatinine, mg/dL	1.08	1.08	0.97	1.19	1.04	0.04	0.070	0.42
Lactate, mg/dL	48.79	51.25	0.75	41.21	44.19	0.70	7.720	0.18
Lactate dehydrogenase, U/L	1333	1314	0.80	1227	1279	0.49	76.810	0.18

Table 5. *Cont.*

Item	GOOD SC [1]			MM + POOR SC [1]				*p*-Value
	CON [1]	AA+ [1]	*p*-Value	CON	AA+	*p*-Value	RSD [2]	SC
Day 42								
Total protein, g/dL	5.59	5.69	0.69	5.45	5.63	0.43	0.235	0.54
Urea, mg/dL	23.38	18.67	0.16	26.06	19.21	0.04	3.310	0.49
Creatinine, mg/dL	1.31	1.34	0.65	1.27	1.22	0.47	0.080	0.14
Lactate, mg/dL	67.28	71.09	0.81	85.75	48.71	0.03	15.990	0.86
Lactate dehydrogenase, U/L	1256	1403	0.09	1281	1253	0.75	87.250	0.31

[1] CON, AA profile diet according to the NRC (2012); AA+, diet with Thr:Lys, Met+Cys:Lys, and Trp:Lys ratios increased by 20% above the CON diet; GOOD SC, facility without mixing piglets from different origins and daily cleaning; MM + POOR SC, facility with mixed management of piglets from different origins and poor sanitary condition. [2] Residual standard deviation.

There was no significant interaction ($p > 0.05$) between SC and D for any of the oxidative metrics analyzed on Day 21 (Table 6). However, the blood plasma of piglets under MM + POOR SC showed a tendency ($p = 0.08$) toward increased circulating ROS levels. Moreover, SOD activity was higher ($p < 0.05$) in pigs under GOOD SC than in pigs under MM + POOR SC. Regarding the diet effect, pigs under GOOD SC fed the AA+ diet had lower GSH:GSSG Eh values (a lower value, i.e., closer to zero, indicates a less oxidized redox status) than that in piglets fed the CON diet (−316.9 vs. −324.3, $p = 0.05$).

Table 6. Plasma redox parameters of piglets fed control or amino-acid-supplemented diet above the requirement and raised under GOOD or MM + POOR sanitary conditions (SC) during the nursery phase (42 days).

Item [3]	GOOD SC [1]			MM + POOR SC [1]				*p*-Value
	CON [1]	AA+ [1]	*p* Value	CON	AA+	*p* Value	RSD [2]	SC
GSH, pmol.mL^{-1}	20.27	22.47	0.46	19.57	22.99	0.31	2.32	0.96
GSSG, pmol.mL^{-1}	8.02	5.92	0.16	5.94	6.89	0.62	1.24	0.64
GSH:GSSG E_h, mV [4]	−316.9	−324.3	0.05	−320.5	−321.4	0.55	3.139	0.89
GST, mU.mL^{-1}	6.82	6.94	0.82	7.21	7.30	0.85	0.42	0.32
LPO, mmol.mL^{-1}	38.75	25.17	0.18	32.79	28.64	0.69	7.28	0.70
SOD, U.mL^{-1}	47.02	46.79	0.91	44.04	44.16	0.90	1.15	0.01
ROS, DCFHA-DA [5]	458.8	336.9	0.21	601.7	440.6	0.27	88.26	0.08
T-AOC, μM.L^{-1}	2.99	3.51	0.80	1.55	2.61	0.49	1.97	0.29

[1] CON, AA profile diet according to the NRC (2012); AA+, diet with Thr:Lys, Met+Cys:Lys, and Trp:Lys ratios increased by 20% above the CON diet; GOOD SC, facility without mixing piglets from different origins and daily cleaning; MM+POOR SC, facility with a mix of piglets from different origins and poor hygiene conditions. [2] Residual standard deviation. [3] ROS: reactive oxygen species; LPO: lipid peroxidation; T-AOC: total antioxidant capacity; SOD: superoxide dismutase; GST: glutathione S-transferase; GSH: reduced glutathione; GSSH: glutathione disulfide, and GSH:GSSH ratio. [4] Nernst equation ($E_h = -264 - (61.5/2) \times \log GSH^2/GSSG$) was applied to determine the redox potential of GSH:GSSG ratio. [5] DCFHA-DA: ROS detection by 2′,7′-dichlorofluorescein diacetate probes emitting fluorescence due to probe oxidation.

3.4. Fecal Microbiome Compositional Characteristics

Fecal microbiome analysis was conducted only on a subset of animals that were not randomly selected after the conclusion of the experiment. Overall, fecal microbiome analyses revealed no major significant differences ($p > 0.05$) in bacterial community composition or individual taxon abundances at the bacterial genus level across treatments over time. The alpha-diversity analysis was performed using both Shannon's and Simpson's D indices as standards. Figure 3A,B and Supplementary Figure S1A,B show the absence of significant differences between diets for GOOD and MM+POOR SC ($p > 0.05$) when measured by Shannon's or Simpson's D indices of alpha-diversity ($p > 0.05$), respectively. Furthermore, no significant changes were found in community dispersion or clustering when using a beta-diversity analysis according to diet in GOOD and MM + POOR SC

(Figure 3C) ($p > 0.05$). Additional community structure analysis based on co-occurrence network mapping revealed the absence of major temporal changes in architecture or keystone nodes (taxa) for HSS animals housed either in GOOD or MM + POOR SC, despite surplus AA+ supplementation (Figures 4–6). Further statistical analysis was carried out to assess taxonomic compositional differences across dietary groups for HSS animals only in both the GOOD and MM + POOR SC while accounting for multiple comparisons (FDR and BC statistical corrections), which ultimately revealed no specific taxon or taxa that were preferentially enriched or diminished over time ($p > 0.05$). Therefore, Figure 7 depicts the major core taxa present across treatments over time while capturing the overall stability in microbial composition, as measured using 16S rRNA sequencing analysis.

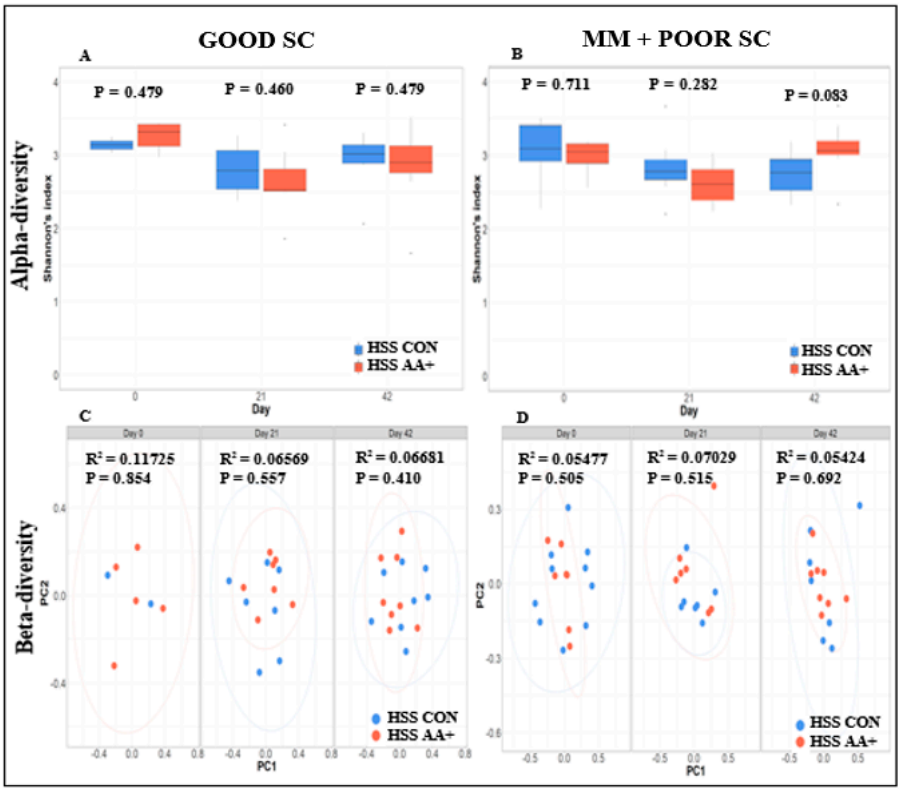

Figure 3. Alpha- and beta-diversity analysis of fecal microbiome samples across piglets with high sanitary status (HSS), housed in GOOD or MM + POOR sanitary conditions (SC), and fed with a control (CON) or extra-supplemented diet with amino acids (AA+). (**A**) shows no significant difference in alpha-diversity (Shannon's Index) between diets (CON vs. AA+) across HSS piglets under GOOD SC over time. (**B**) shows no significant difference in alpha-diversity (Shannon's Index) between diets (CON vs. AA+) across HSS piglets under MM + POOR SC over time. Alpha-diversity comparison between diet groups was conducted using a Welch two-sample T-test ($p < 0.05$). (**C**) shows no significant difference in Bray–Curtis distance matrix between diets across HSS piglets under GOOD SC over time ($p < 0.05$). (**D**) shows no significant difference in Bray–Curtis distance matrix between diets across HSS piglets under MM + POOR SC over time ($p < 0.05$). Two principal components (PC1 and PC2) are shown for fecal samples across 0, 21, and 42 days of experiment. A PERMANOVA model was used to assess diet effect in each sanitary condition (GOOD or MM + POOR) for beta-diversity (Bray–Curtis distances). The sample sizes for the fecal samples were: GOOD SC (**A**,**C**) at day 0, CON (n = 2) and AA+ (n = 5); at day 21, CON (n = 7) and AA+ (n = 7); and day 42, CON (n = 8) and AA+ (n = 8). MM+POOR SC (**B**,**D**) at day 0, HSS CON (n = 10), HSS AA+ (n = 7); at day 21, HSS CON (n = 7), HSS AA+ (n = 7); and day 42, HSS CON (n = 7), HSS AA+ (n = 8). Each piglet was considered an experimental unit throughout the analysis.

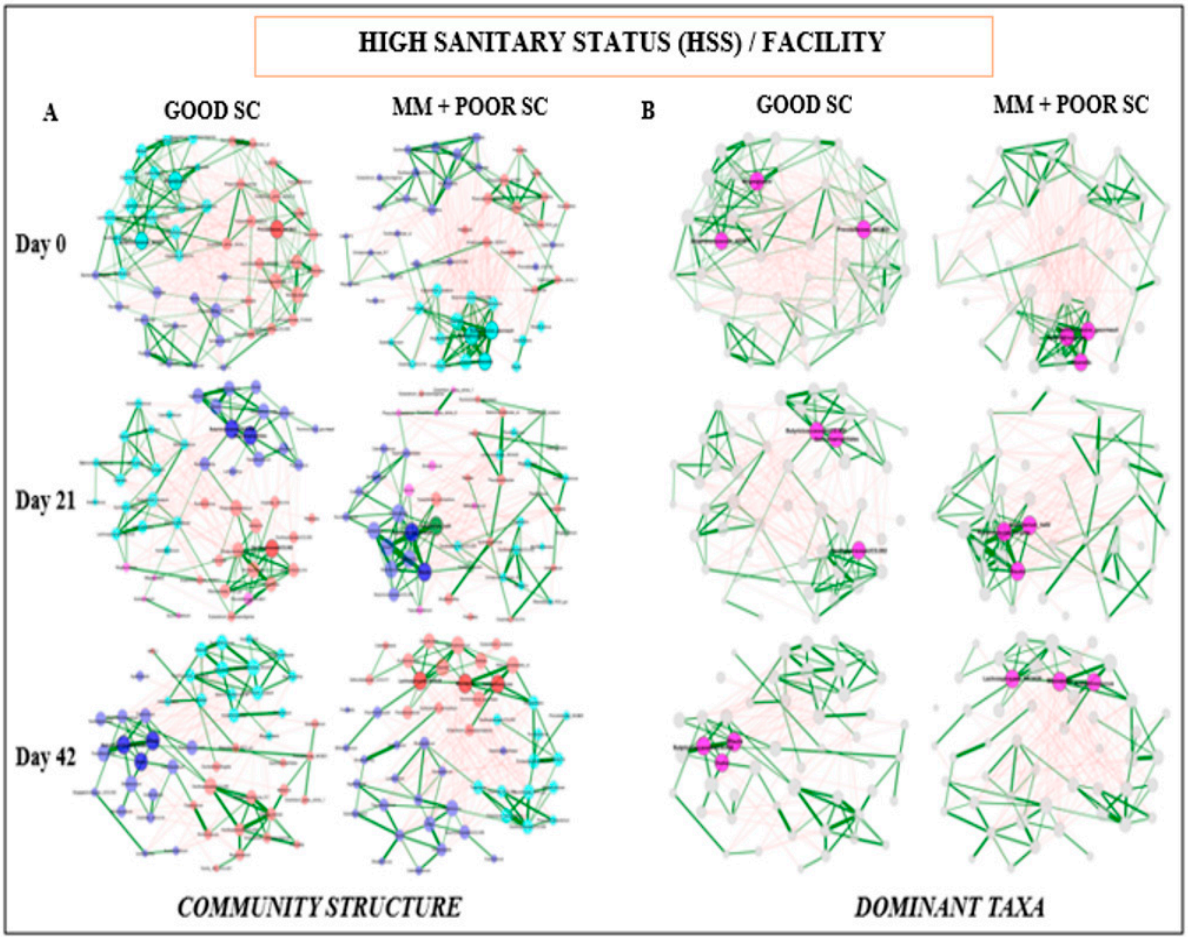

Figure 4. Co-occurrence network analysis of fecal microbiome samples across facilities (GOOD vs. MM+POOR SC) using only HSS animals. Each node represents a bacterial taxon, and the size of each node is scaled according to its centrality. Positive Spearman-based associations between taxa are depicted as green edges (the thicker the line, the stronger the association), whereas red edges are indicative of an anti-correlation between taxa. Edges representing values less than 0.5 are not shown. Taxa are colored based on clusters calculated in the network construction. (**A**) Comparison of co-occurrence networks between GOOD and MM+POOR SC over time. (**B**) Similarly, this plot represents the same correlation structure depicted in plot A, but only highlights the three most important taxa based on the highest degree of centrality (i.e., dominant taxa). The sample sizes for the fecal samples were: GOOD SC (**A**,**B**) at day 0 (n =7); at day 21 (n = 14); and day 42, CON (n = 16). MM+POOR SC (**A**,**B**) at day 0 (n = 17); at day 21 (n = 14); and at day 42 (n = 15). Each animal was considered an experimental unit throughout the analysis.

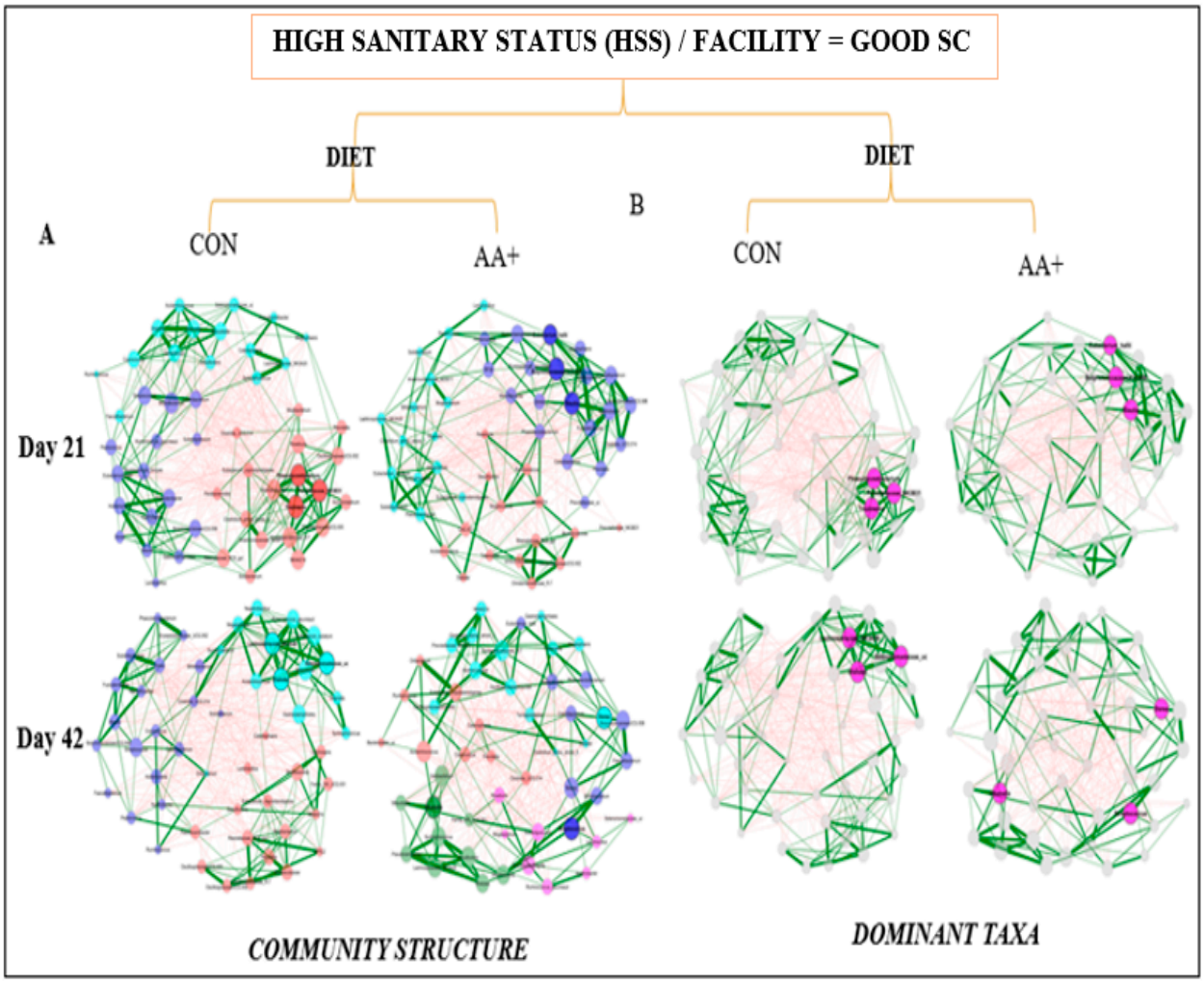

Figure 5. Temporal co-occurrence network analysis of fecal microbiome samples across barns using only HSS animals within the facility with GOOD SC while comparing across dietary treatments (CON for control, and AA+ for amino acid supplementation). Each node represents a bacterial taxon, and the size of each node is scaled according to its centrality. Positive Spearman-based associations between taxa are depicted as green edges (the thicker the line, the stronger the association), whereas red edges are indicative of an anti-correlation between taxa. Edges representing values less than 0.5 are not shown. Taxa are colored based on clusters calculated in the network construction. (**A**) Comparison of co-occurrence networks between CON vs. AA+ diets across days 21 and 42. (**B**) Similarly, this plot represents the same correlation structure depicted in plot A, but only highlights the three most important taxa based on the highest degree of centrality (i.e., dominant taxa). The sample sizes for the fecal samples were: GOOD SC (**A**,**B**) at day 21, CON (n = 7) and AA+ (n = 7); and day 42, CON (n = 8) and AA+ (n = 8). Day 0 was excluded from the analysis because of a small sample size. Each animal was considered an experimental unit throughout the analysis.

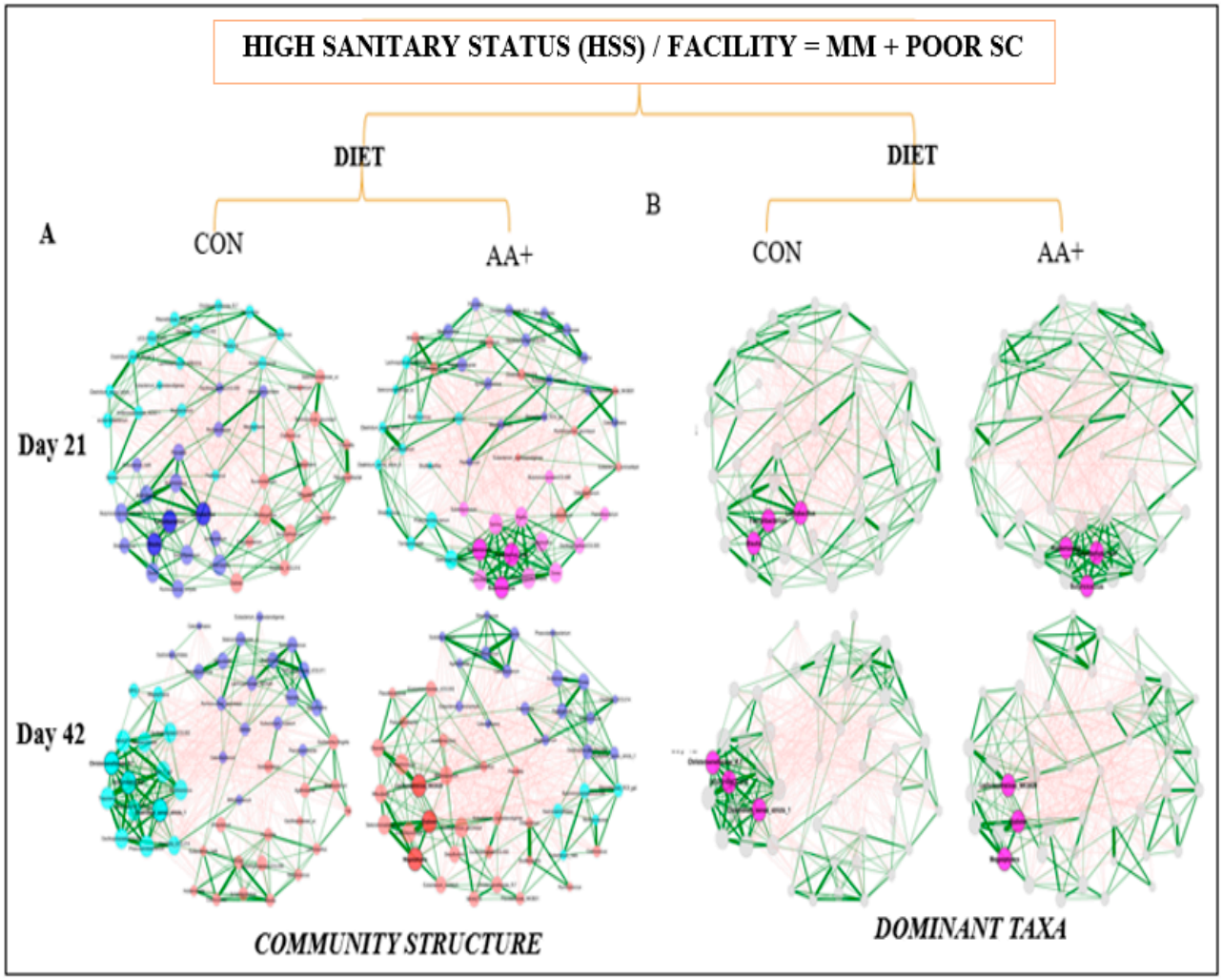

Figure 6. Temporal co-occurrence network analysis of fecal microbiome samples across barns using only HSS animals within the facility with MM+POOR SC while comparing across dietary treatments (CON for control, and AA+ for amino acid supplementation). Each node represents a bacterial taxon, and the size of each node is scaled according to its centrality. Positive Spearman-based associations between taxa are depicted as green edges (the thicker the line, the stronger the association), whereas red edges are indicative of an anti-correlation between taxa. Edges representing a value less than 0.5 are not shown. Taxa are colored based on clusters calculated in the network construction. (**A**) Comparison of co-occurrence networks between CON vs. AA+ diets across days 21 and 42. (**B**) Similarly, this plot represents the same correlation structure depicted in plot A, but only highlights the three most important taxa based on the highest degree of centrality (i.e., dominant taxa). The sample sizes for the fecal samples were: MM + POOR SC (A,B) at day 21, CON (n = 7) and AA+ (n = 7); and day 42, CON (n = 7) and AA+ (n = 8). Day 0 was excluded from the analysis because of a small sample size. Each animal was considered an experimental unit throughout the analysis.

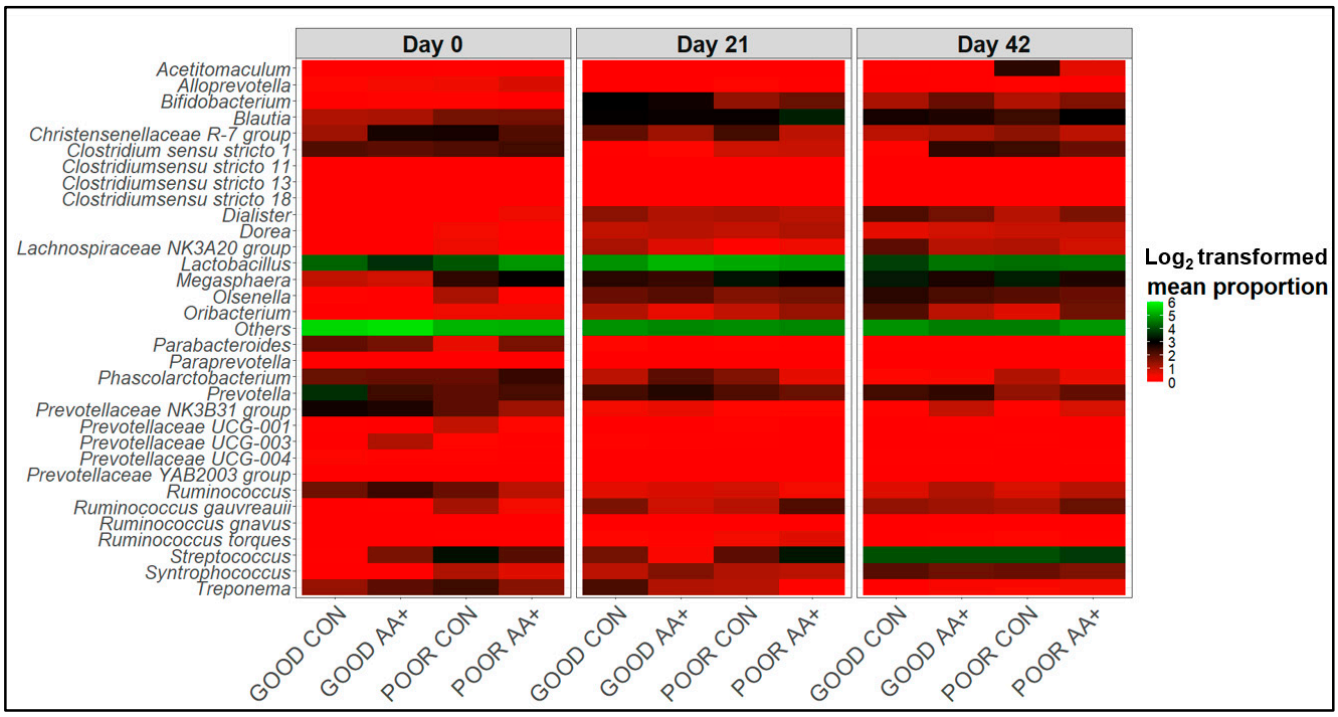

Figure 7. Temporal pattern of fecal microbiome taxa distribution across HSS animals by treatment. Log_2-transformed mean proportion of keystone taxa across HSS animals over time across all treatments, which combined sanitary conditions (GOOD vs. MM + POOR SC) and diets (CON vs. AA+). All GOOD in the Figure refers to GOOD SC, while all POOR refers to MM + POOR SC. The most abundant (>2%) and most dominant taxa depicted across all network analyses were used to construct this heatmap distribution of keystone taxa classified at either the family, genus, or species levels. The stronger the color, the more abundant a given taxon is, on average, across treatments, and over time. The sample sizes for the fecal samples were: GOOD SC at day 0, GOOD CON (n = 2) and GOOD AA+ (n = 5); at day 21, GOOD CON (n = 7) and GOOD AA+ (n = 7); and day 42, GOOD CON (n = 8) and GOOD AA+ (n = 8). MM + POOR SC at day 0, POOR CON (n = 10), POOR AA+ (n = 7); at day 21, POOR CON (n = 7), POOR AA+ (n = 7); and day 42, POOR CON (n = 7), POOR AA+ (n = 8). Each animal was considered an experimental unit throughout the analysis.

4. Discussion

Poor sanitary conditions may be found in commercial swine operations that have inadequate biosecurity protocols, facilities, husbandries, nutritional programs, and endemic respiratory and enteric diseases [1,3]. Nutrition accounts for up to 75% of swine production costs [45] and has the potential to impact individual and herd-based health by maximizing growth and productivity, enhancing host defenses, and altering the GI microbiome [46]. For instance, dietary supplementation with Thr, Met, and Trp has been studied and deployed to enhance the growth and feed efficiency of pigs under sanitary challenges [24,25]. Increasing dietary supplementation of Thr, Met, and Trp in 20% related to Lys was carried out following Valini et al. [24] and Rodrigues et al. [25], in an attempt to achieve similar beneficial results for performance and health as those found by these studies; however, we used piglets under mixed management and poor sanitary conditions. Thus, the present study evaluated the effects of surplus dietary Thr, Met, and Trp supplementation and sanitary conditions on performance, physiological, metabolic, immunological, and oxidative metrics, as well as GI microbiome characteristics and the composition of the piglets. Overall, our study demonstrated that Thr, Met, and Trp supplementation can ameliorate the performance of high-sanitary-status weaned piglets under poor sanitary conditions (mixed piglets and poor housing conditions) while having no effect on counterpart piglets raised under GOOD SC. HSS and LSS farms were specifically selected based on the sanitary protocols adopted

in each farm. A multiplier farm that followed a rigorous sanitary protocol was selected for the HSS origin, while a commercial farm with poor sanitary status resulting in poor performance parameters was selected for the LSS origin. When establishing these conditions to characterize the challenge model, a moderate and recurrent inflammatory process was expected in LSS piglets, which was confirmed by initial immunological screening and fecal microbiome compositional assessment on Day 0. LSS piglets were housed only to compose the challenge model, and additional evaluation (D 21 or D 42) for performance or health was not in our interest. Since weaned piglets from different origins may be mixed under poor housing conditions in multiple-site large-scale swine operations, this study points toward Trp, Thr, and Met having the potential to be strategically deployed to enhance pig performance for piglets undergoing sanitary challenges.

The absence of effects of the AA+ diet on the growth performance of piglets raised under GOOD SC indicates that the nutrient requirements were met in the experimental diets. However, an eventual positive response to the AA+ diet in GOOD SC may be associated with the effect of Trp stimulating the appetite through enhancement of ghrelin expression and secretion [47]. In addition, Trp, Thr, and Met play functional roles in supporting pig health and are especially important for the synthesis of defense molecules related to aiding the integrity of the gut mucosal barrier and regulating the antioxidant and immune responses of challenged pigs [7,48]. In view of this, the model used for sanitary challenge in this study resulted in reduced performance and metabolic changes that may affect the Thr [20], Met [22], and Trp [23] requirements. Reduced plasma Trp availability during the inflammatory process has been attributed to the synthesis of acute phase proteins such as haptoglobin, which have a high Trp content in their composition, and to the increased catabolism of Trp to kynurenine [12,49]. Kynurenine has antimicrobial properties that restrict the proliferation of bacteria, viruses, and parasites [50]. Furthermore, the effects of Trp on piglet performance have mainly been due to its influence on feed intake [51]. For instance, immune response stimulation and inflammatory processes affect the redox status, which demands greater amounts of sulfur AAs (Met + Cys) to reestablish antioxidative homeostasis [52]. In addition, Thr is an indispensable AA for mucin synthesis, which is crucial for preventing pathogen adhesion on gut mucosa, to maintain gut health [19]. Based on these aforementioned functions, the growth performance of MM + POOR SC piglets was attenuated by the AA+ diet, suggesting a beneficial role of Trp, Thr, and Met surplus supplementation in supporting health [11,25,53].

Challenge models creating poor sanitary conditions for pigs, as used here and by others [11,24,54–58], could face strong difficulties in standardization. For instance, differences in the resistance/resilience due to genetic- and sex-related factors, the influence of the sanitary conditions of farms considered to be "mixed" on piglet health status, the pathogenic potential of stool used to dirty the facility, the facility sanitation level before beginning the experiment, the variations in pathogenic threshold pressure, and bacterial strain pathogenicity are factors affecting the standardization of models. However, the present study has presented the negative impact of poor sanitary conditions on pig health and growth while presenting the potential use of Thr, Met, and Trp surplus supplementation in supporting health and attenuating growth reduction, even at contrasting intensities of challenge. Together, the literature data have reinforced that Thr, Met, and Trp are essential for improving the physiological and metabolic responses of challenged pigs.

Due to mixed management and poor sanitary conditions affecting performance during the weaning period, some serum and plasma biomarkers were measured to quantify the dietary effect on the piglets' metabolic changes and immune and redox responses. Notably, an increased serum concentration of haptoglobin is a relevant indicator of ongoing inflammatory processes [11] by pathogenic infections [59]. Moreover, increases in serum haptoglobin levels have been correlated with higher variability in ADG, likely due to the redistribution of nutrients used for muscle deposition being fated to immune molecule biosynthesis [10]. Albumin is also a key health status biomarker, whereby its serum concentration tends to decrease in piglets facing an inflammatory process [25,57], while

oxidative metabolism and free radical formation (reactive oxygen species—ROS) can be imbalanced under such circumstances [60,61]. Free radicals are molecules that contain oxygen atoms with an unpaired electron and have strong bactericidal properties [62]. On the other hand, oxidative stress causes the oxidation of biomolecules such as lipids, proteins, and DNA, as well as the loss of biological functions and/or homeostatic imbalance [63]. For instance, the higher haptoglobin levels, lower albumin synthesis, and the tendency for higher circulating ROS in piglets under MM + POOR SC are indications of changes in immune and redox parameters. As such, the AA+ diet tended to reduce haptoglobin levels in piglets under MM + POOR SC, which may in part explain the supplementation effect in attenuating POOR SC pigs' reduced growth performance.

Additionally, metabolic changes were marked by the AA+ diet's effect on urea and creatinine serum concentrations in the MM + POOR SC group. Urea is a primary metabolite derived from the catabolism of AAs, while creatinine is an indicator of protein degradation in skeletal muscle [64]. Both metabolites have been associated with reduced efficiency of AA utilization and lower protein synthesis during immune system activation [47]. Consequently, AA supplementation can attenuate the immune impact on muscle protein mobilization and degradation in pigs [64]. In fact, piglets under MM + POOR SC fed AA+ had lower serum concentrations of urea (on Days 21 and 42) and creatinine (on Day 21) than those in CON diet piglets. While it was previously considered a metabolic waste product, the metabolic role of lactate has been reconsidered to better understand its involvement as a pro- or anti-inflammatory marker mediating immune cell metabolism [65]. Amino acids that are released from muscle protein breakdown or from the diet may be used as an energy source to support immunometabolism. The inflamed tissue milieu may also change the energy metabolism, increasing lactate production by aerobic glycolysis and attempting to regulate cell immune responses. Accordingly, piglets under MM + POOR SC fed AA+ had a lower lactate concentration than that in CON diet piglets. This finding suggests that an AA+ diet may reduce the changes in cell metabolism, attenuating the impact of sanitary challenge on the immune response, likely by increasing feed intake and, thus, blood AA availability, providing a better AA balance for immunometabolism.

The adoption of good husbandry and sanitary practices during weaning transition is essential for avoiding severe GI dysbiosis, which can lead to diarrhea and reductions in feed efficiency during the nursery stages of production [66]. Surplus dietary AA supplementation for GI microbiome modulation is a potential avenue to be explored for maintaining GI health and homeostasis [54]. For instance, Trp supplementation can increase populations of *Lactobacillus* and/or *Prevotella*, which are linked to short-chain fatty acid (SCFA) production, while reducing opportunistic pathogens such as *Clostridium* sp. and, in turn, improving gut barrier integrity and overall pig health [67,68]. More specifically, the GI microbial production of SCFAs by selective bacterial groups may improve epithelial cell proliferation and GI barrier function. As seen in the present study, which corroborates the findings of Pas et al. [56], sanitary conditions can affect the initial fecal microbial composition of pigs. However, this study suggests that the two treatment diets were similar enough in composition to approximate the HSS and LSS fecal microbiomes, suggesting that at the dose tested, the AA+ treatment did not cause major shifts in bacterial populations at the genus level. Instead, the alpha- and beta-diversity, co-occurrence network analysis, and data mining for identification of unique taxonomic changes revealed that over time, regardless of the treatment, a core microbiome composition and topology was achieved across all treatments (*Alloprevotella*, *Bifidobacterium*, *Blautia*, *Clostridium*, *Lactobacillus*, *Megasphaera*, *Prevotella*, *Ruminococcus*, *Streptococcus*, *Treponema*, etc.), as previously reported [44,69]. This absence of change in the major central taxa suggests a high resistance in the community structure. Obviously, 16S rRNA amplicon sequencing comes with caveats, such as not allowing for microbial absolute counts to be established and not providing enough taxonomic resolution for species-level annotation or strain identification [44,69–71]. Additionally, our fecal microbiome analysis was performed with a limited sample size. Nonetheless, surplus AA supplementation may not be consistent in changing the core GI microbiota composition

at the bacterial genus/family level, but recent work by Beaumont et al. [26] suggests that further studies on microbial metabolic capabilities should be performed to understand the effect of these AAs, potentially including mycobiome data [72,73].

5. Conclusions

The results of this study partially support the hypothesis by demonstrating that supplementation with Trp, Thr, and Met improved the performance, health, and protein metabolism of healthy piglets raised under mixed management and poor housing conditions. On the other hand, it also demonstrated that healthy piglets raised under GOOD SC might not need surplus supplementation with AA for optimal growth, which in turn may lower the cost of production and facilitate management practices. Since piglets often become mixed and form heterogeneous batches in multiple-site production systems, our study suggests that increased Trp, Thr, and Met dietary supplementation could contribute to mitigating the side effects of this known risk factor in modern pig farms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14081143/s1, Figure S1: Alpha-diversity (Simpson's D index) analysis of fecal microbiome samples across piglets with high sanitary status (HSS), housed in a GOOD or POOR sanitary condition (SC), and fed a control (CON) or surplus diet supplemented with amino acids (AA+). Figure S1A shows no significant difference in alpha-diversity (Simpson's D Index) between diets (CON vs. AA+) across HSS piglets under good SC over time. Figure S1B shows no significant difference in alpha-diversity (Simpson's D Index) between diets (CON vs. AA+) across HSS piglets under mixed management and poor SC over time. Alpha-diversity comparison between diet groups was performed using a Welch two-sample T-test ($p < 0.05$). The sample sizes for the fecal samples were: GOOD SC (Figure S1A) on day 0, CON (n = 2) and AA+ (n = 5); on day 21, CON (n = 7) and AA+ (n = 7); and on day 42, CON (n = 8) and AA+ (n = 8); and MM + POOR SC (Figure S1B) on day 0, HSS CON (n = 10), HSS AA+ (n = 7); on day 21, HSS CON (n = 7), HSS AA+ (n = 7); and on day 42, HSS CON (n = 7), HSS AA+ (n = 8). Each piglet was considered an experimental unit throughout the analysis. Table S1: Forward and reverse sequence reads per samples. Table S2: Serological results of the health monitoring program of the high- and low-sanitary-status farms.

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Institutional Review Board Statement: All experimental procedures applied in this study followed the Brazilian National Council of the Control of Animal Experimentation and were reviewed and approved on 24 June 2021 [protocol no. 1541/21] by the Ethical Committee on Animal Use of Faculdade de Ciências Agrárias e Veterinárias (FCAV/UNESP–Jaboticabal, SP, Brazil).

Informed Consent Statement: Not applicable.

Data Availability Statement: All R code and tabular data for microbiome analysis can be found in the following Figshare link: https://figshare.com/projects/Weaned_piglets_under_poor_sanitary_condition/162358, accessed on 17 March 2023. All microbiome 16S rRNA amplicon sequencing data can be found here https://www.ncbi.nlm.nih.gov/bioproject/PRJNA985664, accessed on 20 June 2023. Other data are contained within the article.

Conflicts of Interest: Henrique Gastmann Brand and John Kyaw Htoo are employed at Evonik Brasil Ltd. and Evonik Operations GmbH, respectively. Evonik Operations GmbH has provided partial financial support as well as conducting amino acids analysis of raw materials and experimental diets as in-kind support for this study, which might be a conflict of interest.

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EDITED BY
Jinsu Hong,
University of Minnesota Twin Cities, United States

REVIEWED BY
Janghan Choi,
Texas Tech University, United States
Emily Fowler,
Texas State University, United States

*CORRESPONDENCE
Joao Carlos Gomes-Neto
✉ jgneto84@gmail.com

†These authors have contributed equally to this work and share first authorship

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Temporal changes in fecal swine microbiome primarily reflect *Salmonella* Typhimurium challenge and poor sanitary housing conditions, even with functional amino acid supplementation

Antonio Diego Brandão Melo[1,2†], Graziela Alves da Cunha Valini[1†], Qinnan Yang[2,3†], Marllon José Karpeggiane de Oliveira[1], Danilo Alves Marçal[1], Pedro Righetti Arnaut[1], Ismael França[1], Cleslei Alisson Silva[1], Nate Korth[2,3], Natasha Pavlovikj[4], Paulo Henrique Reis Furtado Campos[5], Henrique Gastmann Brand[6], John Kyaw Htoo[7], Andrew K. Benson[2,3], Luciano Hauschild[1] and Joao Carlos Gomes-Neto[2,3,8*]

[1]Department of Animal Science, School of Agricultural and Veterinary Sciences, State University of São Paulo (UNESP), Jaboticabal, Brazil, [2]Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE, United States, [3]Nebraska Food for Health Center, University of Nebraska-Lincoln, Lincoln, NE, United States, [4]Holland Computing Center, University of Nebraska-Lincoln, Lincoln, NE, United States, [5]Department of Animal Science, Federal University of Viçosa, Minas Gerais, Brazil, [6]Evonik Brazil Ltda, São Paulo, Brazil, [7]Evonik Operations GmbH, Hanau, Germany, [8]Department of Animal Sciences, The Ohio State University, Center for Food Animal Health, Wooster, OH, United States

Nutrition has a significant impact on the gastrointestinal (GI) microbiome, which can influence pig metabolism, nutrient absorption, biomolecule synthesis, and bioavailability (including bile acids and short-chain fatty acids), as well as colonization resistance to GI pathogens and overall disease tolerance through immune maturation and regulation. The aim of this study was to assess the impact of functional amino acid supplementation on the fecal microbiome of pigs allocated into GOOD vs. POOR sanitary conditions (SC) over time, using 16S rRNA data. A total of 120 female growing pigs were randomly assigned in a 2 × 2 factorial arrangement (n = 30/treatment), consisting of two sanitary conditions (GOOD vs. POOR) and two diets [control (CN; 100% NRC, 2012) vs. supplemented with AA (Trp, Thr, and Met+Cys: Lys ratios increased to 20% higher than CN)]. Pigs were allocated to the GOOD SC group and were sham-inoculated, and the barn was kept clean, whereas pigs housed under POOR SC were challenged with *Salmonella* Typhimurium, in addition to the spreading fecal material from a commercial farm undergoing poor growth performance. Fecal samples were collected at day post-challenge (DPC) 0, 10, and 21, and extracted DNA was sequenced for 16S rRNA data analysis. Although alpha-diversity analysis revealed minor, statistically significant changes between groups, beta-diversity analysis demonstrated a significant separation between communities based on sanitary conditions at DPC 21. Accordingly,

the most important taxa differentiating the two groups were the enrichment of the following taxa in the POOR group at DPC 21: *Clostridium sensu stricto 1*, *Dorea*, *Intestinibacter*, *Lactobacillus*, *Romboutsia*, *Ruminococcus torques*, *Subdoligranulum*, *Terrisporobacter*, and *Turicibacter*. Network and correlation structural analysis further revealed a sub-structuring of the data, with positive correlations forming in the POOR SC group: Sub-cluster 1 (*Romboutsia*, *Turicibacter*, *Clostridium sensu stricto 1*, *Terrisporobacter*, and *Intestinibacter*) and Sub-cluster 2 (*Dorea*, *Subdoligranulum*, *Ruminococcus torques*, *Blautia*, *Holdemanella*, and *Solobacterium*). In conclusion, temporal changes in the fecal swine microbiome of growing pigs reflected the *S.* Typhimurium challenge and poor sanitary status despite a dietary surplus of functional amino acids.

KEYWORDS

swine, microbiome, *Salmonella* Typhimurium, amino acids, sanitary condition

1 Introduction

Providing optimal nutrients in swine diets can maximize performance and profitability by improving animal health, potentially through modulation of the gastrointestinal (GI) microbiome (1–3). However, environmental and sanitary factors can affect feed intake, nutrient utilization, and lean meat deposition in modern pig genotypes (4–8). Various feeding and dietary programs, including increased supplementation with functional amino acids (AAs) with or without lowering crude protein, are typically deployed to mitigate the negative impacts of poor sanitary housing conditions and infectious, thereby maximizing pig growth (9–15).

The swine GI microbiome contributes to overall health by participating in, but not limited to, fiber digestion, vitamin and other micronutrient metabolism and biosynthesis (short-chain fatty acids—SCFA and bile acids), nutrient absorption, host metabolism, innate and adaptive immune development and maturation, and colonization resistance against enteric pathogens (2, 3, 16–22). Although the swine GI microbiome is biogeographically variable, growing pigs have a predictable composition at the genus level using 16S rRNA sequencing analysis, with *Acidaminococcus*, *Blautia*, *Clostridium*, *Fusobacterium, Intestinibacter*, *Lactobacillus*, *Megasphera*, *Oscillospira, Prevotella*, *Roseburia*, *Ruminococcus*, *Streptococcus*, *Subdoligranulum*, *Terrisporobacter*, *Treponema*, and *Turricibacter* being among the most dominant fecal taxa (16, 23–25). Many of these core beneficial groups of the swine fecal microbiome are colonizers of the ileum and/or large intestine, with some involved in SCFA production that can, in turn, impact intestinal epithelial health, immune function, and host basal metabolism (1, 26–30). Risk factors that are known for negatively affecting growing pigs' performance, such as poor sanitary housing conditions and *Salmonella enterica subsp*. *enterica* lineage I serovar Typhimurium (henceforth named *S.* Typhimurium) intestinal infection is also associated with disruption of this core fecal microbiome, resulting in predictable changes in SCFA-producing bacteria (9, 11, 12, 22, 28, 31–37).

A surplus of functional amino acids has been used to enhance gut health, promote growth, and maximize feed efficiency in modern pigs (38). More specifically, dietary supplementation with threonine, methionine, and tryptophan has been shown to improve the average performance of *S.* Typhimurium-challenged pigs while supporting pigs' health (9, 39–42), for instance, with an increase in core beneficial members of the swine microbiome such as *Lactobacillus*, *Clostridium*, and *Succinivibrio* (43, 44). Even though studies reporting methionine and threonine effects specifically modulating the swine GI microbiome are still scarce in the literature (3), a recent study demonstrated that *S.* Typhimurium seems to be capable of altering that nutrient metabolic partition by utilizing its virulence apparatus to induce AA (e.g., lysine) malabsorption in the ileum, resulting in a more favorable ecosystem for invasion in the large intestine, where the oxidative metabolism creates a bottleneck for SCFA producers (45).

Therefore, experimental studies assessing temporal changes in the GI microbiome (diversity, compositional structure, and differential taxa) of growing pigs challenged with an endemic enteric pathogen that poses a food safety concern, such as *S.* Typhimurium and raised under poor housing conditions mimicking commercial farms, supplemented with functional AA, pose an opportunity to identify novel, ecologically driven (host-adapted) synbiotic strategies that would help maximize pig performance. Therefore, the current study was designed to assess the impact of increased dietary supplementation with threonine, methionine, and tryptophan on the fecal microbiome over time on group-housed growing pigs under challenging sanitary conditions, expecting specific alterations in the core beneficial members of that microbiota directly linked to SCFA production underlying the microbiome resilience capacity and functional adaptation to *S.* Typhimurium challenge along with exposure to poor hygiene.

2 Materials and methods

2.1 Animal study and housing

A total of 120 female pigs (Pietrain × [Large White × Landrace]) with an initial body weight (BW) of 25.4 ± 3.7 kg were used in this experimental study. Pigs were sourced from SEARA Foods (SEARA Alimentos), Santa Catarina, Brazil. The pigs were distributed in a randomized block design in a 2 × 2 factorial arrangement composed of two sanitary conditions (Barn-SC): good (GOOD) or poor (POOR—*S.* Typhimurium inoculation + poor hygiene) and were fed with one of two diets [control (CN), formulated to meet the nutritional requirements according to (85); or surplus supplemented (AA) with Thr, Met+Cys, and Trp ratios to Lys adjusted to 20% above the CN diet], as previously described (9). The experiment was conducted in two similar barns located at the facilities of the Swine

Research Laboratory at São Paulo State University (UNESP-Jaboticabal, São Paulo, Brazil). Pigs were identified and randomly assigned to one of two similar growing-finishing barns (0.9 m^2/pig), where they had been housed for 14 days in the same facilities prior to the beginning of the experiment. The full concrete floor of both barns underwent the same cleaning and disinfection protocol before allocating all pigs. For both barns, the temperature was controlled (set to 22°C) with the aid of exhaust fans and an automated evaporative system of pad cooling (Big Dutchman, Araraquara, SP, Brazil) and monitored using data loggers (Hobo, Onset Computer Corporation, Bourne, MA, United States). Artificial lights were used to maintain a 12-h photoperiod (7:00 a.m.–7:00 p.m.). Additionally, each room was equipped with four Automatic and Intelligent Precision Feeders (AIPF, University of Lleida, Lleida, Spain) and six nipple drinkers, allowing *ad libitum* access to feed and water, respectively. AIPF's feed delivery is achieved through recognition of the individual radio frequency ear tag (Allflex, Joinville, SC, Brazil) attached to the right ear of each pig, enabling individualized data collection, as previously described (9, 46).

2.2 Experimental challenge

The *S.* Typhimurium strain (RLO971/09, originating from a past swine clinical salmonellosis case) was used to prepare the experimental inoculum. This strain is stored at the Laboratory of Ornitopathology, Department of Veterinary Pathology, UNESP-Jaboticabal, SP, Brazil (9, 11). A single colony was grown overnight to make a total volume of 5 mL of brain heart infusion (BHI, CM 1135, Oxoid, Thermo Fisher Scientific, NH, England) broth, which was used to inoculate 2×10^9 colony-forming units (CFU) of *S.* Typhimurium selected for antibiotic resistance to nalidixic acid (25 μg/mL) by oral gavage in all 60 pigs allocated into the POOR (*S.* Typhimurium inoculation + poor hygiene) SC barn, as previously described (9). The counterpart pigs allocated in the GOOD SC barn were sham-inoculated via oral gavage with 5 mL of brain heart infusion broth solution as negative controls, following the same procedures done on pigs allocated in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC barn.

Under both inoculation conditions, all pigs were fasted for 6 h and had no water consumption for 1 h prior to inoculation. After pigs in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC barn was inoculated with *S.* Typhimurium, fresh manure from a commercial pig farm was spread across all pens to establish the initial sanitary challenge and remained uncleaned throughout the study, as previously described (9). In contrast, in the GOOD SC barn, no manure was spread on the floor, while it was cleaned twice a day with a water jet stream, and potassium monopersulfate (1:200; Virkon; Lanxess, Colony, Germany) was pulverized into the air once a week as part of the biosecurity protocol to improve the hygiene condition. The management teams were required to wear clean clothing and footwear with a bleach solution (1:10 dilution) when entering the building to avoid cross-contamination (9). Regardless of sanitary conditions, pigs did not receive any growth promoters or any other treatment prior to or during the experiment. Cross-contamination between barns was assessed by testing fecal samples at day post-challenge (DPC) 0, 10, and 21 for *Salmonella* spp. growth using selective media (9), and none was found. All experimental procedures applied in this trial followed the guidelines of the Brazilian National Council of 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) (9).

2.3 Experimental diets

As previously described (9), all experimental diets were based on corn-soybean meal and were formulated using the reported nutrient content and analyzed AA composition of ingredients to meet or exceed the nutrient requirements based on pig weight at the beginning of the study, according to (85) and AMINODat® 6.0 (Evonik Operations GmbH, Essen, Germany). AMINODat is a web-based, internationally referenced database for the nutritional composition of feed ingredients. The diets were steam-pelleted (2.5 mm) and provided *ad libitum* without the inclusion of in-feed antibiotics as growth promoters throughout the trial.

2.4 Sample collection, DNA extraction, 16S rRNA amplicon sequencing, and bioinformatic processing

Rectal samples were collected from all pigs at DPC 0, 10, and 21. Specifically at DPC 0, fecal samples were collected prior to *S.* Typhimurium inoculation, and sample aliquots were separated for bacterial counts (fresh samples) and DNA extraction for subsequent 16S rRNA sequencing. All aliquots used for DNA extractions were frozen in liquid nitrogen and then stored at −80°C. DNA extraction was performed using the Qiagen DNeasy® PowerSoil® Pro kit in accordance with the manufacturer's instructions (12). All DNA extracts were eluted in 60 μL of elution buffer and then frozen at −80°C prior to quality assessment and sequencing. DNA concentration was checked using a Nanodrop One spectrophotometer (Thermo Fisher Scientific Inc., Middletown, VA). For 16S rRNA sequencing, the V4 region was amplified from each sample using an in-house modified dual-indexing sequencing strategy on the Illumina MiSeq platform, as previously described (12, 47, 48). Paired-end sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) program (version 2) (49). Sequences were truncated (220 bases for forward reads and 160 bases for reverse reads) and denoised into amplicon sequence variants (ASVs) using DADA2 (50). They were then rarefied to 5,000 reads per sample, as previously done (12). All ASVs were assigned taxonomic information using the pre-fitted sklearn-based taxonomy classifier from the SILVA database (release 138) (12, 48, 51). Only bacterial taxa (contained domain = "Bacteria") with genus-level information in the name (g from QIIME2 output) were filtered for both diversity and taxonomic analyses prior to statistical analysis. Each fecal sample was treated as a unique experimental unit for microbiome sequencing with no replicates added.

2.5 Co-occurrence network analysis of fecal microbiome data

Network construction and visualization were performed using the NetCoMi package within the R statistical framework (version 4.2.2)

(52). Overall, samples were rarefied to 5,000 reads and grouped by sampling at DPC 21 and sanitary conditions [GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene)], as previously described (12). In brief, each network was constructed using a centered log-ratio transformation and Spearman-based correlations, employing the 50 most abundant microbial taxa. Data interpretation was summarized based on each node representing a bacterial taxon, and the size of each node is scaled according to its centrality. Edges represented associations between taxa, with positive correlations being colored in green and negative ones in red. The thickness of each edge corresponds to the strength of the association, and edges representing a value less than 0.5 were excluded from the analysis. Taxa were colored based on clusters calculated in network construction. Therefore, the taxa with the highest degree of centrality were selected to highlight cluster-specific dominance (12).

2.6 Statistical analyses

All bacterial taxonomic outputs were processed for quality control (data distribution) to statistical modeling using R version 4.4.0. All data exploration and statistical analysis were conducted using bacterial genus-level taxonomy only, except for an examination of the most important taxa (>1%) over time, which was also performed using family-level taxonomic resolution when necessary for data amalgamation. In brief, the tidyverse library (version 2.0.0) was used for data exploration, analysis, and plotting. Statistical analysis was conducted at two levels: (1) by comparing Barn-SC GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) by DPC (in the absence of interaction between diet and Barn-SC, with Barn-SC being the main significant effect); and (2) by comparing all treatments [combination between Barn-SC and diet by DPC—GOOD CN, GOOD AA, POOR (*S.* Typhimurium inoculation + poor hygiene) CN, and POOR (*S.* Typhimurium inoculation + poor hygiene) AA]. All taxa data were rarefied to 5,000 counts, as previously described (12). A first-glance analysis of the sampling distribution using mean and median was conducted for taxa with a proportion greater than 1% (major taxa) at both the family and genus levels (hierarchical levels) by DPC, regardless of treatments, to assess data skewness. For alpha-diversity (Simpson's D and Shannon's indexes), either a one-way analysis of variance (ANOVA) (more than two treatments—Barn-SC, dietary condition, and interaction) or a Welch T-test (two treatments only—Barn-SC effect, regardless of diet provided) was used to assess significance ($p < 0.05$). Both Shannon and Simpson's D indexes of alpha diversity were calculated using rarefied counts, with the diversity function in R from the vegan library (version 2.6-6.1).

If no significant effects arose from the interaction between Barn-SC and Diet, only the significant main effect would be further examined. This approach was taken for alpha- and beta-diversity. The rest of the analysis was conducted based on the main significant effect on community diversity, composition, and structure, as previously established by Gomes-Neto et al. (32).

Beta-diversity was calculated using the vegdist function from the vegan library (version 2.6-6.1), employing a Bray-Curtis distance matrix (non-binary data) within a framework previously established (32). For the principal coordinates analysis (PCoA), a classical multidimensional scaling model was first used to reduce the distance matrix to two dimensions (2 principal coordinates—PCs), using the cmdscale function (k = 2) in R from the stats library. Specifically, for DPC 21, principal component analysis (PCA) was employed to reduce the data to three dimensions, thereby enhancing the clustering assessment. Ultimately, PC1 and PC2 were used to present a biplot that captures the effects of either sanitary conditions or all treatment effects on beta diversity each day. A PERMANOVA was used to calculate the effect of treatment groups (p-value and R-squared) on beta-diversity (Bray-Curtis distances), using the 'adonis2' function in R from the "vegan" library (version 2.6-6.1). A volatility analysis (microbiome compositional assessment using each PC independently as an outcome variable—PC1, PC2, or PC3 from the PCoA) was conducted by assessing significant differences between Barn-SC groups across each DPC or across all treatments by DPC as well (a Welch T-test was used to compare Barn-SC by DPC); an ANOVA model was used for all treatments by DPC, with a Tukey Honest Significant Differences test (Tukey HSD) applied for pairwise comparisons, provided a significant effect of treatments ($p < 0.05$) was found in the ANOVA model. An analysis of similarity (ANOSIM) was used to calculate the effect of treatment groups (Barn-SC or all treatments) on beta-diversity (Bray-Curtis distances), using the anosim (method = "bray"—Bray-Curtis's distance matrix) functions in R, respectively, from the vegan library (version 2.6-6.1). Finally, a PCA (without a distance matrix) analysis was also conducted using log2-transformed proportions across bacterial taxa, with rarefied counts as input data, to assess clustering between Barn-SC groups and across all treatments by DPC.

Keystone taxa were identified based on significant ($p < 0.05$) differences in community structure at DPC 21 across Barn-SC groups [GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene)]; by filtering all bacterial genera with (1) mean proportion > 1%; (2) using a LEfSe analysis; (3) using a co-occurrence network analysis to identify central nodes; and (4) using a random forest classifier. Mean and median proportions were compared for skewness, and mean proportions were used to examine the distribution of keystone taxa using either proportion or log2-transformed proportions (adding a pseudo-value of 1 for all data to ensure proper transformation with zero counts). LEfSe analysis for identification of differentiating taxa was done with the Galaxy platform with a Wilcoxon ($p < 0.05$), and upon removal of taxon or features with zero counts across fecal samples, only contrasting sanitary conditions [GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) Barn-SC] were considered (53). The random forest classifier was also applied for DPC 0 and 10. For this purpose, the caTools and randomForest libraries were utilized in R. All random forest models were constructed using rarefied counts (without further transformation), 100 trees, a classification model for a binary outcome, and a 70/30 random split of the training and testing data, respectively. The Gini importance was used to identify the most important predictors of the random forest model, which differentiates between POOR (*S.* Typhimurium inoculation + poor hygiene) and GOOD Barn-SC across each DPC.

Statistical differences across keystone taxa were calculated using both proportion and log2-transformed proportion, all derived from rarefied counts. When comparing GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene), Barn-SC, a Welch T-test ($p < 0.05$), was used by DPC. If comparing across all four treatment groups, then an ANOVA model was used, followed by a pairwise T-test, provided the ANOVA model yielded significant results ($p < 0.05$). Box-and-whiskers and a clustering heatmap were used to visualize the

distribution of taxa across Barn-SC groups or all treatments by DPC. For the clustering heatmap visualizations, a nested approach was applied, considering the 2 × 2 factorial design (diet groups within each barn-SC). All cluster heatmap analysis was performed using log2-transformed proportions (a pseudo-value of 1 was input when zero was present), followed by data scaling (normalization) and visualization using a heatmap.2 functions in R. The correlation structure between keystone taxa was also calculated using log2-transformed proportions, with input values of 1 for zeros. This analysis was conducted using the cor function to calculate the Pearson correlation matrix, followed by the cor.mtest to identify significant correlations ($p < 0.05$). The results were then visualized using a corrplot in R, clustered using a hierarchical clustering approach (hclust). Fold-change (ratio) differences between keystone taxa across Barn-SC or all treatments were made from the endpoint (DPC 21) and baseline (DPC 0). For that, a pseudo-value of 0.01 was initially input for zeros in the mean proportional data (to avoid infinity values in the denominator), followed by a $\log_2$ transformation of the ratio with a pseudo-value imputation of 1 for zeros. Pearson's correlation between keystone taxa relative abundance ($\log_2$ transformed) ratio (DPC 21/DPC 0) and the average daily gain (host-associated trait) for the corresponding period was also calculated. All tests for Pearson's correlation coefficient were calculated using the cor_test function in R ($p < 0.05$). For the average daily gain between DPC 0 and 21 (host-associated trait), an ANOVA model was used to assess the effect of diet, Barn-SC, and interaction between the two ($p < 0.05$). All ANOVA models were done using the aov function in R, while two-group comparisons were made using a T-test by applying a two-sample t-test function in R from the stats library. A sample was removed from the analysis when a missing value (NA) was consistently present across all taxa. Illustrative figures were created using BioRender.

3 Results

3.1 Experimental background and workflow

In our companion study, several aspects of growth, including performance and body composition, among others, were captured in group-housed growing pigs under contrasting sanitary conditions [GOOD SC vs. POOR (*S.* Typhimurium inoculation + poor hygiene) SC] and fed two diets, control (CN) or supplemented with AA (9). Pigs housed under POOR SC (*S.* Typhimurium inoculation + poor hygiene) had a significantly lower average daily gain compared to those in GOOD SC (*S.* Typhimurium inoculation + good hygiene) ($p < 0.05$), underscoring a potential difference in fecal microbial communities. Figure 1 presents the experimental design, sampling, and key microbiome outcomes. Supplementary Table 1A,B describe the fecal sampling scheme throughout the study across all treatments. More specifically, this study assessed microbial diversity, community structure, and enrichment of differentiable taxa across treatments over time using 16S rRNA sequencing from fecal samples. The aim was to identify differences between sanitary conditions and the potential impact of AA surplus in the diets of growing pigs. A first-glance assessment of the keystone fecal taxa (> 1%) both at the family and genus level, over time, regardless of treatment conditions, was done to determine data skewness (mean vs. median) prior to subsequent statistical data mining with all samples rarefied at 5,000 counts (Supplementary Figures 1, 2).

3.2 Alpha-diversity

Differences in alpha diversity across treatments were analyzed using both the Simpson's D (evenness) and Shannon (richness) indexes of diversity. Figures 2A,B demonstrates the results of the ANOVA and T-test-based differences across diet groups by DPC for each SC condition for both Simpson and Shannon indexes, respectively. Significant differences were found for both Simpson ($p = 0.00835$) and Shannon ($p = 0.0104$) indexes of diversity only in barn SC (Figures 2A,B), with no significant differences between diet groups across SC by DPC ($p < 0.05$). Alpha diversity was significantly lower in the POOR SC group (*S.* Typhimurium inoculation + poor hygiene) at both DPC 21 ($p = 0.048$) using Simpson's D index and at DPC 10 ($p = 0.042$) using the Shannon index, in comparison to the GOOD SC group (Figures 2C,D).

3.3 Beta-diversity analysis

Initial assessment of beta diversity across fecal communities showed no clustering by diet across SC conditions by DPC (Figure 3A). A PERMANOVA analysis revealed only a significant effect for barn SC ($p < 0.05$) and not for diet or the interaction between the two, with the R-squared going from ~ 5% at DPC 0 to ~17% at DPC 21, demonstrating a significant biological effect of SC on the community clustering (Figure 3B). Based on this, Figure 3C highlights a clear separation of the fecal communities by SC, starting at DPC 10, with more discrete clustering at DPC 21. A 3D representation of PCoA analysis at DPC 21 confirmed the separation between GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) SC fecal communities (Figure 3D). ANOSIM results mirrored those of the PERMANOVA, underscoring a dissimilarity between GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) SC fecal communities, starting at DPC 10 ($R = 0.196$, $p = 0.01$) and reaching a higher degree at DPC 21 ($R = 0.352$, $p = 0.001$) (Supplementary Figures 3–5). When stratified by all treatment combinations, ANOSIM results corroborated the differences between SC groups, with fecal community dissimilarities most pronounced at DPC 21, irrespective of diet ($R = 0.219$, $p = 0.001$) (Supplementary Figures 6–8). A follow-up analysis using the individual PCs (PC1, PC2, and PC3) as the response variable recapitulated the beta-diversity results, demonstrating significant differences across SC groups at DPCs 10 and 21, not driven by diet ($p < 0.05$) (Supplementary Figures 9A–D, 10). Notably, the POOR (*S.* Typhimurium inoculation + poor hygiene) SC community exhibited higher dispersal, as indicated by PC1- and PC3-based analysis (Supplementary Figures 9A, 10A). An independent PCA analysis (not using a distance matrix) corroborated all results by showing clustering driven by barn SC at DPC 10 and 21 (Supplementary Figures 11–13) and not by diet (Supplementary Figures 14–16).

3.4 Taxa enrichment analysis, temporal changes, and correlation with host phenotype

Given the beta-diversity results clearly demonstrating a separation between fecal communities between SC conditions at DPC 21 (Figures 3B–D) and the absence of a significant dietary effect (Figure 3A), a combination of multiple analytical methods was used

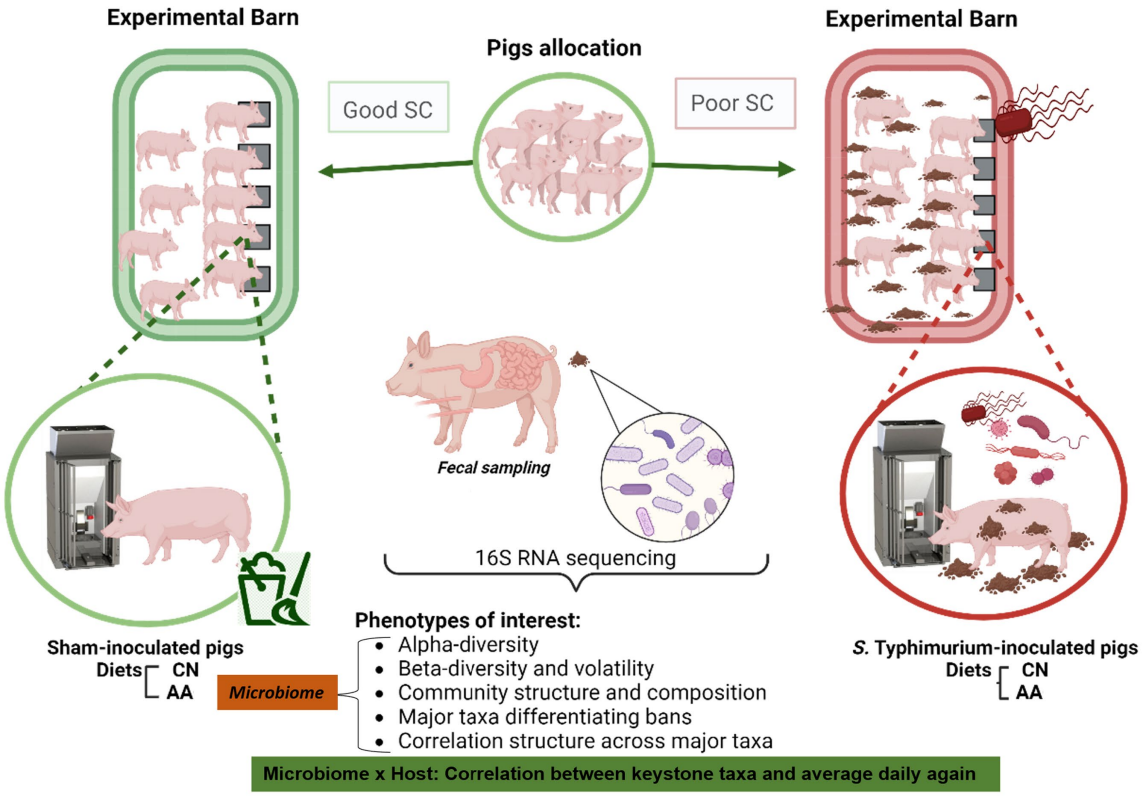

FIGURE 1
Experimental design explaining the experimental and analytical workflow. The experimental workflow describes the random allocation of female, age-matching, growing pigs into two sanitary conditions: GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) (Barn-SC). Fecal samples were longitudinally collected from animals at DPC 0, 10, and 21. The experiment employed a 2 × 2 factorial design, consisting of two housing sanitary conditions (Barn-SC: GOOD vs. POOR) and supplementation, or not, of conventional diets with functional amino acids (AA) (Trp, Thr, and Met+Cys: Lys ratios increased to 20% higher than control). Animals allocated under GOOD sanitary conditions were sham-inoculated, whereas animals housed under POOR sanitary conditions were challenged with *S.* Typhimurium, in addition to using fresh stools collected from a herd with low sanitary status and spread on the floor at DPC 0. Fecal microbiome samples were analyzed using 16S rRNA sequencing for subsequent diversity, community structure, and composition analysis, along with a taxa enrichment analysis to identify differences across treatments (microbiome-associated ecological attributes). Additionally, a fold change in the relative abundances of keystone taxa was correlated with the average daily gain throughout the study (DPC21/DPC0) (Microbiome × Host-associated trait). The fecal sample size for these analyses is presented in Supplementary Table 1A,B. Each animal was considered an experimental unit throughout the analysis.

to identify all taxa differentiating GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) SC groups at DPC 21 first, including network analysis (Figures 4A,B), LEfSe analysis (Figure 4C and Supplementary Figures 17–23), random forest (Supplementary Figures 24–26), and t-test (Figure 5 and Supplementary Figure 29). Additionally, an exploratory analysis was conducted using the overall mean abundance and a cut-off of 1% to select the major taxa across the dataset (Supplementary Figures 1, 2), which allowed for contextual interpretation of the data for the most significantly different taxa between groups ($p < 0.05$).

Based on that, the most important taxa differentiating the two groups are as follows: (1) on average lower in relative abundance in the POOR (*S.* Typhimurium inoculation + poor hygiene) group at DPC 21: *Acidaminococcus*, and (2) on average, higher in relative abundance in the POOR (*S.* Typhimurium inoculation + poor hygiene) group at DPC 21: *Clostridium sensu stricto 1*, *Dorea*, *Intestinibacter*, *Lactobacillus*, *Romboutsia*, *Ruminococcus torques*, *Subdoligranulum*, *Terrisporobacter*, and *Turicibacter*. Network analysis revealed two unique sub-clustering structures (with positive interactions) formed in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group (Figure 4B): Sub-cluster 1 (*Romboutsia*, *Turicibacter*, *Clostridium sensu stricto 1*, *Terrisporobacter*, and *Intestinibacter*) and Sub-cluster 2 (*Dorea*, *Subdoligranulum*, *Ruminococcus torques*, *Blautia*, *Holdemanella*, and *Solobacterium*). LEfSe analysis also indicated enrichment in *Roseburia* for POOR (*S.* Typhimurium inoculation + poor hygiene) SC at DPC 21 (Figure 4C). Average community abundances (proportion or log-transformed) were also examined (Supplementary Figures 27, 28). Following the identification of the most differentiating taxa, a temporal analysis was conducted using relative frequencies (log-transformed or not) for the barn SC effect or across all treatments (Figure 5 and Supplementary Figures 29–31).

The initial clustering separation between GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) SC groups for the beta-diversity analysis at DPC 10 (Figure 3C) could be explained in part by the significantly higher average abundance for *Clostridium sensu stricto 1*, *Intestinibacter*, *Lactobacillus*, *and Terrisporobacter* in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group and significantly lower abundance for *Prevotella*, some *Prevotellaceae* taxa, and *Roseburia* in the same group (Figure 5 and

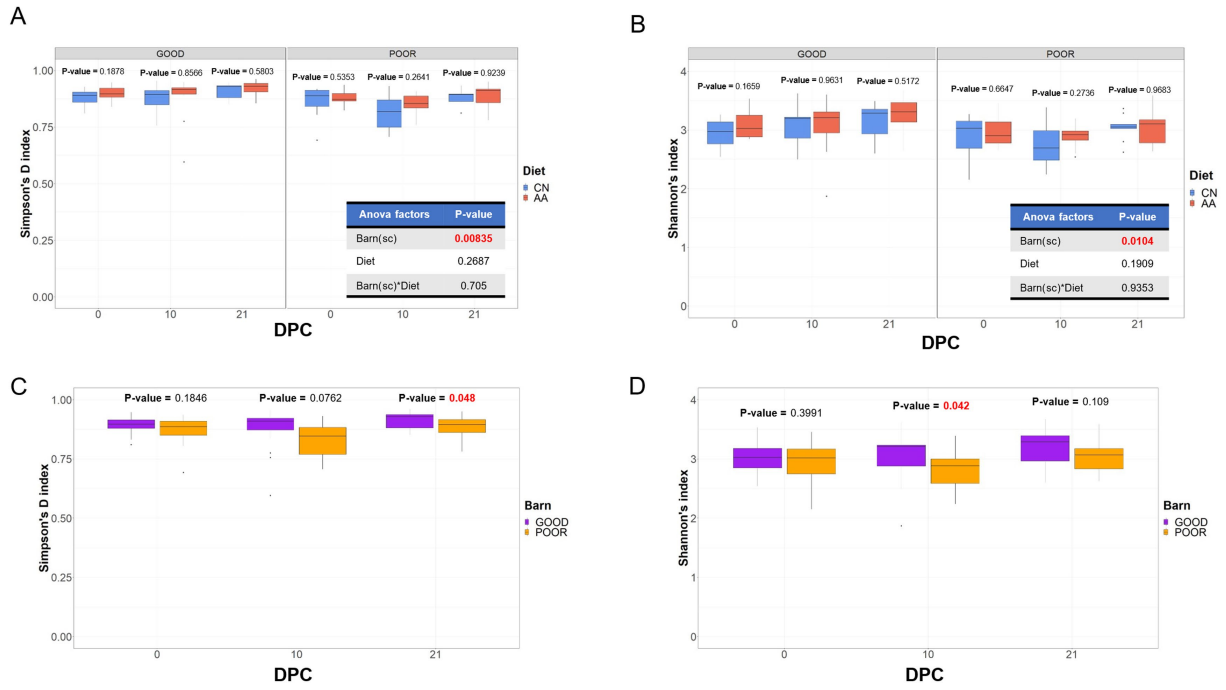

FIGURE 2
Alpha-diversity analysis of the fecal microbiome composition across treatment days post-challenge. **(A,B)** Simpson's D and Shannon's indexes of diversity are analyzed across diets (CN, conventional; AA, supplemented with functional amino acids) based on housing sanitary conditions (Barn-SC), respectively. **(C,D)** Simpson's D and Shannon's indexes of diversity are analyzed across barns with either GOOD or POOR (*S.* Typhimurium inoculation + poor hygiene) sanitary conditions. A one-way ANOVA was used to assess the effects of diet, barn, and their interaction on both Simpson's and Shannon's indexes of alpha-diversity ($p < 0.05$) **(A,B)**. Given the significant effect of "Barn(sc)" (housing sanitary condition) ($p < 0.05$) **(A,B)**, a follow-up analysis (**C,D**) was conducted across DPC time points, comparing sanitary housing groups (GOOD vs. POOR). A two-sided T-test was used for comparison across two groups in all analyses ($p < 0.05$). *p*-values marked in red represent a significant effect for that comparison ($p < 0.05$). All alpha-diversity metrics were calculated using rarefied count data.

Supplementary Figure 29), despite AA surplus (Supplementary Figures 30, 31). Additionally, a fold-change analysis demonstrated that *Clostridium sensu stricto 1*, *Intestinibacter*, *Romboutsia*, *Terrisporobacter*, and *Turicibacter* were among the top enriched taxa in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group at DPC 21 in relationship with DPC 0 (Supplementary Figure 32), despite AA surplus in the diet (Supplementary Figure 33). The host phenotype of interest, average daily gain, was significantly lower in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group and remained unchanged by AA surplus (Supplementary Figure 34). In the GOOD SC group, *Prevotellaceae UCG 001* and *Sphaerochaeta* were significantly positively correlated with average daily gain for the duration of the study (Supplementary Table 2A). On the other hand, *Oscillibacter* and *Turicibacter* were significantly negatively correlated with average daily gain for the study duration in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group, while no significant association was found for the GOOD SC for these two taxa (Figure 6 and Supplementary Table 2B). In aggregate, *Prevotella* was more likely to be positively correlated with the host phenotype for the GOOD SC group (Supplementary Table 1A,B). When stratifying by barn SC, it was found that for GOOD CN, *Moryella* and *Sphaerochaeta* were significantly positively correlated with the outcome (Supplementary Table 3A), while *Lactobacillus* was significantly negatively correlated with it for GOOD AA (Supplementary Table 3B). Finally, for POOR (*S.* Typhimurium inoculation + poor hygiene), AA *Prevotellaceae UCG 003* was found to be significantly positively correlated with average daily gain, while no significant associations were found across taxa for POOR (*S.* Typhimurium inoculation + poor hygiene) CN, even though both *Romboutsia* and *Turicibacter* trended in demonstrating a strong negative correlation in comparison to POOR AA (Supplementary Table 4A,B).

3.5 Temporal clustering and correlation-based analysis for the most differentiable taxa

A temporal clustering and correlation analysis using only the keystone differential fecal taxa confirmed a separation between communities based on barn SC status, most prominently at DPC 21 (Figures 7A–C), with no predictive signatures based on dietary modification (AA surplus) (Supplementary Figures 35–46). At DPC 21 (Figure 7C), there were two groups of taxa more likely to be enriched in the POOR (*S.* Typhimurium inoculation + poor hygiene) than the GOOD SC group: (1) a major sub-cluster comprised of *Clostridium sensu stricto 1*, *Intestinibacter*, *Romboutsia*, *Terrisporobacter*, and *Turicibacter* as previously captured by the temporal analysis of relative abundances (Figure 5 and Supplementary Figure 29); and (2) a secondary sub-cluster comprised of *Blautia*, *Butyricoccus*, *Dorea*, *Faecalibacterium*, *Ruminococcus torques*, and *Subdoligranulum*, with bimodal distribution-like patterns

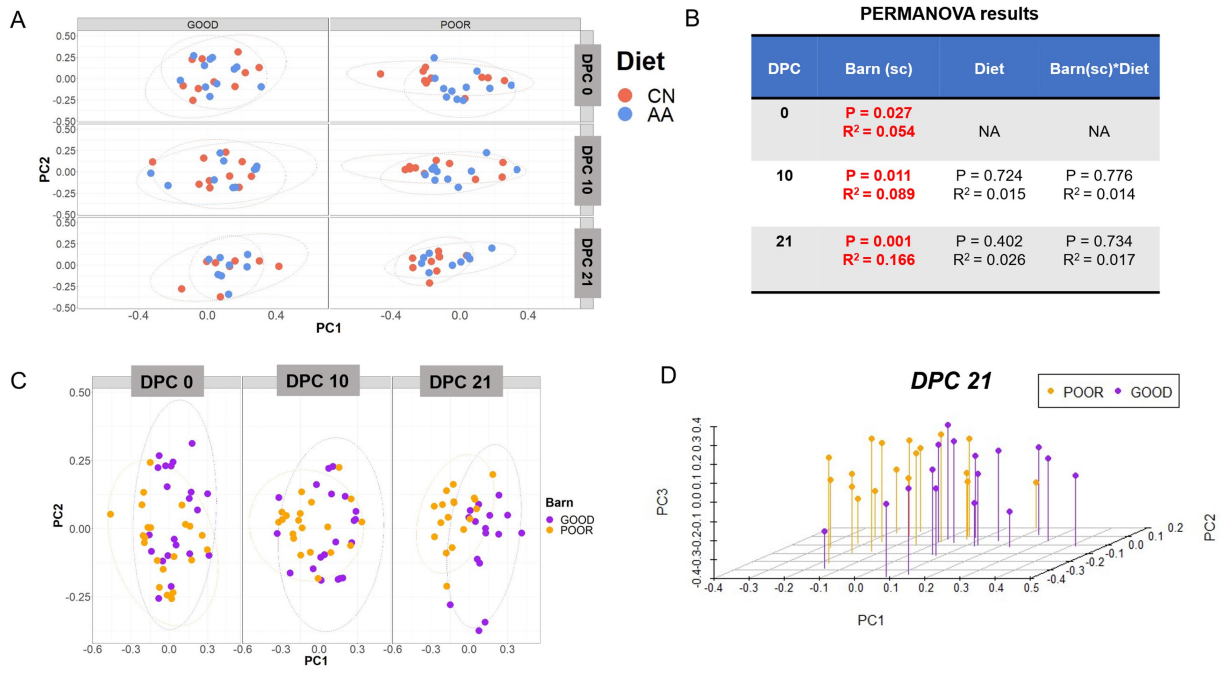

FIGURE 3
Beta-diversity analysis of fecal microbiome composition across diets (CN vs. AA) and housing sanitary conditions (GOOD vs. POOR—*S.* Typhimurium inoculation + poor hygiene) post-challenge. **(A)** Two principal coordinates (PC) are shown across diet and/or barns at DPC 0, 10, and 21. **(B)** A PERMANOVA model was used for variance decomposition across experimental factors, including diet and housing sanitary conditions (Barn-SC) and their interaction effects. PERMANOVA *p*-values and R-squared statistics are depicted with significant differences highlighted in red ($p < 0.05$). **(C)** Beta-diversity analysis was conducted for the "Barn-SC" effect across DPC 0, 10, and 21. **(D)** A three-dimensional visualization of the principal coordinate analysis for the housing sanitary effect is provided. The Bray–Curtis distance matrix was used to calculate beta diversity between diets (CN vs. AA) and/or barns under two distinct sanitary conditions (GOOD vs. POOR). Beta diversity analysis was executed using rarefied count data.

across animals in the POOR SC group. In accordance, these two sub-clusters demonstrated a correlated pattern within themselves, starting at DPC 10 and more clearly at DPC 21 for the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group (Figures 8A–F). The correlation structure among these keystone fecal taxa did not appear to be predictably affected by dietary changes (Supplementary Figures 41–46).

4 Discussion

Pigs have a stable core fecal microbiome composition with the following genus being among the most predominant GI colonizers based on 16S rRNA compositional data: *Acidaminococcus, Blautia, Corynebacterium, Clostridium, Dorea, Eubacterium hallii, Intestinibacter, Lactobacillus, Megasphaera, Oscillospira, Prevotella, Roseburia, Ruminococcus, Solobacterium, Streptococcus, Subdoligranulum, Succinivibrio, Treponema,* and *Turicibacter* (16, 21, 24, 25). The GI microbiome contributes to pig health in a variety of ways, including, but not limited to, the regulation of peristalsis, nutrient digestion and absorption (e.g., fiber fermentation), biosynthesis of vitamins/SCFA, colonization resistance to enteric pathogens, immune development and maturation, and host metabolism in general (2, 17–22). As the GI microbiome composition can also affect inflammatory/immunological responses that are the basis for protective immunity, immunopathology, or disease tolerance (54–56), it is crucial to determine how sanitary housing conditions and infectious challenges with endemic pig pathogens such as *S.* Typhimurium can affect the microbiome community structure and animal performance and whether or not dietary changes commercially used to improve lean meat deposition (AA surplus) can mitigate dysbiosis and positively affect growth. Our previous study demonstrated that AA surplus could help in reducing the negative impact on the growth of pigs exposed to high sanitary pressure (continual exposure to fecal material from low-performance animals plus *S.* Typhimurium infection) (9, 11, 12).

Salmonella Typhimurium cannot only be pathogenic to growing pigs but is also a public health concern due to its zoonotic potential and multidrug resistance (57, 58). It is transmitted through the fecal-oral route across pen-mates and possibly between pens via shared water flow and/or by workers (e.g., contaminated boots) and interacts with the resident GI microbiome (22, 32, 34–36, 45, 59–65). Specifically, in the mouse model, *S.* Typhimurium, through the invasion of the ileal mucosa, can use its virulence apparatus to trigger malabsorption of dietary AA (e.g., lysine, ornithine, methionine, and ascorbic acid), shaping the downstream (ileal-cecal) nutrient landscape that influences its growth and the extent of the inflammatory process (pathology) (45, 66).

The imbalance in AA absorption in the gut is an emerging area of study in the context of *S.* Typhimurium pathogenesis and warrants further investigation. This pathogen is also known to elevate oxygen levels in the gut lumen as a result of epithelial inflammation, which leads to the depletion of SCFA-producing bacteria that typically inhibit *S.* Typhimurium growth. Therefore, it is expected that fiber

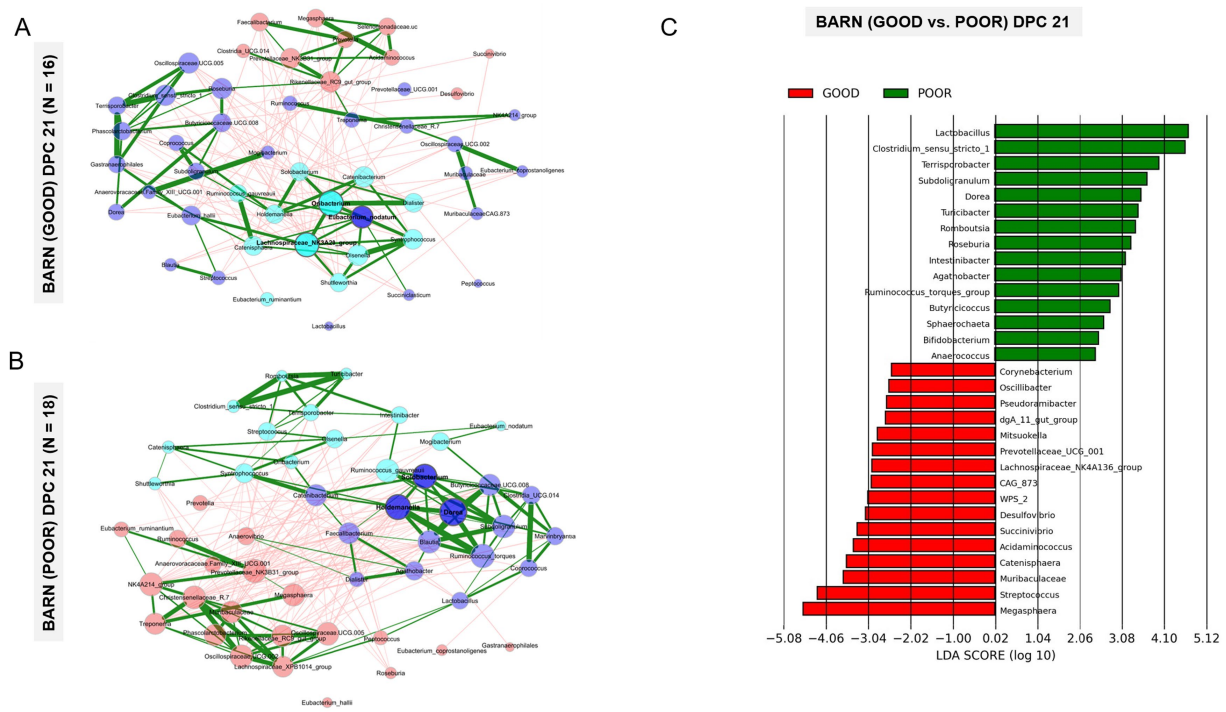

FIGURE 4
Community structure analysis complemented by linear discriminant analysis effect Size (LEfSe) of fecal microbiome samples from pigs housed under either GOOD or POOR (*S.* Typhimurium inoculation + poor hygiene) sanitary conditions at DPC 21. **(A,B)** Network analysis for examination of community structure patterns across fecal microbiome samples collected at DPC 21, highlighting the greatest beta-diversity difference between GOOD and POOR sanitary conditions (**Figure 3C**) for pigs housed under each condition, respectively. Each node represents a bacterial taxon, with the size of each node scaled according to its centrality. Positive Spearman-based associations between taxa are depicted as green edges (the thicker the line, the stronger the association), while red edges are indicative of an anti-correlation between taxa. Edges representing a value less than 0.5 are not shown. Taxa are colored based on clusters calculated during network construction. **(C)** LEfSe results for comparison between GOOD and POOR housing conditions at DPC 21. Both network and LEfSe analyses were used to identify core-microbiome taxa that differentiate pigs housed under GOOD vs. POOR sanitary conditions, as the strongest beta-diversity effect was found at DPC 21. Rarefied taxon counts and proportions (derived from rarefied count data) were used as raw input data for network and LEfSe analysis, respectively.

fermenters (keystone members of the swine GI microbiome, specifically those in the large intestine) would be rapidly and transiently depleted post-*S.* Typhimurium infection can subsequently be restored at the genus level after the first 7–14 days post-infection, as *S.* Typhimurium population decreases in the GI tracts (ileal-cecal niche) and potentially goes silent (sub-clinical infection) in the mesenteric lymph nodes and/or liver (22, 36, 62). The previous data for this study corroborated the expected pattern for *S.* Typhimurium dynamics since most of the pigs shed it with a high load during the first-week post-exposure, with about 50% of the pigs shedding it up to 14 days post-exposure at an average of 1–2 $\log_{10}$ CFU/g of fecal material despite AA surplus in the POOR SC (*S.* Typhimurium inoculation + poor hygiene) group (9).

SCFA producers, such as *Prevotella*, *Clostridium sensu stricto 1*, *and Blautia,* among others, are known to be negatively correlated with either *S.* Typhimurium load and/or intestinal pathology in pigs (32, 67). *Prevotella* relative abundance has been previously shown to be a predictor of high shedders, suggesting that high initial *Prevotella* abundance may increase colonization resistance against *S.* Typhimurium (67). Nevertheless, little is known about *Prevotella* diversity in the swine gut at the species and strain levels. However, *P. copri* and *P. stercorea* are expected to be among the most dominant. It would be expected that a higher diversity of species and strains would be positively correlated with colonization resistance against both *S.* Typhimurium and *S.* Monophasic (29, 68, 69). In the mouse model, restoration of SCFA producers and/or SCFA can ameliorate disease (34, 35, 70). The microbiome's ability to recover (resilience) is crucial for reestablishing SCFA and maintaining a stable community structure due to its pleiotropic effects on gut health and host metabolism (28). Notably, in this study, *Prevotella* and *Roseburia* were, on average, less abundant in the POOR (*S.* Typhimurium inoculation + poor hygiene) group compared to the GOOD SC group at DPC 10, potentially as an initial consequence of the *S.* Typhimurium infection. However, we lacked microbiome data for individual days during the first week, which would have provided a higher resolution of the community structure dynamics. Additionally, despite not reaching the statistical threshold of significance, perhaps due to the small sample size, *Prevotella* groups have a stronger positive correlation coefficient between fold changes from DPC 0 to 21 and average daily gain in this study for GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) SC, suggesting its potential positive impact on pig growth as a core member of the community post-weaning (16, 24, 25, 68).

Across all differentiable taxa between GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene), SC, *Clostridium sensu stricto 1*, *Intestinibacter*, *Romboutsia*, *Terrisporobacter*, and *Turicibacter* were not only among the top hits individually but also formed a primary

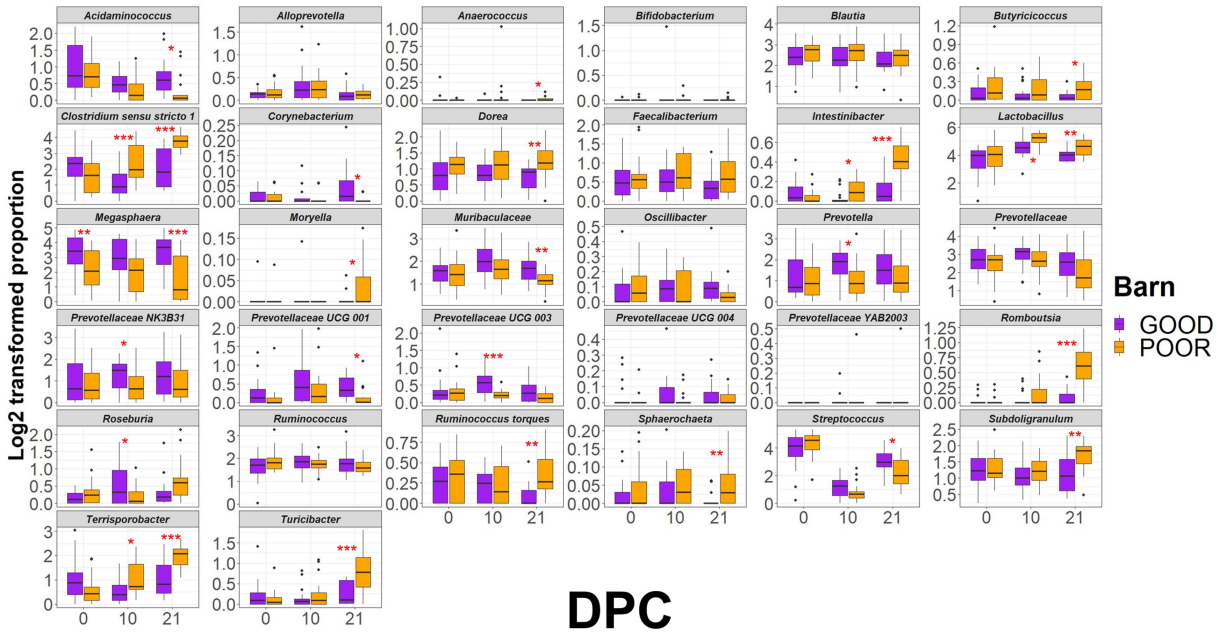

FIGURE 5
Distribution of keystone taxa across fecal samples from pigs housed under GOOD vs. POOR (*S.* Typhimurium inoculation + poor hygiene) sanitary conditions. The log2-transformed proportions of keystone taxa across sanitary conditions (Barn). A two-sided T-test was conducted to assess differences between housing sanitary conditions at each DPC ($p < 0.05$). * For $p < 0.05$; ** for $p < 0.01$, and *** for $p < 0.001$. Rarefied taxon counts transformed to proportions were used as raw data prior to $\log_2$ transformation.

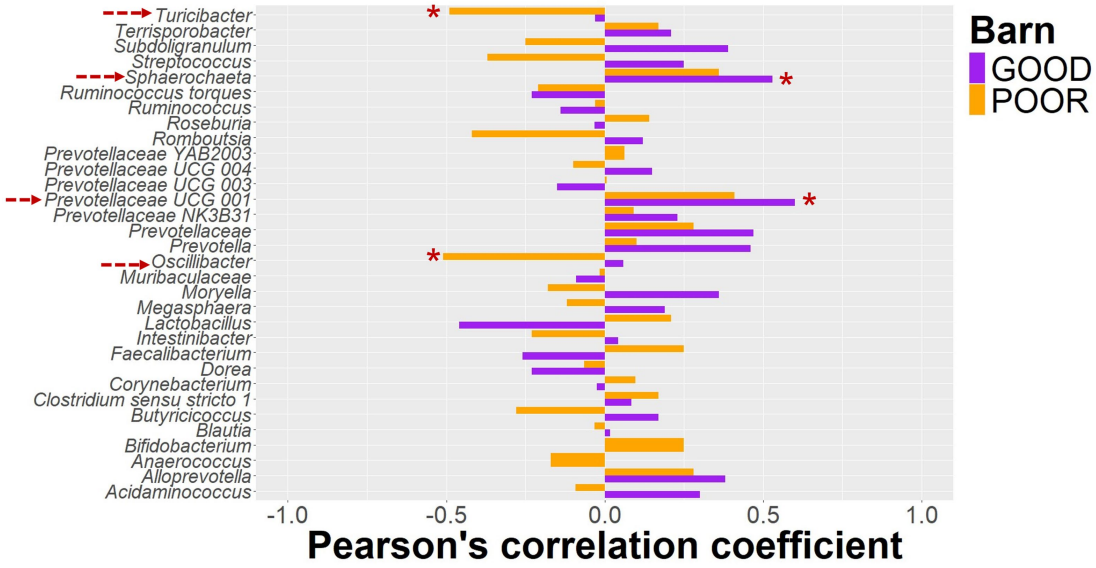

FIGURE 6
Pearson's correlation between keystone taxa using log2 ratios from baseline to endpoint and average daily gain. Pigs were housed under either GOOD or POOR conditions (*S.* Typhimurium inoculation + poor hygiene) in sanitary conditions. The log2 transformed proportional ratio of keystone taxa across the sanitary conditions (barn) was used for the correlation analysis. All log2 transformed ratios were calculated using rarefied data as input, followed by calculating the relative abundance of each taxon by animal DPC 21 in relation to DPC 0. A value of 0.01 was assigned for proportions that were zero at DPC 0. The average daily gain between DPC 0 and 21 was used as the animal phenotype. Red dotted arrows on the left side mark all taxa with a significant Pearson's correlation coefficient based on $p < 0.05$. * For $p < 0.05$; ** for $p < 0.01$, and *** for $p < 0.001$.

correlated network sub-cluster that explained most of the beta-diversity differences at DPC 21. Interestingly, for those five taxa, whether statistically significant or not, the mean relative abundance began to increase already at DPC 10 for the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group. Most likely, these dynamics suggest a compensatory pattern, perhaps revealing the resilience of the resident

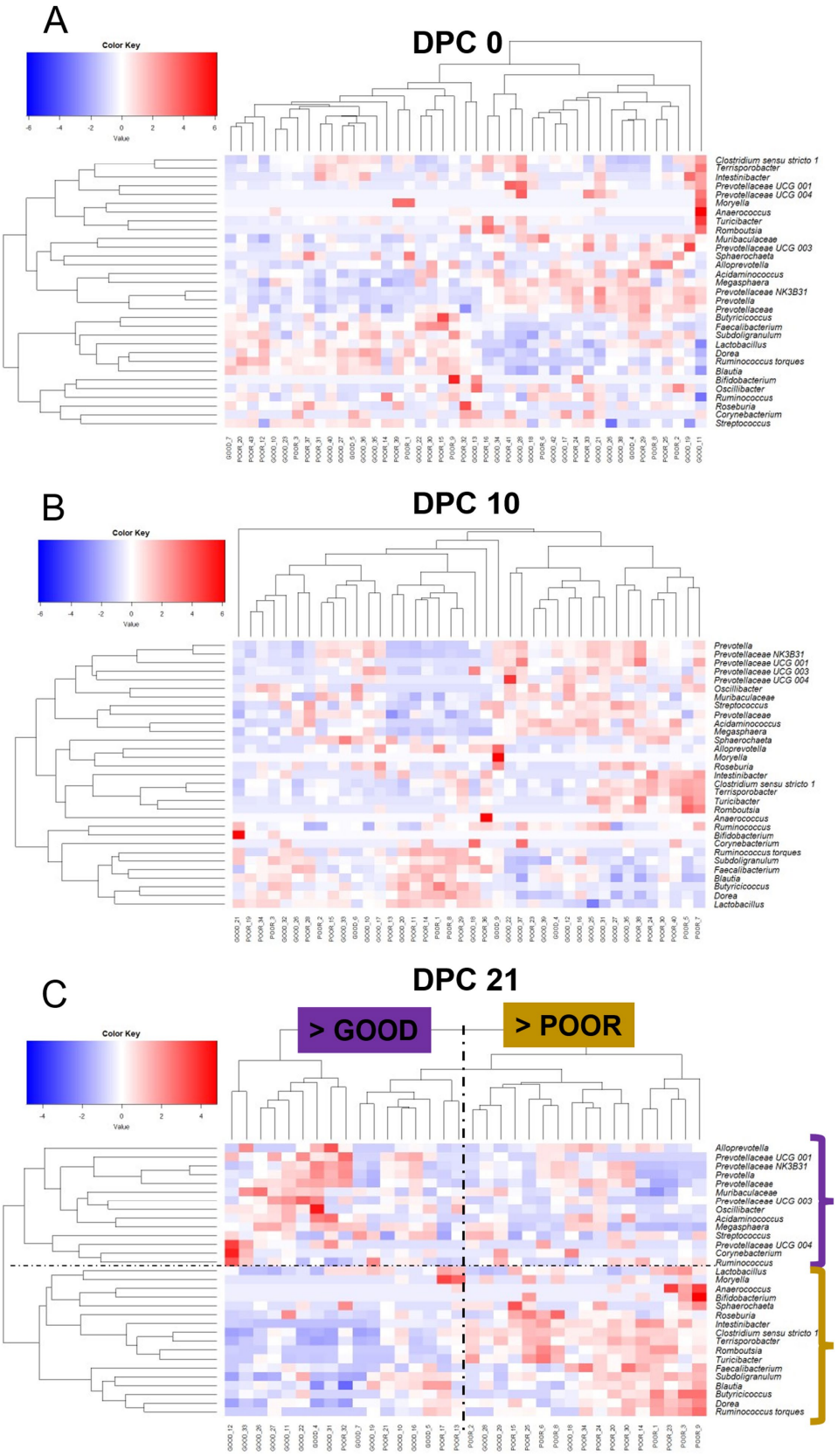

FIGURE 7
Keystone-based heatmap clustering analysis demonstrating changes in fecal microbiome composition across DPC 0 **(A)**, 10 **(B)**, and 21 **(C)** for pigs housed either at GOOD or POOR (*S.* Typhimurium inoculation + poor hygiene) sanitary conditions. Plot **C** is marked with a vertical dotted line to demonstrate a clustering separation between barn GOOD vs. POOR (> representing that the majority of pigs in that group belonged to the respective

(Continued)

FIGURE 7 (Continued)
barn group) and a horizontal dotted line to show the taxa differentially enriched between groups (with two brackets on the side—color-matching pattern based on barn). Each row represents a distinct taxon, while each column represents an individual pig housed in barns under GOOD or POOR sanitary conditions. The input data consisted of rarefied proportions that were log2-transformed prior to scaling (deviation from the mean divided by the standard deviation of the sample). The scaling transformation was applied simultaneously across all taxa.

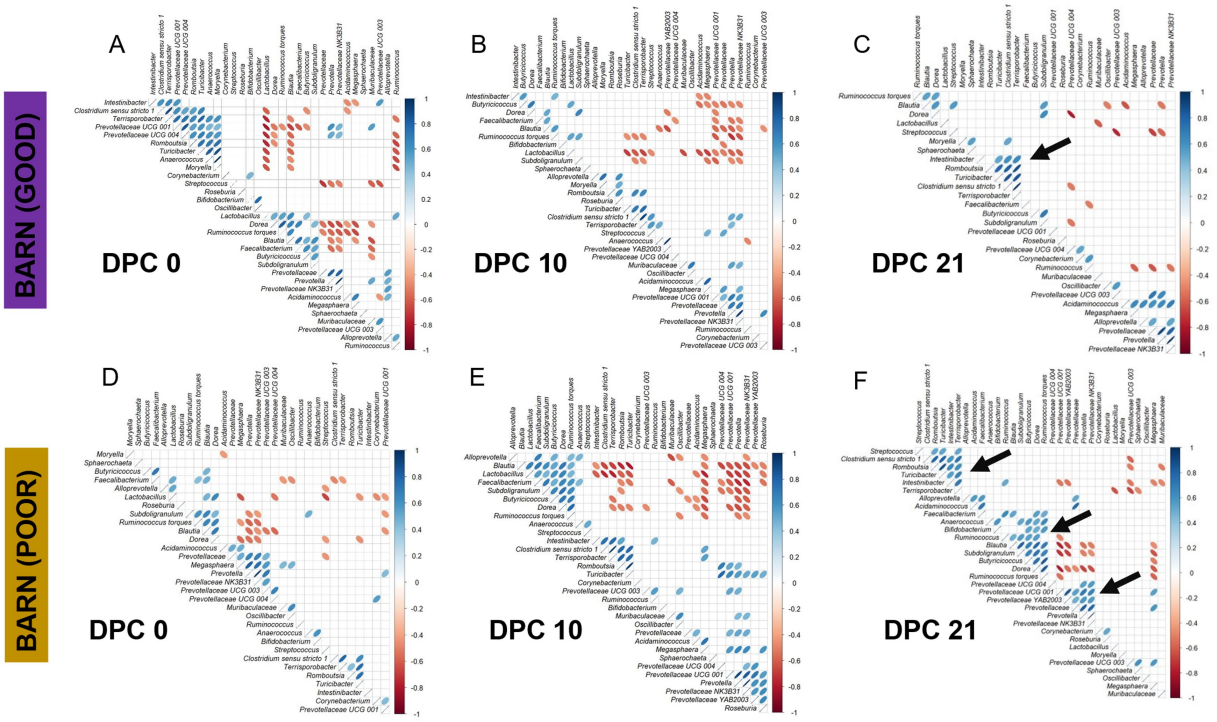

FIGURE 8
Correlation analysis of fecal microbiome taxa among pigs housed in barns under GOOD or POOR (*S.* Typhimurium inoculation + poor hygiene) sanitary conditions across DPC 0, 10, and 21. **(A–C)** Plots for the barn under GOOD sanitary conditions across DPC 0, 10, and 21, respectively. **(D–F)** Plots for the barn under POOR sanitary conditions across DPC 0, 10, and 21, respectively. In all plots, blue ellipses indicate a positive correlation, while red ellipses indicate a negative correlation. The thinner the ellipse, the stronger the correlation or anti-correlation between taxa. Pearson's correlation was used at a p-value of < 0.05 significance level. Only pre-filtered keystone fecal microbiome taxa were included in the analysis (most differentiable between barns at DPC 21). The input data comprised rarefied proportions that were log2-transformed prior to calculating the correlation structure. Black arrows at DPC21 highlight important correlated clusters of taxa for either GOOD **(C)** or POOR (**F**) sanitary conditions.

microbiome for reestablishing GI health and homeostasis post the first 14 days of high exposure to *S.* Typhimurium and continual fecal exposure (poor hygiene). Interestingly, all five major taxa abovementioned tended on average to be lower in relative abundance for POOR AA vs. POOR CN (*S.* Typhimurium inoculation + poor hygiene), suggesting that the AA-supplemented diet did not promote the expansion of these taxa at DPC 21. These five taxa, along with the primary members of the secondary sub-cluster enriched in the POOR (*S.* Typhimurium inoculation + poor hygiene) SC group, comprise *Dorea*, *Ruminococcus*, and *Subdoligranulum, which* are capable of some SCFA production; their correlated pattern may be explained by cross-feeding or the utilization of several carbohydrates, proteins, and AAs (28, 71, 72). For instance, *Ruminococcus torques* can be a mucin degrader that generates oligosaccharides that other species can utilize (73).

Although our analytical approach was comprehensive in identifying the most differential keystone taxa, additional qPCR quantification at the genus level coupled with metagenomics would enhance our ability to assess temporal dynamics both at the genus and species level and the potential metabolic capabilities of the microbiota (74–76). Interestingly, *Turicibacter* appears to be a biomarker for autism spectrum disorder, therefore being associated with the gut–brain axis due to its potential to impact serotonin secretion in the intestine, which requires tryptophan for biosynthesis (77–79). As with *Prevotella copri,* whereby strain diversity differentially impacts polysaccharide utilization (80, 81), the genetic diversity of *Turicibacter* strains may differentially affect their ability to modify bile acids and host lipids (82). Two new studies have also demonstrated that *Turicibacter* is enriched in pigs under both social and heat stress, suggesting its potential role in host physiological adaptation, possibly through serotonin modulation (83, 84).

Despite its uniqueness, our study also presents some limitations, such as the use of 16S rRNA relative abundance data only, having had to rarefy the data at 5,000 counts as previously used by our group and others (12, 23), and the reproducibility of the sanitary conditions applied, which has been successfully used before, albeit with modifications (9, 11, 12). Therefore, we suggest the following opportunities to enhance the quality of this study and for future validating studies: (1) the need to optimize DNA extraction protocols to increase the DNA yield for 16S rRNA

sequencing; (2) the need for qPCR for absolute quantification of target bacteria and temporal analysis based on appropriate power calculations for the phenotype in question; (3) the need to consider sampling different anatomic sites since biogeography can affect microbial dynamics in the *S.* Typhimurium pig infectious study (32); (4) the need to isolate the target taxon and conduct a whole-genome sequencing analysis to assess their genetic diversity and test for relevant phenotypes *in vitro*; (5) the need to consider the use of metagenomics on a subset of samples for further species characterization; (6) the need to consider how randomization might affect the compositional data analysis if a particular taxon is expected to change in relative abundance given the experimental conditions (blocking by enterotypes or specific taxon abundance prior to challenge or dietary changes); (7) the need to assess data distribution and apply a diverse array of methods to increase the confidence on the findings given the multidimensional sparse aspects of the data; (8) the need standardize how poor hygienic challenges will be done using fecal material from pig age-matching barns with low performance and/or other endemic sanitary issues (e.g., screening for predictable endemic pathogens given the geographical locations and historical data); and (9) the need for experimental ecological studies to assess the causative role of specific taxa for colonization resistance against *S.* Typhimurium and to improve pig performance under less optimal housing.

In conclusion, this study identified a collective list of bacterial genus taxa, including *Butyricoccus*, *Clostridium stricto sensu 1*, *Dorea*, *Intestinibacter*, *Romboutsia*, *Ruminococcus torques*, *Subdoligranulum*, *Terrisporobacter*, and *Turicibacter* (many of which are SCFA producers), that were enriched in the POOR SC group at the endpoint of this study, potentially suggesting an ecological recovery of the community post-*S.* Typhimurium infection and poor manure hygiene. That change occurred despite an AA surplus, suggesting that threonine, methionine, cysteine, and tryptophan did not directly affect these core members of the swine fecal microbiome, as indicated by 16S rRNA sequencing data. This suggests a potential role for SCFA producers in stabilizing the large intestine microbiome in the absence of consistently predictable positive associations with increased average daily gain by individual members.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and the accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA985664.

Ethics statement

The animal study was approved by the Ethical Committee on Animal Use (CEUA) of Faculdade de Ciências Agrárias e Veterinárias (FCAV/UNESP—Jaboticabal, SP, Brazil). The study was conducted in accordance with local legislation and institutional requirements.

Author contributions

AB: Writing – review & editing, Project administration, Supervision, Conceptualization, Funding acquisition, Investigation, Writing – original draft, Resources. GA: Conceptualization, Project administration, Writing – original draft, Writing – review & editing, Investigation, Methodology. QY: Data curation, Writing – review & editing, Formal analysis, Writing – original draft. MK: Investigation, Writing – review & editing, Conceptualization, Writing – original draft, Methodology, Formal analysis. DA: Writing – original draft, Methodology, Supervision, Investigation, Conceptualization, Project administration, Writing – review & editing. PR: Writing – original draft, Writing – review & editing. IF: Writing – original draft, Writing – review & editing. CA: Writing – review & editing, Writing – original draft. NK: Writing – original draft, Writing – review & editing. NP: Writing – review & editing, Writing – original draft. PHR: Writing – original draft, Writing – review & editing. HG: Writing – original draft, Writing – review & editing. JH: Writing – review & editing, Writing – original draft. AKB: Writing – review & editing, Writing – original draft, Supervision. LH: Resources, Project administration, Investigation, Conceptualization, Writing – review & editing, Writing – original draft, Funding acquisition, Supervision. JCGN: Writing – review & editing, Conceptualization, Data curation, formal analysis, Funding acquisition, Investigation, Project Administration, Supervision.

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Conflict of interest

HB was employed by Evonik Brazil Ltda. JH was employed by Evonik Operations GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1597857/full#supplementary-material

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