DANTE TEIXEIRA VALENTE JÚNIOR

PROBIOTICS, PREBIOTICS, AND ORGANIC ACIDS AS ALTERNATIVE TO ANTIBIOTICS AND ZINC OXIDE IN DIETS FOR WEANED PIGLETS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Alysson Saraiva

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BIOGRAPHY

Dante Teixeira Valente Júnior, son of Dante Teixeira Valente and Maria de Fátima Teixeira Valente, was born in Viçosa, MG, Brazil, on August 24, 1993. He began his undergraduate studies in Animal Science at *Universidade Federal de Viçosa* (UFV) in 2012 and earned his bachelor's degree in 2018. During his undergraduate program, he participated in the MAST International program at University of Minnesota, USA, from October 2015 to December 2016. After completing his undergraduate degree, he pursued a Master's in Animal Science at UFV, focusing on swine nutrition, and obtained *Magister Scientiae* degree on July 28, 2020.

In the same year, he began his Ph.D. in Animal Science at UFV, specializing in swine nutrition and meat quality. From April 2022 to May 2023, he was a Visiting Ph.D. Student at the Muscle Biology Laboratory at University of Guelph, Canada, where part of his research was conducted under the supervision of Dr. Marcio Duarte. On August 21, 2024, he successfully defended his thesis and obtained the *Doctor Scientiae* degree in Animal Science.

“Sempre acreditei que todo aquele que luta por uma causa justa e nobre alcança o caminho da felicidade"

Expd. Domingos Teixeira Valente

ABSTRACT

VALENTE JÚNIOR, Dante Teixeira, D.Sc., Universidade Federal de Viçosa, August, 2024. **Probiotics, prebiotics, and organic acids as alternative to antibiotics and zinc oxide in diets for weaned piglets**. Adviser: Alysson Saraiva.

Two experiments were carried out with weaned piglets. In experiment I, the study aimed to evaluate the effects of dietary *Bacillus subtilis* DSM 32540 supplementation with or without different fiber sources for piglets. A total of 150 piglets (7.20 ± 0.98 kg) weaned at 21 days old were assigned to one of five treatments in a randomized block design, with ten replicates and three piglets per pen. Treatments consisted of negative control (NC, without ZnO and amoxicillin), positive control (PC, with ZnO and amoxicillin), NC + 0.5 g/kg of probiotic (PRO), NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF + 5 g/kg inulin as SDF (PROCI), and NC + 0.5 g/kg probiotic + 50 g/kg wheat bran (PROWB). From D0 to D14 and D0 to D28, piglets fed NC or PROWB had the lowest ($P < 0.01$) average daily gain (ADG) and final body weight (BW), while piglets fed PRO and PROCI had similar ADG and BW to those fed PC. From D0 to D14, piglets fed PRO and PC had lower ($P < 0.01$) feed conversion ratio (FCR) than those fed NC. From D0 to D28, piglets fed PROWB and NC had the highest ($P < 0.01$) FCR, while there was no difference in FCR among PC, PRO and PROCI groups. Piglets fed PRO, PROCI and PROWB had lower ($P < 0.001$) diarrhea incidence than those fed NC. Piglets fed PC, PRO and PROCI and those fed PRO and PROWB had higher ($P < 0.01$) duodenum and jejunum villus heights (VH) and villus to crypt ratios, respectively, than those fed NC. Piglets fed NC had the highest ($P = 0.045$) serum DAO content, and no difference was observed among groups PC, PRO, PROCI and PROWB on serum concentration of DAO. In conclusion, *B. subtilis* DSM 32540 supplementation improves performance of piglets fed diets with or without pure fiber sources. In addition, regardless of the fiber source, *B. subtilis* DSM 32540 reduces diarrhea incidence and improves intestinal morphology and integrity of nursery piglets. In experiment II, the study investigated the effects of feed additive (FA) containing symbiotic and organic acids on performance and intestinal health of weaned piglets. A total of 288 piglets (BW = 6,53 ± 1,04 kg) were assigned to one of four treatments in a randomized block design, with nine replicates and eight piglets per pen. The treatments were NC (without ZnO), PC (with ZnO), FA1 (NC+1.0 kg/ton FA), and FA2 (NC+2.0 kg/ton FA). Piglets fed FA2 had the lowest ($P = 0.01$) gain:feed (G:F) between 21 and 35 days, while piglets fed FA1 and NC had similar G:F to those fed PC. Piglets fed NC had the lowest (P = 0.04) BW at 35 and 70 days, and those fed FA2 and NC had similar BW compared to PC group. Piglets fed NC had the highest (P = 0.03) fecal score, while no significant difference was observed among groups PC, FA1, and FA2. Piglets fed NC had the lowest (P = 0.03) jejunal VH, whereas VH of piglets fed FA1 and FA2 were comparable to those fed PC. Piglets fed FA1 had higher (P = 0.03) occludin expression compared to those fed NC, while piglets fed PC and FA1 had higher (P < 0.01) TGF-β1 expression compared to piglets fed FA2. In conclusion, although there were no discernible performance benefits, inclusion of 1.0 kg/ton of FA to diet without ZnO helps maintain gut health of piglets by improving intestinal morphology and epithelial integrity and stimulating immune system inactivation.

Keywords: Immune system. Intestinal morphology. Piglets. Prebiotic. Symbiotic. Weaning.

RESUMO

VALENTE JÚNIOR, Dante Teixeira, D.Sc., Universidade Federal de Viçosa, agosto de 2024. **Probióticos, prebióticos e ácidos orgânicos como alternativa aos antibióticos e óxido de zinco em dietas de leitões desmamados**. Orientador: Alysson Saraiva.

Foram realizados dois experimentos com leitões desmamados. No experimento I, objetivou-se avaliar os efeitos da suplementação dietética com *Bacillus subtilis* DSM 32540, com ou sem diferentes fontes de fibra para leitões. Um total de 150 leitões (7,20 ± 0,98 kg), desmamados aos 21 dias de idade, foram distribuídos em cinco tratamentos em delineamento de blocos ao acaso, com dez repetições e três leitões por baia. Os tratamentos consistiram em controle negativo (CN, sem ZnO e amoxicilina), controle positivo (CP, com ZnO e amoxicilina), CN + 0,5 g/kg de probiótico (PRO), CN + 0,5 g/kg de probiótico + 5 g/kg de celulose como fibra insolúvel + 5 g/kg de inulina como fibra solúvel (PROCI), e CN + 0,5 g/kg de probiótico + 50 g/kg de farelo de trigo (PROFT). Do D0 ao D14 e do D0 ao D28, leitões alimentados com CN ou PROFT tiveram menor ($P < 0,01$) ganho de peso diário (GPD) e peso final (PF), enquanto os leitões alimentados com PRO e PROCI apresentaram GPD e PF semelhantes aos alimentados com CP. Do D0 ao D14, os leitões alimentados com PRO e CP tiveram menor ($P < 0,01$) conversão alimentar (CA) em comparação aos alimentados com CN. Do D0 ao D28, os leitões alimentados com PROFT e CN apresentaram o maior ($P < 0,01$) CA, enquanto não houve diferença na CA entre os grupos CP, PRO e PROCI. Leitões alimentados com PRO, PROCI e PROFT tiveram menor ($P < 0,001$) incidência de diarreia do que aqueles do grupo CN. Leitões alimentados com CP, PRO e PROCI e aqueles dos grupos PRO e PROFT tiveram maiores ($P < 0,01$) alturas de vilosidade (AV) no duodeno e jejuno e relações vilo:cripta, respectivamente, do que aqueles do grupo CN. Os leitões alimentados com CN tiveram o maior ($P = 0,045$) concentração sérica de DAO, e nenhuma diferença foi observada entre os grupos CP, PRO, PROCI e PROFT na concentração sérica de DAO. Em conclusão, a suplementação com *B. subtilis* DSM 32540 melhora o desempenho de leitões alimentados com dietas com ou sem fontes de fibra puras. Além disso, independentemente da fonte de fibra, *B. subtilis* DSM 32540 reduz a incidência de diarreia e melhora a morfologia e integridade intestinal de leitões na fase de creche. No experimento II, investigou-se os efeitos de um aditivo (FA) contendo simbióticos e ácidos orgânicos no desempenho e saúde intestinal de leitões desmamados. Um total de 288 leitões (PC = 6,53 ± 1,04 kg) foram distribuídos em quatro tratamentos em delineamento de blocos ao acaso, com nove repetições e oito leitões por baia. Os tratamentos foram CN (sem ZnO), CP (com ZnO), FA1 (CN + 1,0 kg/ton de FA) e FA2 (CN + 2,0 kg/ton de FA). Os leitões alimentados com FA2 tiveram o menor ($P = 0,01$) eficiência alimentar entre 21 e 35 dias, enquanto os leitões alimentados com FA1 e CN apresentaram eficiência alimentar semelhante aos do grupo CP. Os leitões alimentados com CN tiveram o menor ($P = 0,04$) PF aos 35 e 70 dias, e aqueles alimentados com FA2 e CN tiveram PF semelhante ao grupo CP. Os leitões alimentados com CN tiveram o maior ($P = 0,03$) escore fecal, enquanto nenhuma diferença foi observada entre os grupos CP, FA1 e FA2. Os leitões alimentados com CN tiveram a menor ($P = 0,03$) AV jejunal, enquanto as AV de leitões alimentados com FA1 e FA2 foram comparáveis àquelas alimentadas com CP. Os leitões alimentados com FA1 tiveram maior ($P = 0,03$) expressão de ocludina no jejuno em comparação com aqueles alimentados com CN, enquanto os leitões alimentados com CP e FA1 tiveram maior ($P < 0,01$) expressão de TGF-β1 em comparação com leitões alimentados com FA2. Em conclusão, a inclusão de 1,0 kg/ton de FA na dieta sem ZnO ajuda a manter a saúde intestinal de leitões, melhorando a morfologia e integridade intestinal, e estimulando a inativação do sistema imunológico.

Palavras-chave: Desmame. Leitões. Morfologia intestinal. Prebiótico. Simbiótico. Sistema imune.

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General introduction

Weaning is one of the most stressful phases in pig production, leading to significant challenges in intestinal health and overall performance due to abrupt changes in environment, social dynamics, and diet (Pluske et al., 2018). These stressors disrupt the gut microbiota, damage the gastrointestinal tract, and trigger immune responses, often resulting in diarrhea, poor performance, and increased mortality (Moeser et al., 2017; Pluske et al., 2018). Historically, zinc oxide (ZnO) and antibiotics were widely used to mitigate these effects, but growing concerns over antibiotic resistance and environmental pollution have led to restrictions on their use in many countries (Slifierz et al., 2015; Jensen et al., 2016).

As a result, alternatives such as probiotics, prebiotics, dietary fibers, and organic acids (OA) are being actively investigated to enhance gut health and performance in weaned piglets. Probiotics can inhibit the growth of pathogenic bacteria through mechanisms such as competition for nutrients and binding sites in the gut mucosa, along with the production of antibacterial compounds (Dong et al., 2014). Prebiotics, meanwhile, serve as substrates that enhance the growth of beneficial probiotic bacteria, reduce the attachment of harmful pathogens, and modulate immune responses (Gibson et al., 2017). Organic acids, such as butyric and citric acids, are well-known for their bactericidal and bacteriostatic properties (Long et al., 2018; Han et al., 2020; Xu et al., 2020). Previously considered an anti-nutritional factor, dietary fiber is now recognized for its vital role in gut health, with both soluble and insoluble fibers acting as prebiotics. These fibers support the elimination of pathogenic bacteria and the production of short-chain fatty acids (SCFAs), which are crucial for energy production in enterocytes, as well as for regulating inflammation and cell proliferation, ultimately improving intestinal integrity (Molist et al., 2014; Agyekum and Nyachoti, 2017).

Although numerous studies have demonstrated the individual benefits of probiotics, prebiotics, OAs, and dietary fibers on gut health and piglet performance, the synergistic effects of combining these additives remain unclear. Therefore, further research is needed to explore the potential of these feed additives working together to enhance intestinal health and performance, offering an effective alternative to ZnO and antibiotics.

Therefore, this thesis was developed based on three independent experiments with the objective to:

1- Determine if the benefits of using a combination of soluble and insoluble dietary fiber on performance and gut health of piglets are achieved similarly from intact fiber source as wheat bran compared as purified source in combination with *B. subtilis* DSM 32540 addition.
2- Evaluate the effects of dietary ZnO replacement by symbiotic and OA on performance, intestinal morphology and gene expression associated to epithelial integrity and activation of the immune system of weaned piglets.

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CHAPTER 1: Supplementation of *Bacillus subtilis* DSM 32540 improves performance and intestinal health of weaned pigs fed diets containing different fiber sources.

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ABSTRACT

The study aimed to evaluate the effects of dietary *Bacillus subtilis* DSM 32540 supplementation with or without different fiber sources for piglets. A total of 150 piglets (7.20 ± 0.98 kg) weaned at 21 days old were assigned to one of five treatments in a randomized block design, with ten replicates and 3 piglets per pen. Treatments consisted of negative control (NC, without ZnO and amoxicillin), positive control (PC, with ZnO and amoxicillin), NC + 0.5 g/kg of probiotic (PRO), NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF + 5 g/kg inulin as SDF (PROCI), and NC + 0.5 g/kg probiotic + 50 g/kg wheat bran (PROWB). From D0 to D14 and D0 to D28, piglets fed NC or PROWB had the lowest ($P < 0.01$) average daily gain (ADG) and final body weight (BW), while piglets fed PRO and PROCI had similar ADG and BW to those fed PC. From D0 to D14, piglets fed PRO and PC had lower ($P < 0.01$) feed conversion ratio (FCR) than those fed NC. From D0 to D28, piglets fed PROWB and NC had the highest ($P < 0.01$) FCR, while there was no difference in FCR among PC, PRO and PROCI groups. Piglets fed PRO, PROCI and PROWB had lower ($P < 0.001$) diarrhea incidence than those fed NC. Piglets fed PC, PRO and PROCI and those fed PRO and PROWB had higher ($P < 0.01$) duodenum and jejunum villus heights and villus to crypt ratios, respectively, than those fed NC. Piglets fed NC had the highest ($P = 0.045$) serum DAO content, and no difference was observed among groups PC, PRO, PROCI and PROWB on serum concentration of DAO. In conclusion, *B. subtilis* DSM 32540 supplementation improves performance of piglets fed diets with or without pure fiber sources. In addition, regardless of the fiber source, *B. subtilis* DSM 32540 reduces diarrhea incidence and improves intestinal morphology and integrity of nursery piglets.

Keywords*:* fiber, intestinal health, immune response, performance, probiotic, weaned pig.

1. Introduction

Weaning is one of the most stressful periods in the entire pig production cycle, wherein piglets are subjected to a new environment, social interaction and different diet (Pluske et al., 2018). Stressful events associated with weaning cause damage to gastrointestinal tract (GIT) mucosa affecting its morphology and permeability, promotes changes in the intestinal microbiota and activation of the immune system with negative consequences on performance (Moeser et al., 2017; Pluske et al., 2018).

In order to prevent gastrointestinal disorders and improve performance of weaned piglets, dietary antibiotics and zinc oxide (ZnO) as growth-promoters have been used regularly (Dębski, 2016; Satessa et al., 2020). However, the use of ZnO and antibiotics have been banned or restricted in several countries as it can result in antibiotic-resistant bacteria and environmental contamination (Slifierz et al., 2015; Jensen et al., 2016; López-Gálvez et al., 2021).

Thus, nutritional strategies to ameliorate the damage caused by the weaning on intestinal integrity and immune system are needed during this stressful period. In this context, the use of probiotics, such as *Bacillus subtilis* strains and combination of soluble or insoluble fibers from certain ingredients in diets for weaning piglets have been evaluated to modulate the gut microbiota and reduce its colonization by pathogens (López-Gálvez et al., 2021).

Fermentation of substrates such as dietary fibers in the hind gut of pigs can increase short chain fatty acids (SCFAs) production which can be used as energy source by beneficial bacteria and reduce mucosal disorders (Molist et al., 2014; Agyekum and Nyachoti, 2017). Short chain fatty acids synthesized from fiber fermentation are used by enterocytes to obtain energy and are important in modulating the inflammatory response and cell proliferation, reflecting in greater integrity and functionality of the intestinal villi (Pieper et al., 2016; Agyekum and Nyachoti, 2017).

Due to its solubility, fiber sources can be more fermented in the proximal or distal intestine of piglets (Pieper et al., 2014). Therefore, mixture of insoluble (IDF) and soluble (SDF) dietary fiber may result in better performance, reduction of diarrhea, and improve gut health of weaned pigs compared to feeding only insoluble or soluble dietary fiber during the whole nursery period (Pieper et al., 2014; Chen et al., 2020; Li et al., 2020; Slama et al., 2020). Since most studies are done with pure sources of SDF (i.g. inulin) or IDF (i.g. cellulose), a comparison with an integrated combination of both type of fibers in raw materials (e.g. wheat bran and oat bran) should be evaluated.

Probiotics can attenuate the negative effects of weaning through different mechanisms. Modulation of the microbiota through competitive exclusion by adhesion sites and substrates, and direct antimicrobial inhibition, via reduction of luminal pH and production of bactericidal substances (Cao et al., 2019; Luise et al., 2019; Park et al., 2020). In addition, probiotics modulate the host immune responses, with changes in mucus secretion and expression of tight junction proteins, inducing and reducing anti-inflammatory and pro-inflammatory cytokines, respectively (Liao and Nyachoti, 2017).

However, in the literature there are a few reports regarding the association of probiotics and SDF derived from purified sources of fiber, such as inulin (Mair et al., 2010a; Liu et al., 2018; Rodríguez-Sorrento et al., 2020) and to our knowledge, no study has evaluated the combined effects of probiotics and different fiber types for piglets. Therefore, the symbiotic effects of dietary fiber sources (IDF and SDF) and probiotics remains unclear and needs further investigation, as it may have unique effect on gut health and performance of piglets.

It was hypothesized that inclusion of *B. subtilis* DSM 32540 in diets containing intact or purified fiber sources improves performance, morphology and epithelial integrity of the intestine and decrease immune system activation of piglets weaned at 21 days of age. Therefore, the present study was carried out to determine if the benefits of using a combination of SDF and IDF on performance and gut health of piglets are achieved similarly from intact fiber source as wheat bran compared as purified source in combination with *B. subtilis* DSM 32540 addition.

2. Material and methods

2.1. Animal ethic statement

All methods involving the handling of pigs followed the ethical principles of animal research (CONCEA) and were approved by the Commission of Ethics in the Use of Production Animals (CEUAP) of the Universidade Federal de Viçosa (protocol 05/2021).

2.2. Animals, experimental design, housing and diets

A total of 150 mixed-sex piglets (Agroceres PIC) weaned at 21 d of age (D0) with initial body weight (BW) of 7.20 ± 0.98 kg were assigned to one of five treatments in a randomized block design according to initial BW, with ten replicates per treatment and 3 piglets per pen for 28 days (D0 to D28).

During the suckling period, the piglets did not receive any dietary supplement besides sows' milk. After weaning (D0), piglets were fed with corn-soybean meal-based diets (Table 1), formulated to meet the nutritional requirements during the two phases of nursery period (D0 to D14 and D15 to D28), as recommended by Rostagno et al. (2017). The treatments consisted of: negative control (NC, without ZnO and amoxicillin); positive control (PC, with ZnO and amoxicillin); NC + 0.5 g/kg probiotic (PRO); NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF (PROCI); and NC + 0.5 g/kg probiotic + 50 g/kg wheat bran (PROWB).

Zinc oxide and amoxicillin were included at levels of 2,500 ppm and 250 ppm, respectively, from D0 to D14, and 2,000 ppm and 250 ppm, respectively, from D15 to D28. The probiotic product (*B. subtillis* DSM 32540; GutPlus; Evonik Operations GmbH, Hanau-Wolfgang, Germany) supplies the viable *B. subtillis* spore count of 2×10^9 CFU/g.

Piglets were assigned during the experimental period of 28 d in unclean metal cages, with plastic slatted floors, equipped with semiautomatic feeders and nipple drinkers, located in nursery rooms in which the temperature was maintained within the thermoneutral zone during the experimental period by electric heaters.

During the experimental period, average ambient temperature was 26.19 ± 1.57 °C, minimum ambient temperature 25.13 ± 1.10 °C and maximum ambient temperature 27.26 ± 1.22 °C.

Piglets had free access to feed and water throughout the experimental period (D0 to D28). During the trial, feed was weighed before feeding and feed wastage and leftovers were collected and weighed daily to determine average daily feed intake (ADFI). Piglets were individually weighed on D0, D14 and D28 of the experimental period to determine BW, average daily gain (ADG), and feed conversion ratio (FCR).

2.3. Fecal score and diarrhea incidence

Fecal score of each pig was visually assessed from 8 a.m. to 11 a.m. by only one trained observer, during the first phase of the experimental period, D0 to D14 (21 to 35 day of age), using the method described by Liu et al. (2010). Fresh excreta were ranked using the following scale: 1 = solid; 2 = semi-solid; 3 = semi-liquid; and 4 = liquid. The occurrence of diarrhea was defined as production of feces at level 3 or 4 for two continuous days. *Diarrhea incidence (%) = [number of pigs with diarrhea in each pen* × *diarrhea days/(3 pigs* × *14 d)]* × *100*.

2.4. Digesta, intestinal tissue and blood samples collection

On D14 (at 35 d of age), one piglet with BW closest to the pen average from each treatment was selected for blood collection via venipuncture in the orbital sinus into 5 mL uncoated vacuum tubes, and centrifuged (3,500 × *g* at 4 °C for 10 min) to separate serum. Serum was stored at – 20 °C until analysis.

Posteriorly, the same piglet was electrically stunned followed by exsanguination to collect histological samples. Segments of 2.0 × 2.0 cm of the intestine were sampled corresponding to duodenum (10 cm from pylorus), jejunum (the middle portion) and ileum (5 cm proximal to the ileocecal junction), according to Yang et al. (2014).

Histological sections were then washed in physiological solution and fixed in 4.0% paraformaldehyde solution for 24 h at room temperature.

The intestinal contents of the cecum and colon were placed in sterile 15 mL falcon tubes and stored at -20 °C until the analysis of acetate, propionate, isobutyrate, butyrate, isovalerate, valerate and total volatile fatty acids (VFAs).

2.5. Sampling and gene expression analysis

In order to collect jejunum samples for gene expression of *NF-κB*, haptoglobin (*Hap*), cytokines (*IL-1β, IL-6, TNFα, IL-10* and *TGF β1*), SCFAs transporters [monocarboxylate transporter 1 (*MCT1*) and sodium-coupled monocarboxylate transporter (*SMCT2*)] and tight junction proteins (*ZO-1* and *occludin*), instruments that came into contact with the tissue were sanitized with alcohol 70%, dried with paper towel and sprayed with RNAse Exterminator (Protech Technology Enterprise CO., Ltd., Taipei, Nanking Dist., Taiwan) between each sampling. The portion of the sampled jejunum was washed with sterile saline solution for removal of the digesta in contact with the tissue. From the tissue sample, about ten 0.25 cm^3 subsamples were collected, washed individually in sterile saline solution serum, placed in cryotubes with a capacity of 1.8 mL, frozen instantaneously in liquid nitrogen, and stored at -80 °C for further analysis.

Total RNA extraction was performed using TRIzol (Invitrogen) following the manufacturer instructions. The RNA concentration was estimated by NanoDrop Lite (Thermo Fisher Scientific), and RNA integrity was evaluated through 1% agarose gel electrophoresis. Complementary DNA synthesis was performed according to GoScript Reverse Transcription System protocol (Promega Corporation). GenBank numbers to access the primers of the genes are shown in Table 2. Primers were used for reverse transcription quantitative PCR with GoTaq qPCR Master Mix (Promega) in QuantStudio 3 (Applied Biosystems, Thermo Fisher Scientific). Geometric means of Ct values of B2M were used to normalize target genes expression for the jejunum samples. Gene of interest relative expression was calculated by 2-ΔΔCt (Livak and Schmittgen, 2001).

2.6. Serum diamine oxidase (DAO) analysis

Porcine serum DAO level was measured by using commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturers' instructions (Elabscience, E-EL-P1311, USA).

Briefly, 100 µL of standard and serum samples were added to each well and then incubated for 90 min at 37 °C. After that, the liquid was removed and 100 µL of biotinylated detection was added in each well. After 60 min of incubation at 37 °C, the plate was washed 3 times with wash buffer solution, and 100 µL of HRP conjugate was added, and then the plate was incubated for 30 min at 37 °C. Then, the plate was washed five times and 90 µL of substrate reagent was added in each well. Exactly after 15 min of incubation at 37 °C in the dark, 50 µL of stop solution was added in each well in the same order as the substrate solution.

The level of DAO was immediately measured at 450 nm using a spectrophotometer (Thermo Scientific - Multiskan GO) and calculated according to generated standard curve in the assay.

2.7. Intestinal morphology assessment

The histological sections (duodenum, jejunum and ileum) were cross-sectionally cut and dried in ethyl crescent gradients, diaphanized in HistoChoice, and embedded in liquid Paraplast at 65 °C. Five transverse cuts with 5 µm thickness each were placed per slide, and were stained with hematoxylin and eosin. The cuts were semi-serial using one in ten cuts. For morphological readings, an optical microscope EVOS M5000 Imaging System (Invitrogen, Thermo Fisher Scientific) was used with 10× magnification. Afterward, the images were analyzed by image analyzer ImageJ 1.50i; java1.6.0_20 (National Institutes of Health). Heights of 20 villi and their 20 crypts were selected and measured according to Satessa et al. (2020). Villus to crypt ratios using the length data were then calculated. All measurements were made by a single individual.

2.8. Analysis of VFAs content in caecum and colon

For the determination of VFAs, 1 g of the sampled caecum and colon content was weighed in a microtube and 1 mL of distilled water was added. Afterwards, the solution was homogenized in a tube shaker and centrifuged at 12,500 *g* at 4°C for 60 min.

Subsequently, the supernatant extract (approximately 0.4 mL) of each sample was transferred to chromatographic vials, in which 100 µL of 3:1 solution of 25% metaphosphoric acid with 98-100% formic acid and 50 µL of solution of 100 mM 2-ethyl-butyric acid. From this extract, 1 µL was automatically injected by the injector system in a gas chromatograph (CG HP 7890A; Injector HP 7683B, Agilent Technologies) equipped with a capillary column HP-FFAP (1909F-112; 25 m; 0.32 mm; 0.5 µm; JeW Agilent Technologies). The carrier gas used was H_2, maintained at a flow of 31.35 mL/min. The temperature of the injector and detector was 260 °C and the total time of the chromatographic analysis was 16 min, divided into three heating ramps: 80 °C (1 min), 120 °C (20 °C/min; 3min) and 205 °C (10 °C/min; 2 min). The acid concentration (mM) was determined based on an external calibration curve (Ferreira et al., 2016). The analyzes were carried out at the Laboratory of Nutrition of Reproduction of the Luiz de Queiroz School of Agriculture –ESALQ of the Federal University of São Paulo (USP). Total VFAs was determined as the sum of analyzed acetate, propionate, and butyrate. Additionally, three-branched fatty acids, namely, isobutyrate, isovalerate, and valerate were also measured. All procedures were performed in triplicate.

2.9. Statistical analysis

The cage was considered the experimental unit for performance, fecal index and diarrhea incidence analysis. The piglet, slaughtered on D14, was considered the experimental unit for serum DAO, intestinal morphology, gene expression and VFAs content in digesta. Data were analyzed using the ExpDes.pt package of software R Core Team (2020), version 4.1.0. Data were subjected to analysis of variance (ANOVA) and means were compared using a Tukey test. For all statistical analysis probability values less than 0.05 were considered significant.

3. Results

3.1. Performance, fecal score and diarrhea incidence

From D0 to D14 and D0 to D28, piglets fed NC or PROWB diets showed the lowest ($P < 0.01$) ADG and final BW (Table 3), while piglets fed PRO and PROCI diets had similar ADG and BW to those fed PC diet. From D0 to D14, piglets fed PRO and PC diets had lower (P < 0.01) FCR than those fed NC diet. From D0 to D28, piglets fed PROWB and NC diets showed the highest (P < 0.01) FCR, while there was no difference in FCR among PC, PRO and PROCI groups.

Piglets fed PRO, PROCI and PROWB diets had lower (P < 0.001) diarrhea incidence than those fed NC diet (Table 4). Piglets fed NC diet presented the highest (P < 0.001) fecal score values, and no difference was observed in fecal score between PC and PROCI groups.

3.2. Intestinal morphology and serum DAO

Piglets fed NC had the lowest (P < 0.01) duodenum and ileum villus heights (VH) and villus to crypt ratios (VCR), whereas there was no differences among the PC, PRO, PROCI and PROWB groups (Table 5). Piglets fed PC, PRO and PROCI diets and those fed PRO and PROWB diets showed higher (P < 0.01) duodenum and jejunum VCR, respectively, than those fed NC diet. Piglets fed PC and PRO diets had lower (P = 0.01) duodenal crypts depth (CD) compared to those fed NC diet. No effects (P > 0.05) were observed on jejunal VH and CD, and ileal CD.

Piglets fed NC diet had highest (P = 0.045) serum DAO content, while no difference was observed among groups PC, PRO, PROCI and PROWB on serum concentration of DAO (Fig. 1).

3.3. Gene expression

No effects (P > 0.05) on jejunal mRNA gene expression of *NF-κB, Hap*, cytokines (*IL-1β, IL-6, TNFα, IL-10* and *TGF β1*), SCFAs transporters (*MCT1* and *SMCT2*) and tight junction proteins (*ZO-1* and *occludin*) were observed (Table 6).

3.4. VFAs concentrations

Piglets fed PROCI diet had higher (P = 0.029) millimolar concentration of acetate in caecum compared to those consumed PC diet, and no difference was observed among the NC, PRO and PROWB groups. However, no effect ($P = 0.376$) on acetate content in the colon was observed (Table 7).

The content of propionate, isobutyrate, butyrate, isovalerate, valerate and total VFAs in the caecum and colon were not ($P > 0.05$) altered by the treatments.

4. Discussion

Despite the ability of ZnO and antibiotics to improve performance and intestinal morphology, and reduce epithelial barrier disruption and diarrhea incidence in piglets, as reported in the current study and previously (Pei et al., 2019; Sun et al., 2019; Lin and Yu, 2020; Ma et al., 2021), its regular use can result in antibiotic-resistant bacteria and environmental contamination, which increase the demand of feed alternatives.

In this regard, results from the present study demonstrated that piglets fed with *B. subtilis* DSM 32540 (PRO) had similar BW, ADG and FCR compared to those fed PC diets, which was in line with the previous studies (He et al., 2020; Ding et al., 2021; Li et al., 2021). In addition, *Probiotic* supplementation reduced diarrhea incidence of weaned piglets compared to animals fed NC diet. Therefore, the dietary inclusion of *B. subtilis* appears to be important to mitigate the reduced performance and higher incidence of diarrhea when newly weaned piglets receive diets without antibiotics and ZnO.

These beneficial responses promoted by *B. subtilis* supplementation may be attributed to reduction of pathogenic population in the GIT through production of antimicrobials (i.g. bacteriocins, peptides and lipopeptides), that result in reduced competition for nutrients between host and microorganisms, and increased mucin production, which improve gut barrier function against pathogens (Abriouel et al., 2011; Grant et al., 2018; Luise et al., 2019). In addition, the positive influence of *B. subtilis* on performance, diarrhea incidence and gut health could be mediated by other mechanisms, including production of extracellular enzymes, regulation of host immune system, reduction of intestinal pH and production of SCFAs (Cao et al., 2019; Makarenko et al., 2019; Park et al., 2020).

In the present study, unlike the wheat bran diet (PROWB), the inclusion of *Probiotic* in the diet with pure fiber sources (PROCI) promoted similar BW, ADG and FCR results compared to PC diet, suggesting that the association of *Probiotic*, inulin and cellulose may have a better effect on growth performance compared to the inclusion of probiotic in the diet containing wheat bran as fiber source. In addition, piglets fed PROCI were the only group to present similar fecal score and diarrhea incidence compared to PC, which indicates a possible synergic effect between *Probiotic* and purified fiber source.

Besides the *B. subtilis* benefits on intestinal health, the use of inulin and cellulose alone or in combination as SDF and IDF, respectively, for weaning piglets have been reported to modulate the gut microbiota and reduce its colonization by pathogens, which leads to better performance and gut development, and reduced diarrhea incidence (Bosscher et al., 2006; Halas et al., 2009; Pascoal et al., 2015; Chen et al., 2020).

Despite the reduction on diarrhea incidence, a clear synergistic effect of inulin, cellulose and *Probiotic* on piglets' performance were not found, since piglets fed PROCI diets presented similar performance variables compared to those from the PRO group, which is supported by other studies that also did not find synergic effects between probiotics and prebiotics (Mair et al., 2010b; Rodríguez-Sorrento et al., 2020).

It was interesting to observe that adding *B. subtilis* in the diet containing wheat bran (PROWB) tended to reduce performance of piglets fed the diet containing probiotic only (PRO). However, piglets fed PROWB diets had lower diarrhea incidence than those from the NC group.

Previous studies have shown that the inclusion of dietary wheat bran in piglets' diet decreased pathogenic bacteria attachment to the intestinal mucosa, reduced intestinal permeability and system immune activation, thereby reducing diarrhea incidence (Chen et al., 2017; Shang et al., 2020; Shang et al., 2021). Although wheat bran can improve the microbiota and intestinal integrity, as well as attenuate the inflammatory response, its dietary inclusion can negatively affect the digestion of energy, protein and organic matter (Molist Gasa et al., 2010; Yu et al., 2016; Koo et al., 2017; Zhao et al., 2018), since wheat bran increases the passage rate, thereby decreasing the retention time of digesta in the gut for digestion and absorption of nutrients (Flis et al., 2017).

In this way, our results suggest that inclusion of *B. subtilis* DSM 32540 in diets containing 50 g/kg of wheat bran improves intestinal health of piglets, but did not result in improved performance, which is probably due to a lower nutrient digestibility caused by the level wheat bran inclusion.

Intestinal villi are the major sites for the nutrient absorption, and increased VH is positively correlated to enzyme production and surface area for digestion and absorption of nutrients, being an indicator of intestinal health (Wang et al., 2020a). At high rates of intestinal cell renewal, crypts become deeper resulting in increased energy expenditure to support epithelial cell turnover (Shang et al., 2020). In this sense, higher VH and lower the CD indicate better digestion and absorption of nutrients.

In the current study, dietary supplementation of *Probiotic* increased the duodenum and jejunum VCR, and reduced duodenum CD compared to piglets fed NC diet. Evaluating the association between *B. subtilis* DSM 32540 and fiber sources, piglets fed PROCI and PROWB diets had higher duodenum, jejunum and ileum VCR compared to those from the NC group, which is in agreement with the performance and diarrhea incidence results, and indicates that regardless the fiber type, *Probiotic* supplementation attenuates the damage on intestinal epithelium caused by the weaning. In this sense, the improvements on intestinal morphology could be attributed to several mechanisms.

B. subtilis and fiber sources beneficially modulate the intestinal microbiota, favoring acidophilic bacteria growth and controlling the pathogens in the GIT of weaned piglets through competition of nutrients and binding sites in gut mucosa, reduction of intestinal pH and production of antibacterial substances (Halas et al., 2009; Yu et al., 2016; Grant et al., 2018; Luise et al., 2019). In addition, studies evaluating inulin, cellulose, wheat bran or *B. subtilis* in diets of weaned piglets reported increased production of SCFAs in the intestine (Mu et al., 2017; Tang et al., 2019; Shang et al., 2020; Wang et al., 2020b), which plays important role in cells proliferation, differentiation, and reduction of enterocytes apoptosis (Sigalet et al., 2017).

Therefore, the dietary supplementation of *Probiotic* in diets containing different fiber sources may have reduced the intestine colonization by pathogens and increased SCFAs production, resulting in greater intestinal morphology.

The serum concentration of DAO is used as a marker of intestinal permeability, since when there is a disruption of the intestinal barrier, this enzyme that is mainly distributed in the cytoplasm of intestinal epithelial cells is released to the blood circulation (Cai et al., 2019; Wang et al., 2020b).

In the present study, piglets fed NC diet had the highest serum DAO concentration, while the animals that consumed PRO, PROCI and PROWB diets presented similar serum DAO concentration compared to PC group. In this way, regardless of fiber sources, supplementation of *Probiotic* attenuated the higher intestinal disruption caused by ZnO and antibiotics removal from the diets, corroborating that probiotic and dietary fiber plays important role on intestinal barrier function and development.

Weaning is associated with an increased inflammatory response and intestinal permeability, caused by a positive regulation of pro-inflammatory cytokines and acute-phase proteins, and reduction of tight junction proteins (Pié et al., 2004; Sauerwein et al., 2005; Hu et al., 2013).

In this study, although piglets fed PC, PRO, PROCI and PROWB had better intestinal barrier function and development compared to those from the NC group, no effects were observed on gene expression of inflammatory markers, tight junction proteins and SCFAs transporters. However, the pathway that each analyte produced by these genes participates could be altered in order to restore and protect the intestinal epithelium.

As aforementioned, the improvements on intestinal morphology and integrity, as well as the reduction in the diarrhea incidence of piglets with the inclusion of *Probiotic* in diets with different fiber sources (inulin, cellulose and wheat bran) could be due to increased production of SCFAs in the intestine.

In this context, according to Morrison and Preston (2016), acetate can inhibit pathogenic bacteria, beneficially modulating the microbiota. In the current study, piglets fed PROCI diet had the highest acetate content in the caecum, which was in line with the results of the studies evaluating cellulose and inulin (Chen et al., 2020) and *B. subtilis* (Wang et al., 2021) in the diet for weaned piglets. Therefore, these results suggest that combined dietary supplementation with cellulose, inulin and *B. subtilis* may act synergistically to increase the VFAs production, resulting in improved intestinal health and increased performance of weaned pigs.

Overall, our study shows that dietary *B. subtilis* DSM 32540 supplementation has comparable effects to ZnO and antibiotics in improving performance and intestinal health of weaned piglets. However, further studies should be carried out focusing on the interaction between *B. subtilis* and the type of fiber, in order to better understand the effects (singly or in combination) of these feed additives.

5. Conclusion

Bacillus subtilis DSM 34520 supplementation improves performance of piglets fed diets with or without pure fiber sources. In addition, regardless of the fiber source, *B. subtilis* reduces diarrhea incidence and improves intestinal morphology and integrity in nursery piglets without affecting the gene expression of inflammatory markers.

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Table 1. Composition of experimental diets (as-fed basis).[1]

Ingredient, g/kg	Phase I (D0 to D14)					Phase II (D15 to D28)				
	NC	PC	PRO	PROCI	PROWB	NC	PC	PRO	PROCI	PROWB
Corn	463.30	457.70	462.80	445.80	404.60	530.80	525.00	530.30	513.00	470.90
Soybean meal	170.00	170.00	170.00	170.00	170.00	259.50	259.50	259.50	259.50	259.50
Whey powder	160.00	160.00	160.00	160.00	160.00	80.00	80.00	80.00	80.00	80.00
Micronized soybean	110.00	110.00	110.00	110.00	110.00	50.00	50.00	50.00	50.00	50.00
Blood plasma	40.00	40.00	40.00	40.00	40.00	---	---	---	---	---
Dicalcium phosphate	15.60	15.60	15.60	15.60	14.90	18.20	18.20	18.20	18.20	17.50
Soybean oil	14.50	16.50	14.50	21.50	23.50	30.70	33.50	30.70	38.00	41.00
Limestone	11.00	11.00	11.00	11.00	11.20	9.50	9.50	9.50	9.54	9.80
L-lysine HCl	4.10	4.10	4.10	4.10	3.97	5.27	5.27	5.27	5.27	5.10
L-threonine	2.45	2.45	2.45	2.45	2.40	2.93	2.93	2.93	2.93	2.90
DL-methionine	2.07	2.07	2.07	2.07	2.00	2.18	2.18	2.18	2.18	2.12
L-tryptophan	0.48	0.48	0.48	0.48	0.40	0.54	0.54	0.54	0.54	0.48
L-valine	0.80	0.80	0.80	0.80	0.80	1.33	1.33	1.33	1.33	1.25
Salt	0.73	0.73	0.73	0.73	0.73	3.93	3.93	3.93	3.93	3.93
Vitamin premix[2]	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Mineral premix[3]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Zinc oxide 80%	---	3.10	---	---	---	---	2.48	---	---	---
Amoxicillin 50%	---	0.50	---	---	---	---	0.50	---	---	---
GutPlus	---	---	0.50	0.50	0.50	---	---	0.50	0.50	0.50
Cellulose	---	---	---	5.00	---	---	---	---	5.00	---
Inulin	---	---	---	5.00	---	---	---	---	5.00	---
Wheat bran	---	---	---	---	50.00	---	---	---	---	50.00
Calculated nutrient composition[4]										
NE, MJ/kg	10.51	10.51	10.51	10.51	10.51	10.53	10.53	10.53	10.53	10.53
CP, g/kg	215.00	215.00	215.00	215.00	218.00	198.70	198.70	198.70	198.70	198.70
Ca, g/kg	10.00	10.00	10.00	10.00	10.00	9.73	9.73	9.73	9.73	9.73
Available P, g/kg	5.28	5.28	5.28	52.8	5.28	4.81	4.81	4.81	4.81	4.81
Zn, mg/kg	50.00	2,530	50.00	50.00	50.00	50.00	2,034	50.00	50.00	50.00
SID Lys, g/kg	14.50	14.50	14.50	14.50	14.50	13.46	13.46	13.46	13.46	13.46
SID Met + Cys, g/kg	8.13	8.13	8.13	8.13	8.13	7.54	7.54	7.54	7.54	7.54
SID Thr, g/kg	9.72	9.72	9.72	9.72	9.72	9.02	9.02	9.02	9.02	9.02
SID Trp, g/kg	2.76	2.76	2.76	2.76	2.76	2.56	2.56	2.56	2.56	2.56
SID Val, g/kg	10.00	10.00	10.00	10.00	10.00	9.29	9.29	9.29	9.29	9.29
SID Ile, g/kg	7.98	7.98	7.98	7.98	7.98	7.45	7.45	7.45	7.45	7.45

[1] NC, negative control (without zinc oxide and amoxicillin); PC, positive control (with zinc oxide and amoxicillin); PRO, NC + 0.5 g/kg probiotic; PROCI, NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF; PROWB, NC + 0.5 g/kg probiotic + 50 g/kg wheat bran.
[2] Content per kilogram of product: selenium (75.0 mg), folic acid (250.0 mg), niacin (8,750.0 mg), biotin (37.50 mg), pantothenic acid (5,000.0 mg), vitamin A (2,000.0 IU), vitamin B_1 (500.0 mg), vitamin B_{12} (7,500.0 mcg), vitamin B_2 (1,500.0 mg), vitamin B_6 (500.0 mg), vitamin D_3 (375.000.0 IU), vitamin E (6,250.0 IU), vitamin K_3 (750.0 mg).
[3] Content per kilogram of product: Zn (25.0 g), Cu (40.0 g), Fe (15.0g), Mn (13.0 g), Cu (56.0 g), I (350.0 mg).
[4] Values calculated according to Rostagno et al. (2017).

Table 2. Forward and reverse primer sequences used for gene expression analysis of oligonucleotides.

Genes	GenBank number	Primer sequence	Size, bp
NF-kB	NM_001048232.1	F: 5´GAGGTGCATCTGACGTATTC3´ R: 5´AAGTTGCATGGCCTTCTC3’	169
IL-1β	NM_214055.1	F:5′TCTGCCCTGTACCCCAACTG3′ R:5′CCCAGGAAGACGGGCTTT3′	132
IL-6	NM_001252429.1	F:5′CCTGTCCACTGGGCACATAAC3′ R:5′CAAGAAACACCTGGCTCTGAAAC3′	253
IL-10	NM_214041.1	F:5′GAAGGACCAGATGGGCGACTT3′ R:5′CACCTCCTCCACGGCCCTTG3′	256
MCT1	AM286425.1	F: 5’GGTGGAGGTCCTATCAGCAG3’ R: 5’AAGCAGCCGCCAATAATCAT3’	74
SMCT2	XM_003122908.1	F: 5’AGGTCTACCGCTTTGGAGCAT3’ R:5’GAGCTCTGATGTGAAGATGATGACA3’	77
Hap	NM_214000.2	F:5'GCTAAGAATCTCCGCTTGG3' R:5'CAATCTCCACCTCCTGTTTC3'	100
TGF β1	NM_214015.1	F:5′GGACCTTATCCTGAATGCCTT3′ R:5′TAGGTTACCACTGAGCCACAAT3′	133
TNF-α	NM_214022.1	F:5′CATCGCCGTCTCCTACCA3′ R:5′CCCAGATTCAGCAAAGTCCA3′	199
ZO-1	XM_003353439.2	F:5′AAGCCCTAAGTTCAATCACAATCT3′ R:5′ ATCAAACTCAGGAGGCGGC3′	130
Occludin	NM_001163647.1	F:5′ TCCTGGGTGTGATGGTGTTC3′ R:5′ CGTAGAGTCCAGTCACCGCA3′	145
B2M	NM_213978.1	F: 5´TGAAAAACGGGGAGAAGATG3’ R: 5´GTGATGCCGGTTAGTGGTCT3’	189

Abbreviations: *NF-κB*, nuclear factor-κB; *IL-1β*, interleukin 1 beta; *IL-6*, interleukin 6; *IL-10*, interleukin 10; *MCT1*, Monocarboxylate transporter 1; *SMCT2*, Sodium-coupled monocarboxylate transporter 2; *Hap*, haptoglobin; *TGF β1*, transforming growth factor beta 1; *TNFα*, tumor necrosis factor alpha; *ZO-1*, zonula occludens-1; *B2M*, β-2-microglobulin.

Table 3. Growth performance of weaned pigs fed diets supplemented with probiotic and dietary type of fiber.[1]

Item	NC	PC	PRO	PROCI	PROWB	SEM[2] (*n* = 10)	*P*-value
Body weight, kg							
Day 0	7.192	7.194	7.194	7.210	7.202	0.872	0.995
Day 14	10.378 b	11.238 a	10.720 ab	10.744 ab	10.165 b	0.654	0.005
Day 28	18.509 b	20.924 a	19.583 ab	19.136 ab	18.397 b	0.550	0.002
Average daily gain, kg							
Days 0-14	0.228 b	0.289 a	0.259 ab	0.249 ab	0.212 b	0.020	0.006
Days 14-28	0.581 b	0.675 a	0.631 ab	0.591 ab	0.594 ab	0.026	0.019
Days 0-28	0.402 b	0.490 a	0.443 ab	0.424 ab	0.399 b	0.024	0.002
Average daily feed intake, kg							
Days 0-14	0.324	0.348	0.328	0.328	0.285	0.019	0.109
Days 14-28	0.818	0.897	0.885	0.834	0.818	0.028	0.110
Days 0-28	0.571	0.624	0.606	0.584	0.552	0.023	0.103
Feed conversion ratio, kg/kg							
Days 0-14	1.459 a	1.207 c	1.272 bc	1.323 abc	1.374 ab	0.054	<0.001
Days 14-28	1.418	1.330	1.409	1.414	1.380	0.029	0.134
Days 0-28	1.437 a	1.278 b	1.375 ab	1.380 ab	1.388 a	0.035	0.002

[a,b] Means followed by different lowercase letters in the lines differ ($P < 0.05$) from each other by Tukey test.

[1] NC, negative control (without zinc oxide and amoxicillin); PC, positive control (with zinc oxide and amoxicillin); PRO, NC + 0.5 g/kg probiotic; PROCI, NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF; and PROWB, NC + 0.5 g/kg probiotic + 50 g/kg wheat bran.

[2] SEM, standard error of the mean.

Table 4. Effect of supplementation with probiotic and dietary type of fiber on fecal scores and diarrhea incidence of weaned pigs from D0 to D14.[1]

Item	NC	PC	PRO	PROCI	PROWB	SEM[2] (*n* = 10)	*P*-value
Fecal score[3]	1.7 a	1.2 c	1.5 ab	1.4 bc	1.5 ab	0.066	<0.001
Diarrhea incidence (%)	25.0 a	4.5 c	14.0 b	10.9 bc	11.9 bc	2.565	<0.001

[a,b,c] Means followed by different lowercase letters in the lines differ ($P < 0.05$) from each other by Tukey test.

[1] NC, negative control (without zinc oxide and amoxicillin); PC, positive control (with zinc oxide and amoxicillin); PRO, NC + 0.5 g/kg probiotic; PROCI, NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF; and PROWB, NC + 0.5 g/kg probiotic + 50 g/kg wheat bran.

[2] SEM, standard error of the mean.

[3] Fecal score: 1 = solid; 2 = semi-solid; 3 = semi-liquid; and 4 = liquid.

Table 5. Effect of supplementation with probiotic and dietary type of fiber on intestinal morphology of weaned pigs on D14 (35 days of age).[1]

Item	NC	PC	PRO	PROCI	PROWB	SEM[2] (*n* = 10)	*P*-value
Duodenum							
VH, µm	408.54 b	495.73 a	457.95 ab	481.10 ab	432.55 ab	19.89	0.047
CD, µm	298.09 a	258.29 b	260.62 b	267.33 ab	272.58 ab	7.51	0.010
VH:CD	1.39 b	1.95 a	1.80 a	1.84 a	1.62 ab	0.08	<0.001
Jejunum							
VH, µm	388.94	408.29	450.13	415.06	457.43	23.21	0.182
CD, µm	184.49	166.99	159.13	165.27	163.20	7.71	0.083
VH:CD	2.14 b	2.50 ab	2.85 a	2.56 ab	2.87 a	0.13	0.004
Ileum							
VH, µm	284.64 c	398.16 a	326.69 bc	387.65 ab	365.77 ab	20.06	<0.001
CD, µm	161.21	138.01	148.44	154.39	142.62	5.59	0.069
VH:CD	1.79 c	3.02 a	2.26 bc	2.59 ab	2.63 ab	0.14	<0.001

[a,b,c] Means followed by different lowercase letters in the lines differ ($P < 0.05$) from each other by Tukey test.

[1] NC, negative control (without zinc oxide and amoxicillin); PC, positive control (with zinc oxide and amoxicillin); PRO, NC + 0.5 g/kg probiotic; PROCI, NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF; and PROWB, NC + 0.5 g/kg probiotic + 50 g/kg wheat bran.

[2] SEM, standard error of the mean.

Abbreviations: VH = Villous height; CD = Crypt depth.

Table 6. Effect of supplementation with probiotic and dietary type of fiber on relative gene expression in the jejunum of weaned pigs on D14 (35 days of age).[1]

Item	**NC**	**PC**	**PRO**	**PROCI**	**PROWB**	**SEM (n = 10)**	***P*-value**
NF-κB	1.855	2.084	1.865	2.083	2.190	0.282	0.898
IL-1β	2.861	2.835	2.883	2.826	3.151	0.380	0.979
IL-6	10.518	12.807	9.402	10.391	9.475	2.171	0.868
IL-10	2.936	3.510	3.241	3.465	4.295	0.433	0.368
MCT1	1.665	1.991	1.716	2.148	1.735	0.279	0.689
SMCT2	2.928	3.462	3.087	3.385	3.528	0.369	0.678
Hap	2.413	2.053	2.509	2.807	2.477	0.214	0.264
TGF β1	2.088	2.272	2.062	2.356	2.435	0.207	0.703
TNFα	3.246	2.861	2.621	2.750	3.075	0.269	0.526
ZO-1	1.617	2.081	2.059	1.973	1.795	0.168	0.384
Occludin	2.618	2.623	2.836	2.836	2.941	0.296	0.898

[a,b] Means followed by different lowercase letters in the lines differ ($P < 0.05$) from each other by Tukey test.

[1] NC, negative control (without zinc oxide and amoxicillin); PC, positive control (with zinc oxide and amoxicillin); PRO, NC + 0.5 g/kg probiotic; PROCI, NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF; and PROWB, NC + 0.5 g/kg probiotic + 50 g/kg wheat bran.

Abbreviations: *NF-κB*, nuclear factor-κB, *IL-1β*, interleukin 1 beta; *IL-6*, interleukin 6; *IL-10*, interleukin 10; *MCT1*, Monocarboxylate transporter 1; *SMCT2*, Sodium-coupled monocarboxylate transporter 2, *Hap*, haptoglobin; *TGF β1*, transforming growth factor beta 1; *TNFα*, tumor necrosis factor alpha; *ZO-1*, zonula occludens-1; and SEM, standard error of the mean.

Table 7. Effect of supplementation with probiotic and dietary type of fiber on volatile fatty acids (VFA) millimolar (mM) concentration in digesta of weaned pigs on D14 (35 days of age).[1]

Item	NC	PC	PRO	PROCI	PROWB	SEM[2] ($n = 10$)	*P*-value
Cecum							
Acetate (mM)	38.894 ab	36.123 b	43.542 ab	46.767 a	37.851 ab	2.482	0.029
Propionate (mM)	21.414	17.638	19.077	21.989	20.023	4.159	0.324
Isobutyrate (mM)	0.908	0.652	0.847	0.804	0.908	0.157	0.631
Butyrate (mM)	9.330	8.834	9.979	10.196	9.218	1.224	0.944
Isovalerate (mM)	1.299	1.019	1.196	1.197	1.373	0.319	0.818
Valerate (mM)	1.460	1.172	1.143	1.197	1.532	0.189	0.440
Total VFA (mM)	72.442	67.885	73.557	82.276	64.943	7.417	0.166
Colon							
Acetate (mM)	23.728	22.725	19.635	23.357	18.574	2.959	0.376
Propionate (mM)	9.353	8.431	7.379	9.569	6.934	1.132	0.241
Isobutyrate (mM)	0.694	0.594	0.506	0.636	0.492	0.104	0.303
Butyrate (mM)	8.015	6.994	5.775	7.727	5.713	0.980	0.113
Isovalerate (mM)	1.202	1.003	0.835	1.016	0.823	0.214	0.306
Valerate (mM)	1.044	0.749	0.660	0.827	0.602	0.178	0.221
Total VFA (mM)	43.282	40.494	34.788	43.131	33.138	5.317	0.296

[a,b] Means followed by different lowercase letters in the lines differ ($P < 0.05$) from each other by Tukey test.

[1] NC, negative control (without zinc oxide and amoxicillin); PC, positive control (with zinc oxide and amoxicillin); PRO, NC + 0.5 g/kg probiotic; PROCI, NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF; and PROWB, NC + 0.5 g/kg probiotic + 50 g/kg wheat bran.

[2] SEM, standard error of the mean.

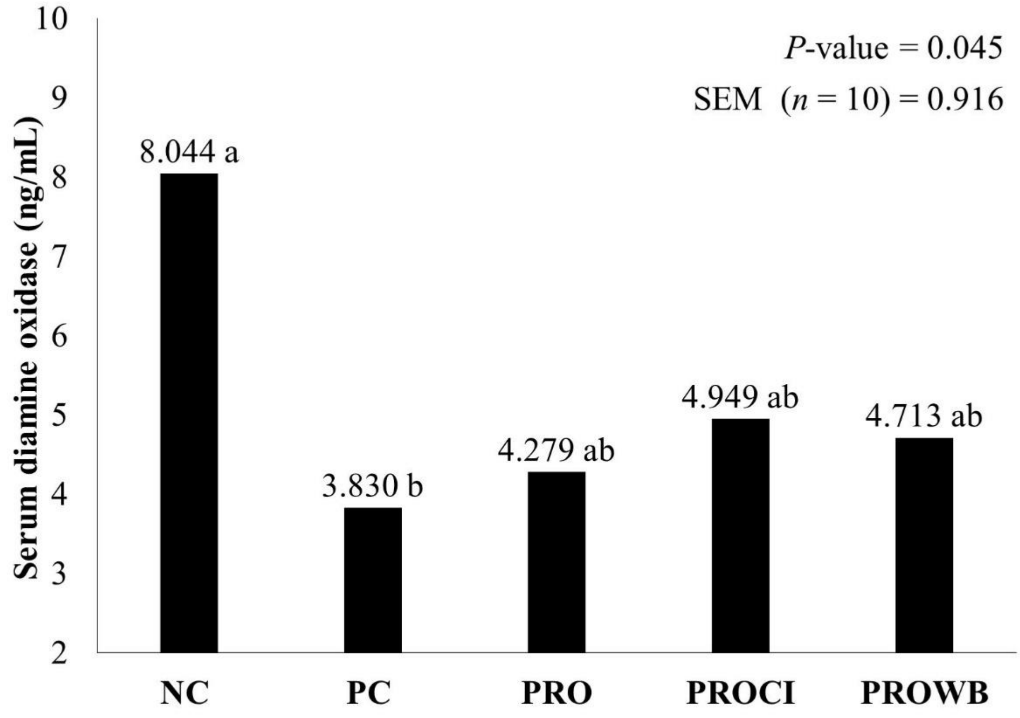

Fig. 1. Effect of dietary type of fiber and probiotic on serum diamine oxidase (DAO) concentration of piglets at D14 (35 days of age). [a,b] Means followed by different lowercase letters in the lines differ ($P < 0.05$) from each other by Tukey test. Abbreviations: NC, negative control (without zinc oxide and amoxicillin); PC, positive control (with zinc oxide and amoxicillin); PRO, NC + 0.5 g/kg probiotic; PROCI, NC + 0.5 g/kg probiotic + 5 g/kg cellulose as IDF and 5 g/kg inulin as SDF; PROWB, NC + 0.5 g/kg probiotic + 50 g/kg wheat bran; SEM, standard error of the mean.

Ethics Committee Certificate

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

Campus Universitário – Viçosa, MG – 36570-900 – Telefone:(31) 3899.3275 – e-mail: ceuap@ufv.br – site: www.ceuap.ufv.br

Viçosa, 01 de abril de 2021

CERTIFICADO

Certificamos que o projeto intitulado **"Efeito do tipo de fibra e probiótico no desempenho e na saúde intestinal de leitões dos 21 aos 49 dias de idade "**, protocolo **nº 05/2021**, sob a responsabilidade de **Alysson Saraiva,** que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi apreciado pela Comissão de Ética no Uso de Animais de Produção da Universidade Federal de Viçosa (CEUAP-UFV) em reunião de **01 de abril de 2021.**

Finalidade: (x)Pesquisa
Vigência do Projeto: de Maio de 2021 a Julho de 2021
Espécie/linhagem: Porcos/Suínos (Sus scrofa domesticus)
Nº de animais: 150 **Peso: +/- 6 kg** **Idade: 21 dias** **Sexo: Macho/Femea**
Origem:Setor Suinocultura - DZO /UFV - CNPJ/CPF: 25.944.455/0001-96

CERTIFICATE

We certify that the project entitled **"Effect of dietary type of fiber and probiotic on performance and gut health of piglets from 21 to 49 days of age "**, protocol **nº 05/2021**, under the responsibility of **Alysson Saraiva -** which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **April 01st of 2021.**

Finality: (x)Research
Duration of the Project: from May of 2021 to July of 2021
Species / strain: Pig/Swine (Sus scrofa domesticus) **Nº of animals: 150**
Weight: +/- 6 kg **Age: 21 days** **Sex: Male/ Female**
Source: Setor Suinocultura - DZO /UFV - CNPJ/CPF: 25.944.455/0001-96

Luciana Navajas Rennó
Coordenadora da CEUAP/UFV

CHAPTER 2: Symbiotic and organic acids as alternative to zinc oxide in diets for weaned piglets

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ABSTRACT

This study investigated the effects of feed additive (FA) containing symbiotic and organic acids on performance and intestinal health of weaned piglets. A total of 288 piglets [body weight (BW) = 6,53 ± 1,04 kg] were assigned to one of four treatments in a randomized block design, with nine replicates and eight piglets per pen. The treatments were NC (without ZnO), PC (with ZnO), FA1 (NC+1.0 kg/ton FA), and FA2 (NC+2.0 kg/ton FA). Piglets fed FA2 had the lowest ($P = 0.01$) gain:feed (G:F) between 21 and 35 days, while piglets fed FA1 and NC had similar G:F to those fed PC. Piglets fed FA2 had the lowest ($P = 0.04$) BW at 35 and 70 days, and those fed FA1 and NC had similar BW compared to PC group. Piglets fed NC had the highest ($P = 0.03$) fecal score, while no significant difference was observed among groups PC, FA1, and FA2. Piglets fed NC had the lowest ($P = 0.03$) jejunal villous height (VH), whereas VH of piglets fed FA1 and FA2 were comparable to those fed PC. Piglets fed FA1 had higher ($P = 0.03$) *occludin* expression compared to those fed NC, while piglets fed PC and FA1 had higher ($P < 0.01$) *TGF-β1* expression compared to piglets fed FA2. In conclusion, although there were no discernible performance benefits, inclusion of 1.0 kg/ton of FA to diet without ZnO helps

maintain gut health of piglets by improving intestinal morphology and epithelial integrity and stimulating immune system inactivation.
Keywords: immune response; intestinal morphology; organic acids; piglets; symbiotic.

1. Introduction

Weaning is the most stressful event experienced by pigs on commercial farms, which result in an imbalance of the intestinal microbiota, increasing the susceptibility of piglets to enteric pathogens (Alexopoulos et al. 2004) and causing acute activation of the immune system, intestinal morphological damage and barrier dysfunction with consequent diarrhea, poor performance and increased mortality rate (Estrada et al. 2001; Pié et al. 2004; Moeser et al. 2017; Wen et al. 2018).

In order to prevent gastrointestinal disorders and improve performance of weaned piglets, the use of dietary zinc oxide (ZnO) as growth-promoter has been done regularly (Dębski 2016; Satessa et al. 2020). Despite the proven ability to improve intestinal health, the use of pharmacological doses (2,500 to 3,000 ppm) of ZnO has stimulated restrictions/bans in several countries as it can result in resistant bacteria and environmental contamination (Slifierz et al. 2015; Jensen et al. 2016). In this way, many researchers have suggested the use of probiotics, prebiotics and organic acids (OA) as potential alternatives to ZnO to maintain intestinal health (Wang W et al. 2018; San Andres et al. 2019; Pearlin et al. 2020; Valente Júnior et al. 2023).

Probiotics may inhibit the growth of pathogenic bacteria by several pathways, such as competition for nutrients and binding sites in gut mucosa; and production of antibacterial substances (Dong et al. 2014). In other hand, prebiotics are substrates that improve the growth of probiotics bacteria, reduce the adhesion of pathogenic bacteria in the epithelium and regulate the immune response (Gibson et al. 2017). Therefore, an appropriate combination of both additives (symbiotic) in the diets without ZnO may promote a superior effect on intestinal health in weaned piglets, compared to diets formulated with only probiotic or prebiotic (Markowiak and Śliżewska 2018).

Organic acids, e.g. butyric and citric acids, have been widely studied due to their bactericidal and bacteriostatic actions (Long et al. 2018; Han et al. 2020; Xu et al. 2020). Thus, OA may enhance the positive effects of prebiotics and probiotics (Bomba et al. 2002; Ren et al. 2019; Salah et al. 2019). However, there is limited information about the synergistic effect of the simultaneous inclusion of these three dietary additives for weaned piglets on intestinal health, inflammatory response and performance.

Hence, our hypothesis is that the addition of symbiotic and OA in the diet replaces ZnO as growth-promoter, improves performance, morphology and epithelial integrity of the intestine besides decreasing immune system activation of piglets weaned at 21 days of age. Therefore, the present study was carried out to evaluate the effects of dietary ZnO replacement by symbiotic and OA on performance, intestinal morphology and gene expression associated to epithelial integrity and activation of the immune system of weaned piglets from 21 to 70 days of age.

2. Material and methods

All methods involving the handling of pigs followed the ethical principles of animal research (CONCEA) and were approved by the Commission of Ethics in the Use of Production Animals (CEUAP) of the Universidade Federal de Viçosa (protocol 03/2020).

2.1. Feed additive (FA) description

Composed of a combination of probiotics (*Bacillus subtillis* and *Bacillus licheniformis*), prebiotic [chicory (*Cichorium intybus*) root extract] and OA (citric and butyric acids).

2.2. Animals, experimental design, housing and diets

A total of 288 castrated male and female piglets (Agroceres PIC) weaned at 21 days of age with initial body weight (BW) of 6,53 ± 1,04 kg were assigned to a randomized block design according to the initial BW, in 4 diets, with 9 replicates and 8 piglets per experimental unit (4 males and 4 females), represented by the cage.

The experimental diets (Tables 1 and 2) were formulated to meet the nutritional requirements of pigs during the 3 phases of nursery period (21 to 35, 36 to 49 and 50 to 70 days), as recommended by Rostagno et al. (2017). The diets consisted of: negative control (NC, without ZnO as growth-promoter); positive control (PC, with ZnO as growth-promoter); Feed additive 1 (FA1, NC + 1,0 kg/ton of feed additive); and Feed additive 2 (FA2, NC + 2,0 kg/ton of feed additive). In the last phase (50 to 70 days), ZnO was not added to PC diet in pharmacological dose.

Piglets were housed during the experimental period of 49 days in metal cages, equipped with feeders and nipple drinkers, in climatic-controlled nursery rooms in which ambient temperature was maintained within the thermoneutral zone by the use of furnace-automated system. During the experimental period, average ambient temperature was 27.6ºC ± 1.6ºC, minimum ambient temperature 24.6 ± 1.8ºC and maximum ambient temperature 31.3 ± 1.6ºC.

Piglets had free access to feed and water throughout the experimental period (21 to 70 days of age). During the trial, feed was weighed before feeding and feed wastage and leftovers were collected and weighed daily to determine average daily feed intake (ADFI). Piglets were individually weighed at 21, 35, 49 and 70 days of age (end of the experimental period) to determine BW, average daily gain (ADG), and gain:feed (G:F).

2.3. Fecal score and diarrhea incidence

Fecal score of each pig was visually assessed each morning by only one observer, during the first phase of the experimental period, 21 to 35 days of age, using the method described by Liu P et al. (2010). Fresh excreta were ranked using the following scale: 1 = solid; 2 = semi-solid; 3 = semi-liquid; and 4 = liquid. The occurrence of diarrhea was defined as production of feces at level 3 or 4 for 2 continuous days. *Diarrhea incidence (%)* = [*number of pigs with diarrhea in each pen* × *diarrhea days* ÷ (*8 pigs* × *14 d*)] × *100*.

2.4. Slaughter procedures and intestinal samples collection

At 35 days of age, one piglet from each experimental unit, with average BW closest to the average weight of the piglets from its respective cage, was electrically stunned followed by exsanguination to collect histological samples. Segments of 2.0 × 2.0 cm of the intestine were sampled corresponding to duodenum (10 cm from pylorus), jejunum (the middle portion) and ileum (5 cm proximal to the ileocecal junction), according to Yang et al. (2014). Histological sections were then washed in physiological solution and fixed in 4.0% paraformaldehyde solution for 24 h at room temperature.

2.5. Sample collection for gene expression analysis

In order to collect jejunum samples for gene expression of cytokines and tight junctions proteins, instruments that came into contact with the tissue were sanitized with alcohol 70%, dried with paper towel and sprayed with RNAse Exterminator (Protech Technology Enterprise CO., Ltd., Taipei, Nanking Dist., Taiwan) between each sampling. The portion of the sampled jejunum was washed with sterile saline solution for removal of the digesta in contact with the tissue. From the tissue sample, about ten 0.25 cm^3 subsamples were collected, washed individually in sterile saline solution serum, placed in cryotubes with a capacity of 1.8 mL, frozen instantaneously in liquid nitrogen, and stored at - 80°C for further analysis.

2.6. Intestinal morphology assessment

The histological sections (duodenum, jejunum and ileum) were cross-sectionally cut and dried in ethyl crescent gradients, diaphanized in HistoChoice (Clearing Agent, Sigma-Aldrich, Oakville, ON, Canada), and embedded in liquid Paraplast (Sigma-Aldrich, Barueri, SP, Brazil) at 65°C. Five transverse cuts with 5 µm thickness each were placed per slide, and were stained with hematoxylin and eosin. The cuts were semi-serial using 1 in 10 cuts. For morphological readings, an optical microscope EVOS M5000 Imaging System (Invitrogen, Thermo Fisher Scientific) was used with 10x magnification. Afterward, the images were analyzed by image analyzer ImageJ 1.50i; java1.6.0_20 (National Institutes of Health). Heights of 20 villi and their 20 crypts were selected and measured according to Satessa et al. (2020). Villus to crypt ratios (VCR) using the length data were then calculated. All measurements were made by a single individual.

2.7. Gene expression analysis

Total RNA extraction was performed using TRIzol (Thermo Fisher Scientific, Burlington, ON, Canada) following the manufacturer instructions. The RNA concentration was estimated by NanoDrop Lite (Thermo Fisher Scientific), and RNA integrity was evaluated through 1% agarose gel electrophoresis. Complementary DNA synthesis was performed according to GoScript Reverse Transcription System protocol (Promega Corporation). GenBank numbers to access the primers of the genes are shown in Table 3. Primers were used for reverse transcription quantitative PCR with GoTaq qPCR Master Mix (Promega, Madison, WI) in QuantStudio 3 (Applied Biosystems, Thermo Fisher Scientific). Geometric means of Ct values of β-2-microglobulin (*B2M*) were used to normalize target genes expression for the jejunum samples. Gene of interest relative expression was calculated by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

2.8. Statistical analysis

The cage was considered the experimental unit for performance, fecal score and diarrhea incidence analysis. One piglet per pen, slaughtered at 35 days of age, was considered the experimental unit for intestinal morphology and gene expression analysis. Data were analyzed using the following model:

$$Y_{ijk} = \mu + T_i + \beta_j + e_{ijk}$$

where Y_{ijk} is the observed variable of k^{th} animal in the i^{th} treatment and j^{th} block; μ is the overall mean; T_i is the fixed effect of the i^{th} treatment; β_j is the fixed effect of the j^{th} block effect; and e_{ijk} is the random residual associated with Y_{ijk}, assuming $e_{ijk} \sim (0, \sigma_e^2)$. Data were analyzed using the ExpDes.pt package of software R Core Team (2020), version 4.1.0. Means were compared by Tukey test. For all statistical analysis probability values less than 0.05 were considered significant and probability values between 0.05 and 0.10 were considered as trend.

3. Results

3.1. Performance, diarrhea incidence and fecal score

There were no effects ($P > 0.05$) of ZnO and FA diets on ADG and ADFI during the nursery period (Table 4). The diets did not influence ($P > 0.05$) the G:F of the piglets from 21 to 49 and 21 to 70 days of age. However, from 21 to 35 days of age, piglets fed FA2 had the lowest ($P = 0.01$) G:F, while piglets fed FA1 and NC had similar G:F to those fed PC. No effect (*P* > 0.05) of the diets was observed on BW of the piglets at 49 days of age. However, piglets fed FA2 showed the lowest (*P* = 0.04) BW at 35 and 70 days, and those fed FA1 and NC had similar BW compared to PC group.

From 21 to 35 days old, no dietary effect (*P* > 0.05) was observed on diarrhea incidence (Fig. 1). The fecal score was highest (*P* = 0.03) among piglets fed NC, while no difference in fecal score was observed among groups PC, FA1, and FA2

3.2. Intestinal morphology

There were no differences (*P* > 0.05) in duodenum for villous height (VH), crypt depth (CD) and VCR (Fig. 2). In jejunum, the diets did not alter the CD and VCR values, however, piglets fed NC exhibited the lowest (*P* = 0.03) jejunal villous height (VH), whereas the VH of piglets fed FA1 and FA2 were comparable to those fed PC (Fig. 2d and Fig. 3). It was observed a trend (*P* = 0.09) on ileum VCR (Fig. 2i), and piglets fed NC had the lowest value of ileum VCR, and no difference was observed among groups PC, FA1 and FA2. No effects (*P* > 0.05) were observed in ileum VH and CD values.

3.3 Gene expression

No differences were observed (*P* > 0.05) in jejunum mRNA expression for zonula occludens-1 (*ZO-1*), tumor necrosis factor alpha (*TNF-α*), nuclear factor-κB (*NFκ*B), interleukin 10 (*IL-10*) and *haptoglobin* genes (Fig. 4). Piglets fed FA1 diet had higher (*P* = 0.03) mRNA expression of *occludin* compared to those fed NC diet, and piglets in PC and FA2 groups had intermediate relative expression of *occludin*. Piglets in PC and FA1 groups had higher (*P* < 0.01) mRNA expression of transforming growth factor beta 1 (*TGF-β1*) in jejunum compared to piglets fed FA2 diet. Piglets fed NC diet had intermediate relative expression of *TGF-β1*.

4. Discussion

Although ZnO have been shown in the current study and previously to improve piglet performance, intestinal morphology, epithelial barrier function, and reduce diarrhea (Wang C et al. 2017; Zhu et al. 2017; Pei et al. 2019; Sun et al. 2019), its pharmacological doses use can lead to the emergence of antibiotic-resistant bacteria and environmental contamination. This has prompted the search for alternative feed additives to meet the growing demand for safer options.

Symbiotics and OA have been widely studied as alternatives to ZnO in the diet to minimize the intestinal disorders caused by weaning (Bonetti et al. 2021). Chicory root extract (*Cichorium intybus* L.) contain inulin-type fructan and oligofructose, which are used as energy source by acidophilic bacterial communities in the gastrointestinal tract, stimulating its growth and the production of short chain fatty acids (SCFA) in weaned piglets (Modesto et al. 2009; Liu H et al. 2012; Uerlings et al. 2021). Probiotics such as, *Bacillus subtilis* and *Bacillus licheniformis* competes with pathogens for nutrients and binding sites in gut mucosa, produces antibacterial substances and regulates the immune response and barrier function of piglets (Cao et al. 2019; Wang X et al. 2021; Sampath et al. 2022; Valente Júnior et al. 2023). Therefore, an appropriate combination of chicory root extract, *Bacillus subtilis* and *Bacillus licheniformis* may promote a superior effect on intestinal health and performance of weaned piglets.

In addition, butyric and citric acids, have been studied for weaned piglets due to their bactericidal and bacteriostatic actions, which inhibit the growth of pathogenic bacteria and improve intestinal function (Grilli et al. 2016; Feng et al. 2018; Deng et al. 2021). However, there is limited information about the synergistic effect of the simultaneous inclusion of symbiotic and OA for weaned piglets on intestinal health, inflammatory response and growth performance.

Our results showed that the dietary supplementation of FA did not improve the piglets’ performance variables. Furthermore, the inclusion of 2 kg/ton of FA reduced the BW of piglets at 45 and 70 days of age and the G:F during the first two weeks of the experimental period.

According to Liu Yanhong et al. (2018), the effectiveness of dietary additives for weaned piglets can be influenced by several factors, including the animals' health status and the level of sanitary challenges. Our study was conducted at a research facility with high sanitary standards, which likely contributed to the overall good performance of the piglets. Therefore, the impact of feed additive (FA) supplementation on performance may have been diminished, as the piglets were not exposed to poor sanitary conditions. However, the reasons for the negative effects of FA2 on performance is unclear.

Organic acids, especially butyric acid, present an offensive odor and lower palatability that may reduce the feed intake of the piglets (Bedford and Gong 2018; Deng et al. 2021). In this way, even though with no significant difference in ADFI during the experimental period, piglets may have initially rejected the feed with higher FA content (2.0 kg/ton), leading to reduce the BW and G:F from 21 to 35 days old.

Evaluating the oral administration of probiotics for weaned piglets challenged with *Escherichia coli*, Zhang et al. (2017) reported that overconsumption of *Bacillus subtilis* and *Bacillus licheniformis* may disrupt the colonic microbial ecology, leading to an expansion of *Proteobacteria* and impaired goblet cell function in the ileum. Therefore, this could be another possible factor contributing to the negative impact of the FA2 diet on piglet performance. Additionally, the inclusion of chicory root extract in the diet may have resulted in an overgrowth of probiotics in the intestine, leading to nutrient competition with the host and reduced growth of the piglets (Rodríguez-Sorrento et al. 2020).

Although FA did not improve the piglets' performance, adding FA to their diet led to fecal score values similar to those of piglets fed with ZnO as a growth promoter. This suggests that the supplementation of FA may enhance the intestinal morphology and epithelial integrity of weaned piglets.

Villous height is positively correlated to enzyme activity and surface area for digestion and absorption of nutrients, being an indicator of intestinal health (Wang M et al. 2020). However, weaning is characterized by increased intestinal permeability and villous atrophy (Hu et al. 2013), leading to poor piglet performance. Regarding this matter, piglets that received a supplement of 1.0 or 2.0 kg/ton showed comparable jejunum VH and ileum VCR to the PC group. These results are consistent with the fecal score findings and suggest that the addition of FA can mitigate the damage to the intestinal epithelium caused by weaning in the absence of ZnO as a growth promoter in the diet. Therefore, the enhancement of intestinal morphology can be attributed to at least two factors.

Firstly, these additives beneficially modulate an intestinal microbiota. Symbiotics supplementation can favor the growth of acidophilic bacteria and control the pathogens in the gastrointestinal tract in weaned piglets through competition of nutrients and binding sites in gut mucosa, reduction of intestinal pH and production of antibacterial substances (Mair et al. 2010; Wang W et al. 2018; López-Gálvez et al. 2021). In addition, OA have bactericidal and bacteriostatic actions, which inhibit the growth of pathogenic bacteria (Long et al. 2018; Han et al. 2020). As bactericidal, OA can penetrate as non-dissociated form into pathogenic bacteria cells altering its metabolism, and as a bacteriostatic component, the OA dissociate in the gastrointestinal tract and reduce the pH environment by releasing hydrogen ions (Nguyen et al. 2020). Therefore, the dietary supplementation of OA and symbiotics could reduce the intestine colonization by pathogens and the turnover of intestinal epithelial cells, resulting in greater VH and VCR.

Secondly, studies with inulin, *Bacillus subtilis* and *Bacillus licheniformis* supplementation in diets of weaned piglets reported increased production of SCFA in the intestine (Cao et al. 2019; Wang W et al. 2020; Wang X et al. 2021). Among SCFA, butyrate is associated with increased secretion of glucagon-like peptide-2, which plays important role in proliferation, differentiation, and reduction of enterocytes apoptosis (Liu Y. et al. 2013; Sigalet et al. 2017). In addition, butyrate is the main source of energy for colonocytes, being important to cell proliferation (Singh et al. 1997). Therefore, besides containing butyric acid in its composition, FA1 and FA2 diets may have contributed to a greater SCFA concentration in the intestine, resulting in better intestinal morphology.

Weaning is associated with an increased inflammatory response, contributing to intestinal barrier disruption through the positive regulation of pro-inflammatory cytokines and acute-phase proteins (Pié et al. 2004; Sauerwein et al. 2005; Hu et al. 2013). In the present study, no dietary effects on the mRNA expression of pro-inflammatory markers (*TNF-α*, *NFκb*, and *haptoglobin*) were observed. However, the piglets fed PC and FA1 diets had greater gene expression of *TGF-β1*.

The effect of ZnO and FA1 on the gene expression of *TGF-β1* is probably due to the stimuli of probiotics growing and SCFA production, which stimulates regulatory T cells to produce *TGF-β1* (Licciardi et al. 2012; Levy et al. 2017).

Tight junction proteins (TJ) such as *occludin* and *ZO-1* are responsible to keep the barrier function of the intestinal cells and prevent the passage of bacteria, toxins and antigens to subepithelial tissues, maintaining the integrity and functionality of the intestinal epithelium (He et al. 2017). However, weaning increases the corticotropin releasing factor release by the hypothalamus, which through mast cells activation and proliferation reduces the TJ expression and epithelial integrity of piglets (Moeser et al. 2007; Smith et al. 2010; Hu et al. 2013).

Our results showed no dietary effects on the mRNA abundance of *ZO-1*. However, piglets fed FA1 diets had the highest mRNA abundance of *occludin*, and the animals that consumed the PC and FA2 diets presented intermediate expression of *occludin*. These results suggest that the supplementation of FA and ZnO in the diets increased the barrier function of the intestinal epithelium, which is consistent with the improvements on fecal score and intestinal morphology in the present study.

Possibly the greater abundance of *occludin* mRNA in the current study may be related to two factors. First, *TGF-β1* attenuates the immune response, reducing the production of pro-inflammatory cytokines that promote intestinal barrier disruption (Al-Sadi et al. 2009; Xiao et al. 2017). Furthermore, *TGF-β1* is responsible for regulate the activation and proliferation of mast cells in the epithelium (Gebhardt et al. 2005; Biancheri et al. 2014). Thus, the higher gene expression of *TGF-β1* observed in piglets fed FA1 and PC diets may explain the increased *occludin* expression in the jejunum. Second, as mentioned above, dietary FA supplementation may increase the SCFA content in the intestine. Among the SCFA, butyrate can inhibit mast cell activation through the JNK signaling pathway, improving the intestinal integrity (Wang CC et al. 2018). Therefore, besides the butyric acid presented in the FA1 and FA2 diets, the supposed SCFA production in the intestine promoted by the FA may have contributed to greater *occludin* expression.

5. Conclusion

Although there were no discernible performance benefits, the addition of 1.0 kg/ton of FA (mix of symbiotics and OA) to the diet of post-weaned piglets without ZnO as a growth promoter helps maintain gut health by improving intestinal morphology and epithelial integrity, as well as stimulating immune system inactivation. As such, FA may serve as a viable substitute for ZnO as a growth promoter, mitigating the adverse impacts of weaning.

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Table 1. Composition of experimental diets from 21 to 35 days of age (as-fed basis).[1]

Ingredient, %	NC	PC	FA1	FA2
Corn	33.50	33.14	33.40	33.30
Soybean meal	14.50	14.50	14.50	14.50
Soybean oil	2.00	2.000	2.00	2.00
Feed concentrate 50%[2]	50.00	50.00	50.00	50.00
Zinc oxide 80%	-----	0.36	-----	-----
Feed Additive	-----	-----	0.10	0.20
Nutritional composition[3]				
ME, kcal/kg	3,398	3,391	3,395	3,391
Crude protein, %	21.110	21.065	21.104	21.097
Calcium, %	0.839	0.839	0.839	0.839
Available P, %	0.554	0.554	0.554	0.554
Zn, mg/kg	110.00	3,000	110.00	110.00
SID Lys, %	1.402	1.402	1.402	1.402
SID Met +Cys, %	0.785	0.785	0.784	0.784
SID Thr, %	0.939	0.939	0.939	0.939
SID Trp, %	0.267	0.267	0.267	0.267
SID Val, %	0.968	0.968	0.967	0.967
SID Ile, %	0.769	0.767	0.769	0.768

[1] Negative control (NC, without zinc oxide as growth-promoter); positive control (PC, with zinc oxide as growth-promoter); Feed additive 1 (FA1, NC + 1,0 kg/ton of feed additive); and Feed additive 2 (FA2, NC + 2,0 kg/ton of feed additive).

[2] Content per kilogram of product: folic acid (3.29 mg), glutamic acid (17.30 g), pantothenic acid (55.00 mg), biotin (0.44 mg), calcium (13.50 g), copper (44.0 mg), choline (2.0 mg), ether extract (55 g), iron (220.0 mg), crude fiber (20.0 g), phytase (4,000.0 FTU), phosphorus (8,000.0 mg), iodine (0.59 mg), lysine (19.3 g), L-glutamine (2,500.0 mg), manganese (66.0 mg), mineral matter (150 g), methionine (6,500.0 mg), niacin (165.0 mg), nucleotides (1950.0 mg), crude protein (230.0 g), selenium (0.60 mg), sodium (7,000.0 mg), threonine (11.8 g), tryptophan (3,100.0 mg), humidity (130.0 g), valine (10.90 g), vitamin A (17,600.0 IU), vitamin B12 (77.0 mcg), vitamin B2 (16.49 mg), vitamin B6 (4.39 mg), vitamin D3 (4400.0 IU), vitamin E (132.0 IU), vitamin K (8.8 mg), zinc (220.0 mg), chlorohydroxyquinoline (240.0 mg).

[3] Values calculated according to Rostagno et al. (2017).

Table 2. Composition of experimental diets from 36 to 70 days of age (as-fed basis).[1]

Ingredient, %	Phase II (36 to 49 days of age)				Phase III (50 to 70 days of age)			
	NC	PC	FA1	FA2	NC	PC	FA1	FA2
Corn	47.40	47.10	47.30	47.20	58.50	58.50	58.40	58.30
Soybean meal	29.00	29.00	29.00	29.00	34.00	34.00	34.00	34.00
Soybean oil	3.60	3.60	3.60	3.60	3.50	3.50	3.50	3.50
Feed concentrate 20%[2]	20.00	20.00	20.00	20.00	-----	-----	-----	-----
Feed concentrate 4%[3]	-----	-----	-----	-----	4.00	4.00	4.00	4.00
Zinc oxide 80%	-----	0.30	-----	-----	-----	-----	-----	-----
Feed aditive	-----	-----	0.10	0.20	-----	-----	0.10	0.20
Nutritional composition[4]								
ME, kcal/kg	3,352	3,351	3,349	3,346	3,318	3,318	3,314	3,311
Crude protein, %	20.28	20.24	20.27	20.27	21.08	21.08	21.07	21.07
Calcium, %	0.901	0.901	0.901	0.901	0.780	0.780	0.780	0.780
Available P, %	0.489	0.489	0.489	0.489	0.404	0.404	0.404	0.404
Zinc, mg/kg	82.48	2,500	82.48	82.48	82.74	82.74	82.74	82.74
SID Lys, %	1.378	1.378	1.378	1.378	1.342	1.342	1.342	1.342
SID Met + Cys, %	0.784	0.784	0.784	0.784	0.765	0.765	0.765	0.765
SID Thr, %	0.897	0.897	0.897	0.896	0.872	0.872	0.872	0.872
SID Trp, %	0.261	0.261	0.261	0.261	0.255	0.255	0.255	0.255
SID Val, %	0.952	0.952	0.951	0.951	0.926	0.926	0.926	0.925
SID Ile, %	0.787	0.785	0.787	0.786	0.813	0.813	0.813	0.813

[1] Negative control (NC, without zinc oxide as growth-promoter); positive control (PC, with zinc oxide as growth-promoter); Feed additive 1 (FA1, NC + 1,0 kg/ton of feed additive); and Feed additive 2 (FA2, NC + 2,0 kg/ton of feed additive).

[2] Content per kilogram of product: folic acid (6.15 mg), pantothenic acid (103 mg), biotin (0.82 mg), calcium (35.0 g), copper (385.0 mg), choline (1,800.0 mg), ether extract (40.0 g), iron (400.0 mg), crude fiber (20 g), phytase (10,000.0 FTU), phosphorus (14.0 g), iodine (1.12 mg), lysine (27.0 g), manganese (176.0 mg), mineral matter (220.0 g), methionine (13.0 g), niacin (309.0 mg), crude protein (150.0 g), selenium (1.12 mg), sodium (14.50 mg), threonine (17.0 g), tryptophan (3,400.0 mg), humidity (130.0 g), valine (13.4 g), vitamin A (32,990.0 IU), vitamin B12 (144.0 mcg), vitamin B2 (30.50 mg), vitamin B6 (8.24 mg), vitamin D3 (8,240.0 IU), vitamin E (247.0 IU), vitamin K (16.49 mg), zinc (412.4 mg), chlorohydroxyquinoline (600.0 mg).

[3] Content per kilogram of product: folic acid (31.0 mg), pantothenic acid (517.30 mg), biotin (4.125 mg), calcium (16.00 g), copper (1,985.0 mg), choline (9,006.0 mg), phytase (50,000.0 FTU), iron (2,000.0 mg), phosphorus (48.25 g), fluorine (482.50 mg), iodine (5.60 mg), lysine (88.50 g), manganese (620.00 mg), methionine (44.70 g), niacin (1,547.0 mg), selenium (5.60 mg), sodium (48.12 g), threonine (56.0 g), tryptophan (6,460.0 mg), vitamin A (165,000.0 IU), vitamin B12 (722.0 mcg), vitamin B2 (155.0 mg), vitamin B6 (41.25 mg), vitamin D3 (41,000.0 IU), vitamin E (1,241.0 IU), vitamin K (82.50 mg), zinc (2,068.5 mg).

[4] Values calculated according to Rostagno et al. (2017).

Table 3. Oligonucleotides used on gene expression analysis.

Genes[1]	GenBank number	Sequence	Size, bp
B2M	NM_213978.1	F: 5´TGAAAAACGGGGAGAAGATG3’ R: 5´GTGATGCCGGTTAGTGGTCT3’	189
TNF-α	NM_214022.1	F:5′CATCGCCGTCTCCTACCA3′ R:5′CCCAGATTCAGCAAAGTCCA3′	199
IL-10	NM_214041.1	F:5′GAAGGACCAGATGGGCGACTT3′ R:5′CACCTCCTCCACGGCCCTTG3′	256
NF-κB	NM_001048232.1	F: 5´GAGGTGCATCTGACGTATTC3´ R: 5´AAGTTGCATGGCCTTCTC3’	169
Hap	NM_214000.2	F:5'GCTAAGAATCTCCGCTTGG3' R:5'CAATCTCCACCTCCTGTTTC3'	100
TGF-β1	NM_214015.1	F:5′GGACCTTATCCTGAATGCCTT3′ R:5′TAGGTTACCACTGAGCCACAAT3′	133
Occludin	NM_001163647.1	F:5′ TCCTGGGTGTGATGGTGTTC3′ R:5′ CGTAGAGTCCAGTCACCGCA3′	145
ZO-1	XM_003353439.2	F:5′AAGCCCTAAGTTCAATCACAATCT3′ R:5′ ATCAAACTCAGGAGGCGGC3′	130

[1]*B2M*, β-2-microglobulin; *TNF-α*, tumor necrosis factor alpha; *IL-10*, interleukin 10; *NF-κB*, nuclear factor-κB; *Hap*, haptoglobin; *TGF-β1*, transforming growth factor beta 1; *ZO-1*, zonula occludens-1.

Table 4. Effect of additives fed to piglets from 21 to 70 days of age on performance.[1]

Item	NC	PC	FA1	FA2	SEM[2] (n = 9)	*P*-value
			Body weight, kg			
21 days	6.53	6.53	6.53	6.53	0.67	0.99
35 days	9.38 ab	9.47 a	9.27 ab	9.15 b	0.82	0.04
49 days	16.15	16.22	15.97	15.932	1.03	0.71
70 days	30.41 ab	30.89 a	30.58 ab	29.44 b	1.47	0.04
			Average daily gain, kg			
21 - 35 days	0.204	0.205	0.193	0.185	0.02	0.12
21 - 49 days	0.344	0.348	0.345	0.336	0.01	0.63
21 - 70 days	0.476	0.491	0.480	0.461	0.02	0.33
			Average daily feed intake, kg			
21 - 35 days	0.294	0.284	0.294	0.282	0.02	0.64
21 - 49 days	0.494	0.502	0.504	0.489	0.02	0.82
21 - 70 days	0.758	0.773	0.776	0.735	0.03	0.33
			Gain:Feed			
21 - 35 days	0.69 ab	0.72 a	0.67 ab	0.66 b	0.04	0.01
21 - 49 days	0.70	0.69	0.69	0.69	0.03	0.88
21 - 70 days	0.63	0.64	0.64	0.63	0.04	0.97

[1] Negative control (NC, without zinc oxide as growth-promoter); positive control (PC, with zinc oxide as growth-promoter); Feed additive 1 (FA1, NC + 1,0 kg/ton of feed additive); and Feed additive 2 (FA2, NC + 2,0 kg/ton of feed additive).

[2] SEM, standard error of the mean.

[a.b] Means followed by different lowercase letters in the lines differ ($p < 0.05$) from each other by Tukey test.

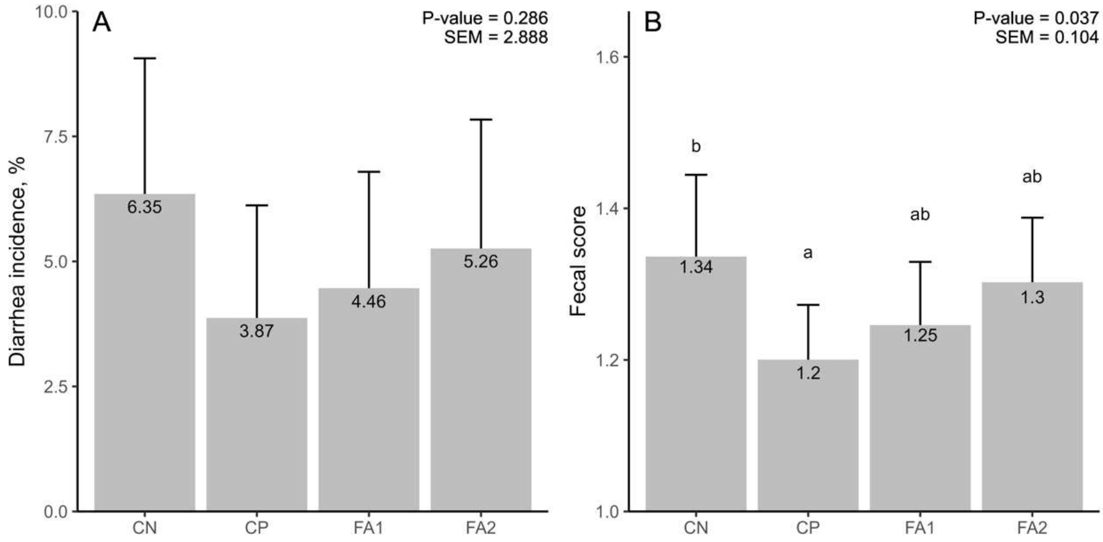

Figure 2. Effect of additives fed to piglets from 21 to 35 days of age on diarrhea incidence (A) and fecal score (B). Negative control (NC, without zinc oxide as growth-promoter); positive control (PC, with zinc oxide as growth-promoter); Feed additive 1 (FA1, NC + 1.0 kg/ton of feed additive) and Feed additive 2 (FA2, NC + 2.0 kg/ton of feed additive). SEM (*n* = 9), standard error of the mean. Fecal score: 1 = solid; 2 = semi-solid; 3 = semi-liquid; and 4 = liquid. [a.b] Means followed by different lowercase letters differ ($P < 0.05$) from each other by Tukey test.

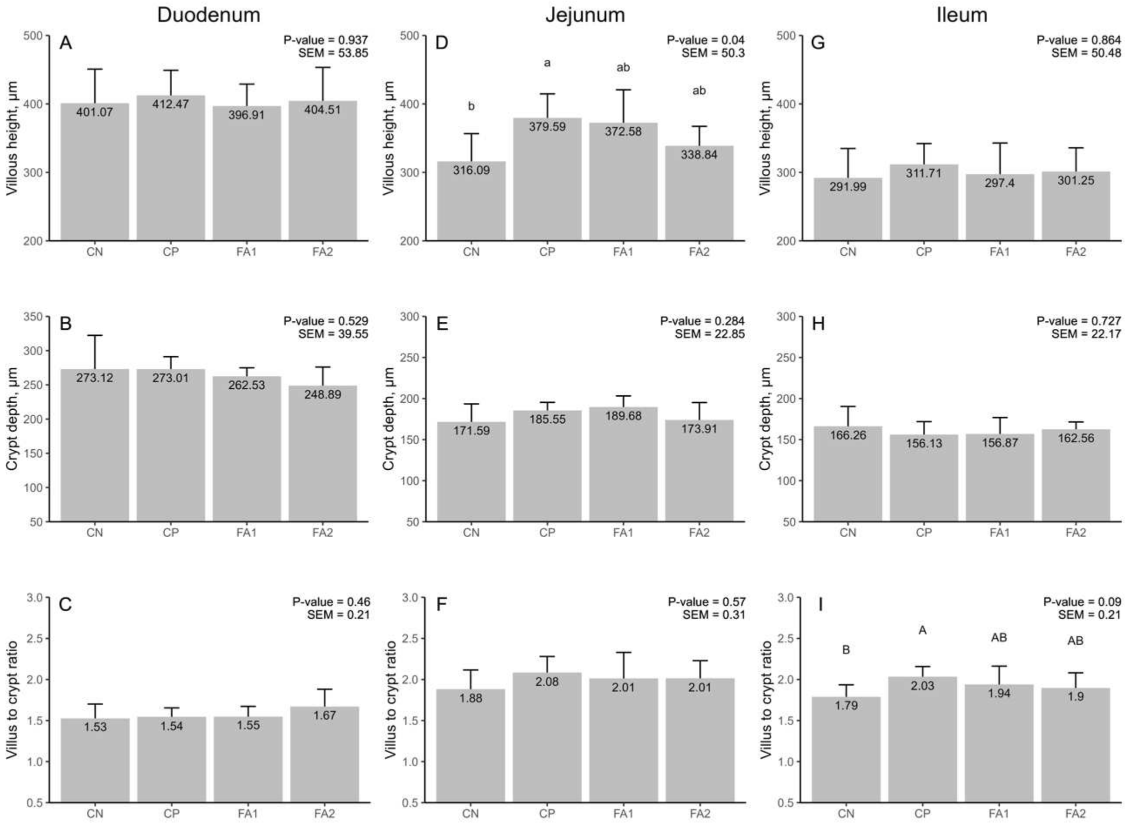

Figure 2. Effect of additives fed to piglets from 21 to 35 days of age on intestinal morphology of duodenum (A, B, C), jejunum (D, E, F) and ileum (G, H, I). Negative control (NC, without zinc oxide as growth-promoter); positive control (PC, with zinc oxide as growth-promoter); Feed additive 1 (FA1, NC + 1.0 kg/ton of feed additive) and Feed additive 2 (FA2, NC + 2.0 kg/ton of feed additive). SEM (n = 9), standard error of the mean. [a,b] Means followed by different lowercase letters differ ($P < 0.05$) from each other by Tukey test. [A,B] Means followed by different capital letters differ ($P < 0.10$) from each other by Tukey test.

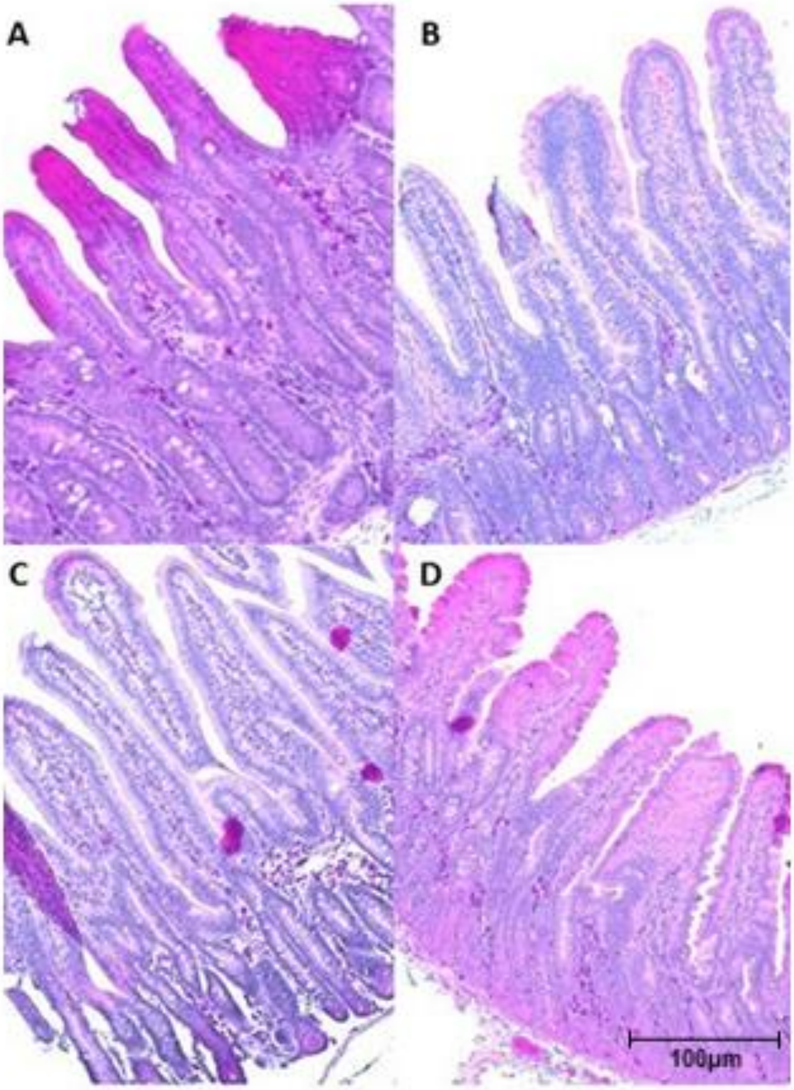

Figure 3. Representative images of jejunal morphology of piglets at 35 days old fed (A) negative control (NC, without zinc oxide as growth-promoter), (B) positive control (PC, with zinc oxide as growth-promoter), (C) feed additive 1 (FA1, NC + 1.0 kg/ton of feed additive) and (D) feed additive 2 (FA2, NC + 2.0 kg/ton of feed additive) diets. Cuts were stained with hematoxylin and eosin. Magnification of 10x.

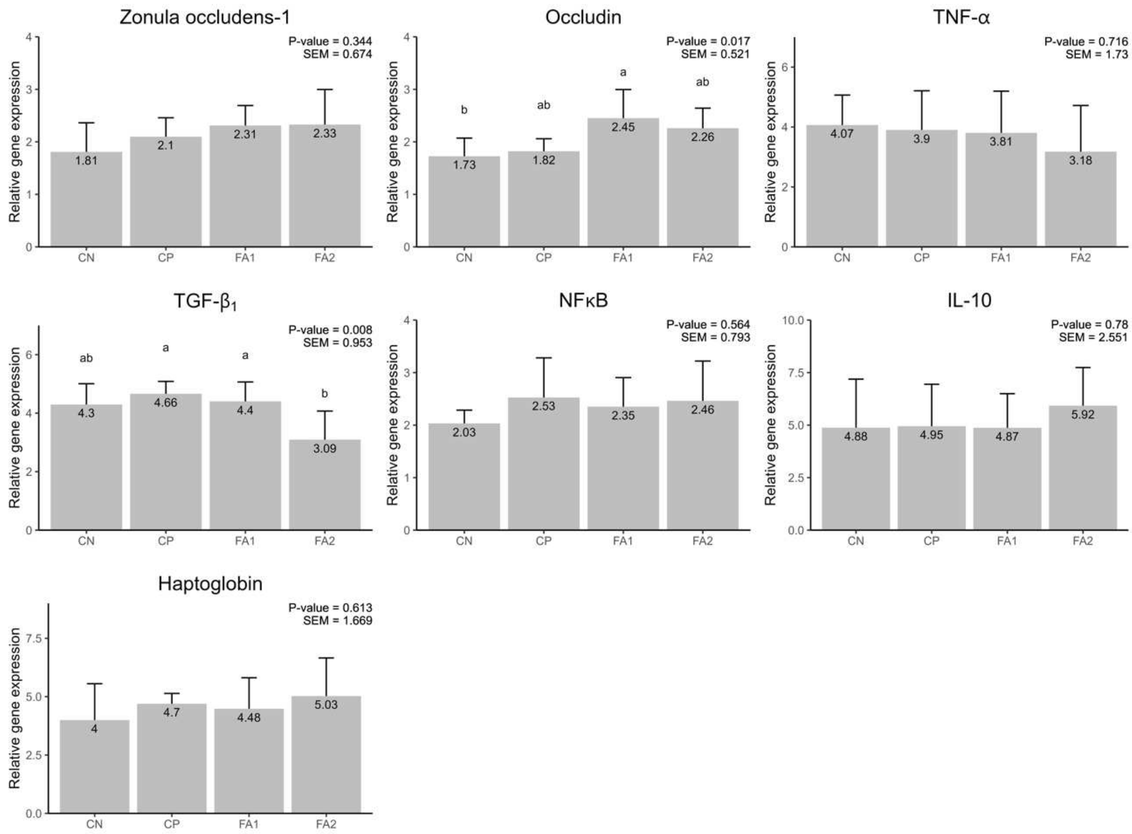

Figure 4. Effect of additives fed to piglets from 21 to 35 days of age on jejunal gene expression of cytokines and tight junction proteins. Negative control (NC, without zinc oxide as growth-promoter); positive control (PC, with zinc oxide as growth-promoter); Feed additive 1 (FA1, NC + 1.0 kg/ton of feed additive) and Feed additive 2 (FA2, NC + 2.0 kg/ton of feed additive). SEM (*n* = 9), standard error of the mean; *TNF-α*, tumor necrosis factor α; *TGF-β1*, transforming growth factor β1; *NF-κB*, nuclear factor-κB; *IL-10*, interleukin 10. [a.b] Means followed by different lowercase letters differ ($P < 0.05$) from each other by Tukey test.

Ethics Committee Certificate

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

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Viçosa, 05 de fevereiro de 2021

CERTIFICADO

Certificamos que o **projeto** intitulado **"Substituição de óxido de zinco e sulfato de cobre por simbiótico na ração de leitões desmamados"**, protocolo **nº 03/2020**, sob a responsabilidade de **Alysson Saraiva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi apreciado pela Comissão de Ética no Uso de Animais de Produção da Universidade Federal de Viçosa (CEUAP-UFV) em reunião de 05 de fevereiro de 2021.

Finalidade: (x)Pesquisa ( )Ensino Vigência do Projeto: de Março de 2021 a 31 de Maio de 2021
Espécie/linhagem:Porcos/Suínos (Sus scrofa domesticus) Nº de animais: 28 Peso: ± 6 kg
Idade: 21 dias Sexo: Macho/Fêmea
Origem:Centro de Pesquisa da Agroceres Multimix Cnpj/CPF: 28.622.744/0001-67 Endereço: Fazenda Serra Negra – BR 365 Km 465,1 s/n – Zona Rural- Patrocínio/MG – 38740-000 Responsável : Fabrício de Almeida Santos

CERTIFICATE

We certify that the project entitled **"Replacement of zinc oxide and copper sulfate by symbiotic in diets for weaned piglets"**, protocol nº **03/2021,** under the responsibility of **Alysson Saraiva -** which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **Feb, 05th, 2021**.

Finality: (x)Research ( )Education
Duration of the Project: from March 2021 a May 31th of 2021
Species / strain: Pig/Swine (Sus scrofa domesticus) Nº of animals: 288 Weight: ± 6 kg Age: 21 days Sex: Male/Female Source: Centro de Pesquisa da Agroceres Multimix Cnpj/CPF: 28.622.744/0001-67 Endereço: Fazenda Serra Negra – BR 365 Km 465,1 s/n – Zona Rural- Patrocínio/MG – 38740-000 Responsável : Fabrício de Almeida Santos

Luciana Navajas Rennó
Coordenadora da CEUAP/UFV

APPENDIX: Do carcass traits influence consumer perception of pork eating quality?

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ABSTRACT
The objective of this study was to assess carcass traits' influence on pork eating quality as evaluated by consumers. A total of 1,360 pork chops were used, with 824 from the sirloin end and 536 from the butt end of the loin *(Longissimuss thoracis et lumborum*), to produce 340 packages, each containing four pork chops. Untrained participants received one package of either sirloin or butt chops, being two pork chops from barrows and two from gilts. Participants answered a survey rating the tenderness, juiciness, flavour, and overall acceptability of each chop on an 8-point scale. Correlation analysis was conducted between carcass traits and pork eating quality attributes. For the descriptive analysis, classes (low, medium, and high) for carcass traits, Warner-Bratzler shear force (WBSF) and cooking loss were created based on our consumer responses dataset for palatability attributes. No significant correlations (P > 0.05) were observed between carcass traits and pork eating quality traits. Tenderness and overall acceptability were negatively correlated

($P < 0.05$) with cooking loss and WBSF. Loin intramuscular fat (IMF) content showed a weak negative correlation ($P < 0.05$) with WBSF and cooking loss. Consumers rated chops from the high and medium/high backfat thickness and loin IMF classes slightly higher for tenderness and juiciness, respectively. Additionally, chops from the low and/or medium WBSF and cooking loss classes received slightly higher scores for tenderness and juiciness than pork chops in the high classes. In conclusion, the study indicated that carcass traits had minimal impact on overall acceptability of pork by consumers.

Keywords: carcass traits, consumer panel, in-home evaluation, intramuscular fat, pork quality.

1. Introduction

Over the last three decades, selection pressure for greater feed efficiency and lean growth for commercial pigs has resulted in carcasses with greater levels of leanness and lower levels of fat (Patience, Rossoni-Serão, & Gutiérrez, 2015; Wu et al., 2017). These compositional changes can affect the consumer satisfaction of meat products as intramuscular fat (IMF) and back fat thickness (BF thickness) content plays an important role on pork eating quality (Font-i-Furnols, Tous, Esteve-Garcia, & Gispert, 2012; V.-B. Hoa et al., 2021; Lebret & Čandek-Potokar, 2022; Moeller, Miller, Edwards, et al., 2010). Thus, given the growing interest in the swine industry to develop better carcass segregation practices that enhance pork eating quality, production of carcasses with greater intramuscular fat content may be a viable option if producers are incentivized appropriately (Bosch, Tor, Reixach, & Estany, 2012; García-Gudiño et al., 2021; Miar et al., 2014).

Controversy exists in the pork industry regarding the influence of carcass and meat quality traits on important sensory attributes for meat products. On one hand, a strong correlation between overall carcass fatness and pork eating quality could influence future genetic selection and the choice of the terminal sire lines for greater IMF deposition (Khanal, Maltecca, Schwab, Gray, & Tiezzi, 2019; Miar et al., 2014; Soares et al., 2022). On the other hand, the absence of strong correlations between IMF deposition and pork eating quality as shown in recent studies (Channon, D'Souza, & Dunshea, 2018; Honegger, Richardson, Schunke, Dilger, & Boler, 2019; Klehm, King, Dilger, Shackelford, & Boler, 2018; Redifer, Beever, Stahl, Boler, & Dilger, 2020) implies that relying solely on higher carcass fatness to optimize pork eating quality for consumers may not necessarily be effective. However, the lack of a strong correlation between carcass fatness and pork eating quality may be explained by the low variability in IMF content across the pork chops, as well as the utilization of diverse cooking methods in the sensory panels (Channon et al., 2018).

Previous studies have shown that high pork fat content, whether subcutaneous or intramuscular, can enhance sensory properties such as tenderness, juiciness, flavour, and overall acceptability (Ba et al., 2019; DeVol et al., 1988; Fortin, Robertson, & Tong, 2005; V.-B. Hoa et al., 2021; V. B. Hoa et al., 2019; Moeller, Miller, Aldredge, et al., 2010; Ventanas, Ruiz, García, & Ventanas, 2007). However, those studies assessed the influence of carcass traits on pork eating quality using trained sensory panels, which may not accurately represent consumer preferences (Claborn et al., 2011; Sveinsdóttir et al., 2010). Instead, in-home evaluations of pork by consumers who are not limited to using specific cooking methods to prepare pork to a standard degree of doneness may provide real-world information on pork eating quality.

As such, the aim of this study was to perform a final consumer evaluation conducting an in-home assessment aiming to evaluate how carcass characteristics impact the pork eating quality.

2. Material and methods

This study was approved by the University of Guelph's Research Ethics Board (REB# 21-01-021) for compliance with federal guidelines for research involving human participants.

2.1. Acquisition of samples and carcass measurements.

Full details regarding collection of pork samples and measurement of carcass parameters were described by Dorleku et al. (2023). A total of 17 slaughter events consisting of approximately 3,950 carcasses were utilized to select the 343 carcasses (174 barrows and 179 gilts) that were used in the study. The pigs (crossbred progeny from Large White dams and Duroc sires) were sourced from nine different producers, and were slaughtered under the supervision of the Canadian Food Inspection Agency (CFIA) at a commercial pork packing plant in southwestern Ontario.

Back fat thickness and loin muscle (LL, *Longissimus lumborum*) depths measurements were collected by experienced operators from the Ontario Pork Grading Authority using a Destron PG-100 grading probe (International Destron Technologies).

The grading probe was inserted perpendicularly at the grading site between the 3rd and 4th last ribs, 7 cm off the mid-line according to Canadian grading standards (Pomar & Marcoux, 2003).

Backfat thickness and muscle depth measurements were used to obtain the predicted lean yield (PLY) value for each carcass using the following equation according to Canadian National Cut-Out (CPC, 1994):

$$PLY\ (\%) = 68.1863 - (0.7833 \times BF\ thickness) + (0.0689 \times muscle\ depth) + (0.0080 \times BF\ thickness^2) - (0.0002 \times muscle\ depth^2) + (0.0006 \times BF\ thickness \times muscle\ depth)$$

Predicted Lean Yield is the Canadian Lean Yield value for each carcass, and BF thickness (mm) and the muscle LL depth (mm) were collected at the grading site for each carcass (CPC, 1994; Pomar & Marcoux, 2003).

Hot carcass weight (HCW) was recorded by study personnel using a voice recorder and a GoPro Hero 8 Black camera. Following the grading station, carcasses were chilled using a conventional chilling system for 22 to 24 hours at ambient temperatures of -4 to 2°C.

The left sides from selected carcasses were placed in meat combos (i.e., plastic bag lined 8-sided cardboard bulk meat containers placed on top of a wooden pallet) approximately 20 to 24 hours following slaughter and immediately delivered to the University of Guelph Meat Laboratory using a commercial refrigerated truck that met CFIA guidelines for transportation.

2.2. Carcass fabrication

Upon arrival at the University of Guelph Meat Laboratory (approximately 24 to 28 hours post-mortem), pork carcass sides were removed from combos and refrigerated at ≤ 4°C in the processing room cooler until fabrication. After ~30 minutes in the cooler, the first carcass sides were fabricated into primals (butt, picnic, loin, ham, and belly) and sub-primals according to the North American Meat Processors Association, and the Institutional Meat Purchase Specifications (IMPS, 2014; NAMP, 2006) guidelines with further separation into lean, fat, and bone components. The carcasses were fabricated between 24- and 48-hours post-mortem.

Nine 3.0 cm-thick loin chops were cut from the *Longissimus thoracis et lumborum* (LTL) muscle, trimmed of epimysium and external fat, and individually identified for various analyses. The first four loin chops originating from the loin's sirloin end were designated for the in-home consumer evaluation. Similarly, the last two loin chops at the butt end of the loin were designated for the consumer evaluation. The samples were individually vacuum packaged and assigned between 14 to 15 d post-mortem ageing at ≤ 4°C prior to evaluation. The remaining loin chops in the middle of the LTL were allocated based on the following arrangement: Two chops were individually vacuum sealed and then aged at ≤ 4°C for 14 to15 d and then frozen at -20°C until used to determine Warner-Bratzler shear force (WBSF). One chop was used to determine intramuscular fat (IMF) content in which each chop was aged for 3 d and then frozen at -20°C until further analysis.

2.3. Intramuscular fat content analysis

Designated chops for determination of IMF content were thawed over 24 h at ≤ 4°C after which any epimysium and subcutaneous fat were fully removed prior to cubing and freeze-drying in a Thermo Scientific (Fisher Scientific, ON, Canada) freeze dryer for approximately 96 h. After drying, samples were ground in a commercial coffee grinder and mixed thoroughly before sampling. Intramuscular fat content was determined in the freeze-dried meat samples using the Ankom XT20 Fat Analyzer (ANKOM Technology Corporation, Macedon, NY, USA) for ether extraction of fat (AOAC, 2000), in which the 2.0-gram freeze-dried sample was extracted using petroleum ether for 30 min.

2.4. Warner-Bratzler shear force and cooking loss analysis

Designated chops for determination of WBSF were thawed over 24 h at ≤ 4°C after which any epimysium and subcutaneous fat were fully removed. Once trimmed, the chops were weighed before being cooked to an internal temperature of 72 °C on a clamshell Garland Grill (Ed-30B: Garland Commercial Ranges LTD, Mississauga, ON) set to a surface temperature of 105 °C. Cooking temperatures were continually monitored using a thermocouple inserted in the geometric center of each chop with initial and final temperatures recorded. Chops were turned after reaching an internal temperature of approximately 40°C. The final endpoint temperature of the chops after being removed from the grill was approximately 74°C. Once the chop was cooked to the target temperature endpoint, the cooked weight was recorded to determine cooking losses. Following cooking, samples were placed in a cooler filed with ice and later chilled in a carcass cooler at ≤ 4°C for 24 h to achieve an internal temperature of approximately 4 °C before coring. Prior to coring, chops were allowed to equilibrate to room temperature (approximately 22°C). Three 1.27 cm meat cores were removed parallel to the muscle fibers from each chop using a press-mounted drill. Cores were sheared perpendicularly to the muscle fibers using a Warner-Bratzler blade on a TA-XT Plus texture analyzer (Texture Technologies Corp., Scarsdale, USA) with a crosshead speed set at 3.3 mms^{-1}. Peak shear force was determined using a customized macro program in Stable Microsystems Exponent software, and the average of the 6 peak force values was taken as the shear force value for the 2 loin chops from each evaluated carcass side.

2.5. In-home consumer evaluation

A total of 1,360 pork chops, 824 from the posterior (sirloin) end of the loin and 536 from the anterior (butt) end of the loin were sorted based on sex (barrow or gilt) and loin IMF content to produce 340 packages containing four individually vacuum-sealed pork chops. The process of allocating pork chops to each package is explained in detail below.

Based on the data set, distinct levels of loin IMF content were distinguished across both sexes, categorized as low (L; < 1.5% of IMF), intermediate (I; 1.5% to 2.43% of IMF), and high (H > 2.43% of IMF). For each pair of chops within their respective sex groups, random allocation was employed to facilitate comparisons encompassing H vs H, H vs I, H vs L, I vs I, and L vs L. Due to the notable prevalence of L and I chops and the relative scarcity of H chops, efforts to achieve comparison balance for each package of chops based on sex were not pursued. Consequently, it's conceivable that one consumer's package might comprise H vs L gilt chops and H vs L barrow chops, while another's could include H vs L gilt chops and L vs L barrow chops, and so forth.

These packages were distributed to untrained participants for evaluation at home, with each participant receiving one package of chops that exclusively contained 4 chops from the sirloin end of the loin or 4 chops from the butt end of the loin, with two chops from barrows and two chops from gilts.

Along with the pork chops, participants received a consumer questionnaire with instructions, a cooked pork color guide, and a stamped, self-addressed envelope. Participants were asked to prepare the pork chops in the normal manner that they were accustomed to and to complete a questionnaire online or on paper which asked for cooking method used, degree of doneness that the pork chops were cooked, the approximate cooking time for each pork chop, and evaluation of tenderness, juiciness, flavour, and overall acceptability attributes for each pork chop. A pork chop color guide (National Pork Board, Pork, USA) was provided to help participants accurately describe degree of doneness.

Sensory properties (tenderness, juiciness, flavour and overall acceptability) for the cooked pork chops were evaluated on a 8-point categorical intensity scale modified from Glanc, Campbell, Cranfield, Swanson, and Mandell (2015). The range in scales included: Tenderness, 1 = Extremely tough, 5 = Slightly tender, and 8 = Extremely tender; Juiciness, 1 = Extremely dry, 5 = Slightly juicy, and 8 = Extremely juicy; Flavour, 1 = Extremely unpork-like, 5 = Slightly pork-like, and 8 = Pork-like; and Overall acceptability, 1 = Dislike extremely, 5 = Like slightly, and 8 = Like extremely.

Participants also provided demographic details (gender, age, and pork consumption frequency) for every package of pork chops received. Importantly, it's worth noting that consumers were under no obligation to respond to the survey concerning sensory evaluation or personal information.

2.6. Statistical analysis

Data were analyzed using R Core Team (2020) software, version 4.1.0. Carcass information (HCW, BF thickness, PLY, loin IMF content, WBSF and cooking loss for LL muscle) and pork eating quality traits (tenderness, juiciness, flavour, overall acceptability) were subjected to correlation analysis and probability values less than 0.05 were considered significant.

Initially, we decided to model the different scores for sensory properties as a function of independent variables using a random forest analysis or a generalized linear model. However, both approaches led to an overfitted adjustment, which did not allow for the obtainment of a functional and inferential interpretation of response patterns (see discussion for more details). We decided to evaluate the pattern of sensory properties by using a non-inferential statistical analysis. To perform a descriptive analysis, classes (low, medium and high) for carcass traits and instrumental analyses (WBSF and cooking loss) were created based on our consumer responses dataset for palatability attributes. For each variable, the data were sorted in ascending order and the categories were divided as follows: data below the 33rd percentile were assigned to the "Low" class, data between the 33rd and 67th percentiles were assigned to the "Medium" class, and data above the 67th percentile were assigned to the "High" class.

3. Results and Discussion

3.1. In-home consumer evaluation response data

From the 340 packages distributed, 182 participants responded to the survey regarding consumer assessment of pork eating quality (53.5% response rate), totaling 728 observations (Unfiltered data; Table 1). Since there was a limited response rate for "degree of doneness" and "cooking method" categories, the total data were unbalanced, which impaired the statistical analysis. To improve the data balance, medium rare responses for "degree of doneness", along with air fryer, indoor grilling, and roasting responses for "cooking methods" were deleted from the data set prior to analysis due to their lower frequency. As a result, the number of observations decreased to a total of 646 (Filtered data; Table 1).

The demographic information gathered from survey respondents is presented in Table 2. Among the 182 participants, only 153 responded to the survey concerning their personal information.

The distribution of received survey response observations for specific carcass traits are presented in Fig. 1, while the proportion of scores for each pork eating quality trait (tenderness, juiciness, flavour and overall acceptability) from survey responses are presented in Fig. 2. According to Barducci et al. (2020), carcass characteristics for commercial pork in Ontario had the following attributes: 106.3 kg, 18.11 mm, and 61.1% for HCW, BF thickness, and PLY, respectively, which was similar to the mean values in this study (107.2 kg, 17.0 mm, and 61.8%, respectively). Furthermore, other studies have reported comparable values for carcass characteristics in lean genotypes (V.-B. Hoa et al., 2021; Li et al., 2021; Soares et al., 2022; Van den Broeke, Leen, Aluwé, Van Meensel, & Millet, 2020; Zhang et al., 2016). In addition, the average of loin IMF content used was 2.18 ± 0.9 % (Fig. 1). Therefore, the pork chops delivered to the participants may be assumed as representative of the pork that is purchased by the final consumer in the market.

3.2. Correlation between carcass traits and pork eating quality

The sensory experience during consumption consists of several palatability attributes: the more important ones are tenderness, juiciness, flavour and aroma (Fortin et al., 2005). Positive correlations between those pork eating quality traits and overall acceptability were previously reported in sensory panel studies (Channon et al., 2018; Channon, Taverner, D'Souza, & Warner, 2014; V.-B. Hoa et al., 2021). Indeed, the present study found that overall acceptability of pork chops was strongly correlated ($P < 0.05$) with tenderness (r = 0.710) and juiciness (r = 0.691), and moderately correlated (P

< 0.05) with flavour (r = 0.461) (Fig. 3). Previous trained panel studies reported that higher pork IMF content improved the sensory properties of loin chops (DeVol et al., 1988; Fortin et al., 2005; Moeller, Miller, Aldredge, et al., 2010; Ventanas et al., 2007). While IMF content was positively correlated with sensory properties for pork in past studies (Ba et al., 2019; Cannata et al., 2010; Fortin et al., 2005; Moeller, Miller, Aldredge, et al., 2010), there was no correlation ($P > 0.05$) between loin IMF content and pork eating quality traits (tenderness, juiciness, flavour and overall acceptability) for the consumer evaluation in the present study (Fig. 3).

To ensure optimal eating quality for consumers, a minimum 2.5% IMF content is recommended for pork (DeVol et al., 1988; Fernandez, Monin, Talmant, Mourot, & Lebret, 1999). However, based on the total number of received responses in our in-home consumer evaluation, only approximately 30.0% of observations were from assessment of pork chops with a loin IMF content ≥ 2.5%, which may explain the absence of any relationship between IMF content and pork eating quality.

Furthermore, it is well reported in the literature that cooking method and degree of doneness affect consumer perceptions and preferences for pork loin chops (Channon, D'Souza, & Dunshea, 2016; Lebret & Čandek-Potokar, 2022; Moeller, Miller, Aldredge, et al., 2010; Rincker, Killefer, Ellis, Brewer, & McKeith, 2008). Additionally, it has been observed that IMF reduces the cooking water loss, with this effect becoming more pronounced as the endpoint temperature increases up to 80°C (Aaslyng, Bejerholm, Ertbjerg, Bertram, & Andersen, 2003), potentially enhancing pork eating quality traits (Channon et al., 2013). However, our in-home consumer evaluation findings also suggest that any importance of IMF content in pork chops on meat quality parameters could not be detected by consumers, which could be influenced by different cooking methods, degree of doneness, and the general lack of training for the panelists, mirroring what occurs on a daily basis for consumers preparing and eating pork. It should be emphasized that our study differed from a taste panel involving trained panelists and from a consumer panel using standardized cooking methods. This difference, when associated with our previous argument, provides evidence that consumer cooking techniques and preferences could be more relevant for overall acceptability of pork than do changes in carcass parameters. When a consumer is allowed to cook according to their preferences, carcass parameters may have less impact on acceptability when compared to the discrimination through acceptability characteristics under a controlled taste panel or a consumer panel with standardized cooking methods.

Carcass traits such as HCW and BF thickness have been found to be positively correlated with IMF content, while PLY has shown a negative correlation with IMF content (Aymerich, Gasa, Bonet, Coma, & Solà-Oriol, 2019; Bosch et al., 2012; V.-B. Hoa et al., 2021). Consequently, these traits are anticipated to indirectly affect the sensory characteristics of meat products through their influence on IMF content, as supported by findings in the literature (Ba et al., 2019; Blanchard, Willis, Warkup, & Ellis, 2000; V.-B. Hoa et al., 2021). In the present study, weak to moderate correlations were observed ($P < 0.05$) between carcass traits (HCW, BF thickness and PLY) and IMF content. However, there were no correlations ($P > 0.05$) observed between these carcass traits and pork eating quality. This lack of correlation may be explained by the different cooking methods, endpoint temperatures and degree of doneness adopted by individual consumers based on their respective preferences and habits.

According to Moeller, Miller, Aldredge, et al. (2010) and Lee et al. (2012), WBSF and cooking loss are the best objective indicators for assessing sensory properties of pork chops. In the current study, there were negative correlations ($P < 0.05$) for WBSF and cooking loss with tenderness and overall acceptability (Fig. 3). These results were in agreement with previous trained panel studies (Cannata et al., 2010; V.-B. Hoa et al., 2021; Lee et al., 2012; Moeller, Miller, Aldredge, et al., 2010), and suggest that instrumental analysis have a place in indicating pork eating quality traits.

The deposition of IMF between muscle fiber fascicules results in the disruption of the endomysium structure. This results in separation and dilution of perimysial collagen fibers, as well as the disorganization of the intramuscular connective tissue; these all contribute to reduced shear force and increased meat tenderness (Hocquette et al., 2010; Wood, 1990). In addition, IMF can increase the water holding capacity of the meat by acting as a physical barrier to water loss during cooking, leading to lower cooking losses and higher juiciness perception (Cannata et al., 2010; Miller, 2002; Warner, 2023). Despite the lack of correlations between IMF content and pork eating quality traits in our in-home consumer evaluation, IMF content was negatively correlated ($P < 0.05$; Fig. 3) with WBSF and cooking loss, implying that not performing the sensory panel might overstate the importance of IMF on pork eating quality traits.

Laboratory procedures for determination of WBSF and cooking loss were standardized for each pork chop, using the identical cooking method, monitoring of internal temperature and degree of doneness endpoint. As previously stated, the use of these standardized methods resulted in IMF content being negatively correlated with

WBSF and cooking loss. This contrast to the absence of similar relationships between IMF and consumer evaluation of tenderness and juiciness in the present study, which seemed to be more impacted by participants' cooking habits (or preferences), as they were instructed to cook the pork chops using their preferred cooking method and degree of doneness.

3.3. Carcass trait classes on pork eating quality

To better understand consumers' perceptions regarding pork quality due to animal-to-animal differences in carcass characteristics, WBSF and cooking loss, box plots were drawn according to the classes (i.e., low, medium and high) for each variable studied to examine how the range in values for a given carcass trait affected sensory traits.

The pork eating quality traits were analyzed for their response to HCW (Fig. 4) and PLY (Fig. 5) classes, but no clear patterns were observed. While higher HCW and PLY classes led to improved juiciness scores, they were also associated with reduced tenderness, which was unexpected given the strong correlation (r = 0.683) between these two variables. Consequently, these results suggested that HCW and PLY had no discernible impact on the perception or preference of consumers, despite their correlations with loin IMF content.

However, we did observe a pattern of responses among BF thickness (Fig. 6), loin IMF (Fig. 7), WBSF (Fig. 8), and cooking loss (Fig. 9) regarding consumer preferences for tenderness and juiciness. Consumers rated pork chops from the high and medium/high classes for BF thickness and loin IMF categories to have slightly higher average scores for tenderness and juiciness, respectively than pork chops in the medium/low or low classes. Interestingly, a complementary pattern was found regarding WBSF, where consumers rated pork chops from the low and low/medium classes to have slightly higher scores for tenderness and juiciness, respectively than pork chops in the medium/high or high classes. With respect to cooking losses, consumers rated pork chops from the low and medium classes to have a slight improvement in tenderness and juiciness than pork chops in the high class.

As mentioned previously, WBSF and cooking loss are accurate and repeatable laboratory analyses that were negatively correlated ($P < 0.05$) with tenderness and overall acceptability. In addition, there were negative correlations ($P < 0.05$) for IMF with WBSF and cooking loss. Although loin IMF content was not correlated with pork eating quality traits in the present study, our in-home consumer evaluation suggested that there were benefits of the high class of pork IMF content (3.2%) on laboratory analyses (Fig. 8 and 9). This suggests that consumers might perceive enhancements in tenderness and juiciness due to higher intramuscular fat content, depending on degree of doneness and cooking method used.

Regardless of the carcass or meat quality trait assessed, there were no apparent effects of class (low, medium, high) on flavour and overall acceptability (Figures 4 to 9). As shown in Fig. 2, the distribution of scores exhibits a pronounced right-skew within the 8-point categorical intensity scale (specifically, between scores of 5 and 7) across all pork eating quality traits. Notably, this skew is even more evident for flavour and overall acceptability, posing challenges in discerning any shifts in consumer preference patterns related to these specific palatability traits. Taken as a whole, the pronounced concentration of scores in the right skew reinforces that consumer cooking habits or preferences may hold greater significance in determining the overall acceptability of pork eating quality compared to improvements in carcass parameters.

In support of this argument, cooking method (Fig. 10) and degree of doneness (Fig. 11) used by the consumer caused variability in consumer evaluation ratings regarding pork quality, particularly tenderness, juiciness, and flavour, across the different loin IMF content classes. This highlights that both the choice of cooking method and degree of doneness according to individual consumer preferences may diminish the impact of IMF content on meat quality. Therefore, in addition to improving meat quality through selecting carcasses with higher IMF content, the industry can implement other interventions to enhance pork tenderness while continuing to communicate the most appropriate cooking methods and degree of doneness to optimize consumer satisfaction of pork meals.

4. Conclusion

Collectively, the results highlight the importance of cooking methods used and degree of doneness on the overall acceptability of pork, which seems to overshadow the influence of carcass traits on consumer perception of pork eating quality. Importantly, it should be noted that this study is based on final consumer's perception and not on studies involving trained panelists and standardized cooking methods and is therefore considered to provide realistic and insightful information for the pork industry.

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Table 1. Data summary of in-home consumer survey responses.

Item		Unfiltered data	Filtered data[1]
Total packages (n)		182	162
Total chops (n)		728	646
Sex	Barrow	50.4%	49.5%
	Gilt	49.9%	50.5%
Loin cut position	Posterior (Sirloin)	74.2%	74.8%
	Anterior (Butt)	25.8%	25.2%
Degree of doneness	Well done	44.1%	45.5%
	Medium well	33.4%	35.3%
	Medium	17.6%	19.2%
	Medium rare	4.9%	-
Cooking method	Outdoor grilling	42.0%	44.7%
	Fry pan	35.2%	36.7%
	Oven broil	16.5%	18.6%
	Air fryer	5.5%	-
	Indoor grilling	0.5%	-
	Roasting	0.3%	-

[1]Filtered data, consisting of the total dataset excluding observations of medium rare for “degree of doneness”, as well as air fryer, indoor grilling, and roasting for "cooking methods" due to their lower frequency, were used for all analyses.

Table 2. Demographic information collected from survey respondents in this study.

Gender distribution (n) of participants within each age group

	20-30 years	31-40 years	41-50 years	51-60 years	> 60 years	Tota l
Male	19	18	10	7	15	69
Female	31	16	13	11	10	81
Prefer not to respond	1	0	0	1	1	3
Total	51	34	23	19	26	153

Distribution (n) of participants based on their current involvement in farming

Involved	51
Non-involved	102
Total	153

Weekly pork consumption frequency (%) of consumers

None	1 to 2 times	3 to 5 times	6 or more times	Don't know
10.46	76.47	11.76	0	1.31

Frequency (%) of preferred package sizes among study participants when purchasing pork

< 0.25 kg	0.26 to 0.50 kg	0.51 to 1.00 kg	1.01 to 1.50 kg	> 1.5 kg	Don't know
3.92	27.45	32.03	14.38	4.58	17.64

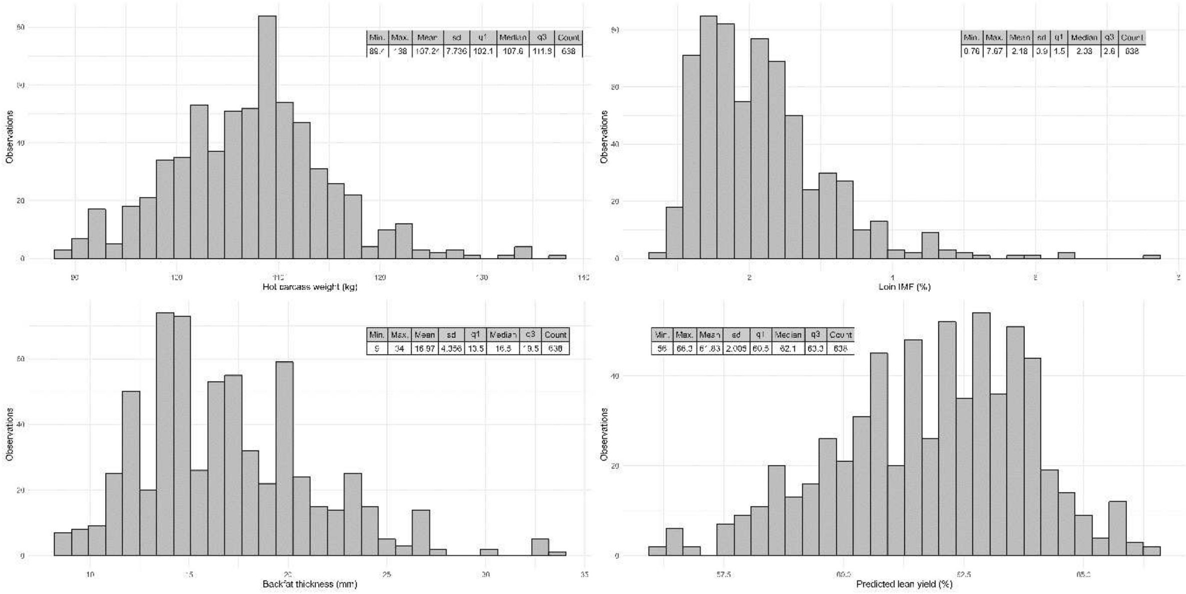

Fig. 1. Distribution of received survey response observations for specific carcass traits used in filtered data analysis. Abbreviations: min = minimum value; max = maximum value; sd = standard deviation; q1 = first quartile; and q3 = third quartile.

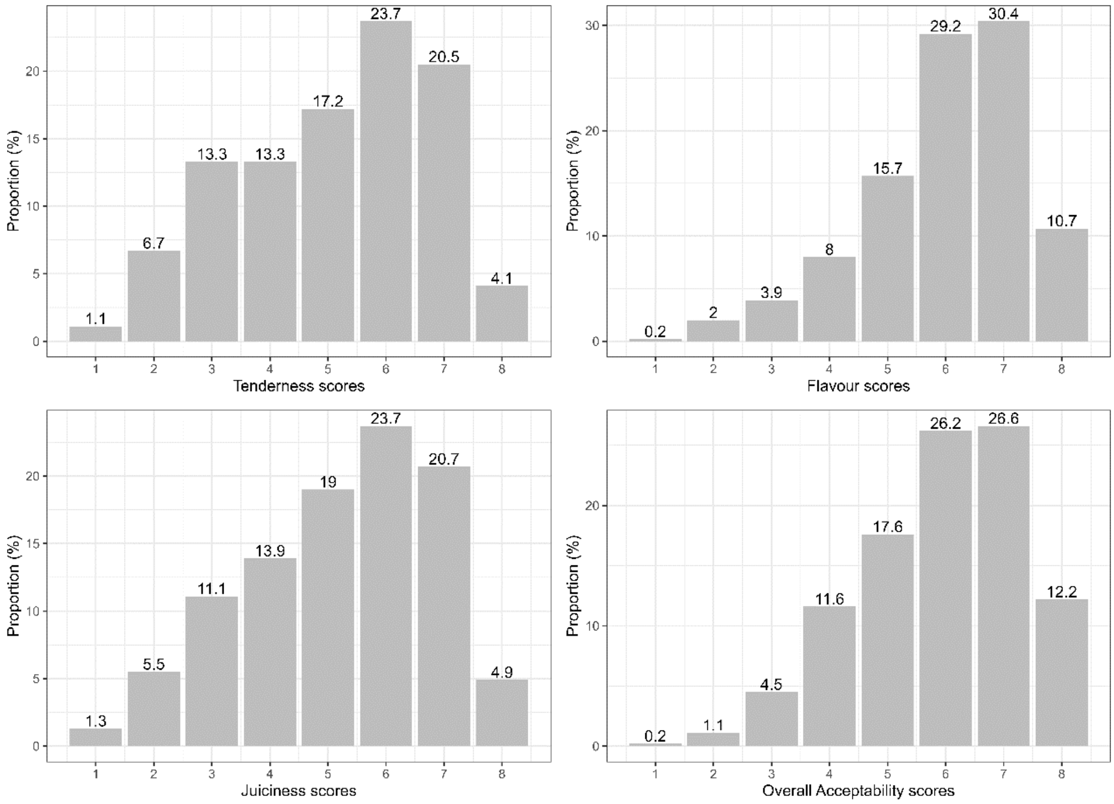

Fig. 2. Proportions of scores for rating pork chops in the in-home consumer evaluation regarding eating quality traits (tenderness, juiciness, flavour and overall acceptability).

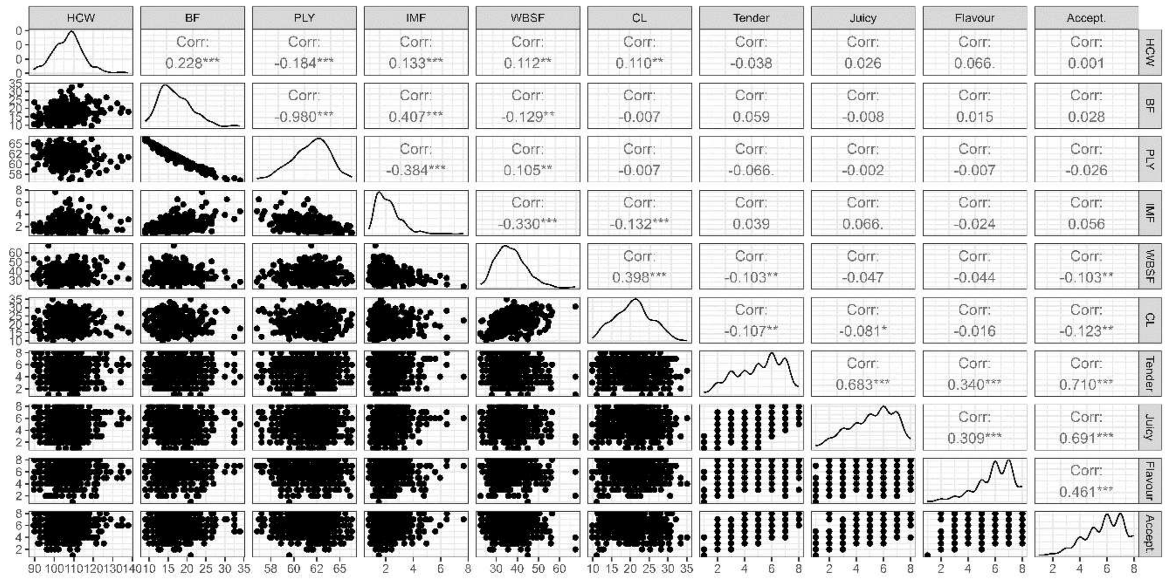

Fig. 3. Correlation coefficients between carcass traits, cooking loss, shear force and pork eating quality traits. Abbreviations: HCW = hot carcass weight (kg); BF = backfat thickness (mm); PLY = predicted lean yield (%); IMF = loin intramuscular fat (%); WBSF (N) = Warner-Bratzler shear force; CL = cooking loss (%); Tender = tenderness; Juicy = juiciness; and Accept = overall acceptability. *** $P < 0.001$; ** $P < 0.01$; and * $P < 0.05$.

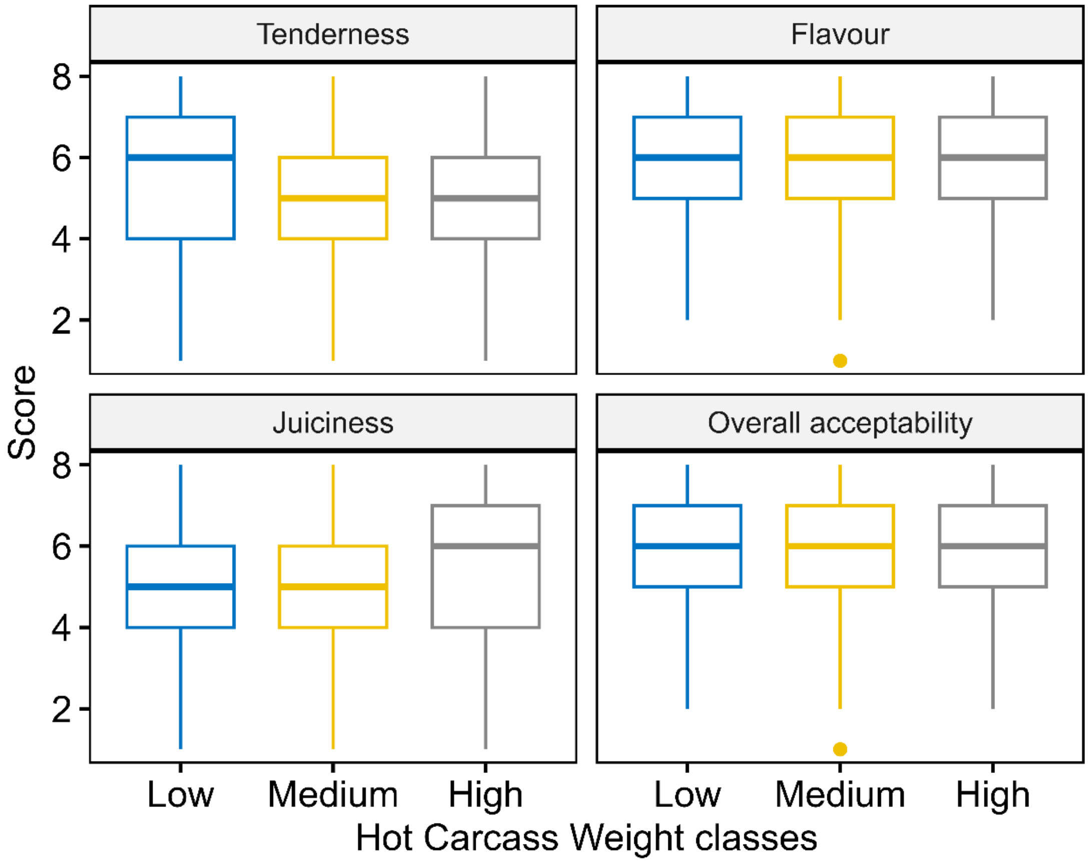

Fig. 4. Descriptive results for the influence of hot carcass weight classes on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for hot carcass weight (kg) classes: low (98.5 ± 3.78 kg); medium (107.4 ± 1.76 kg) and high (115.5 ± 5.23 kg).

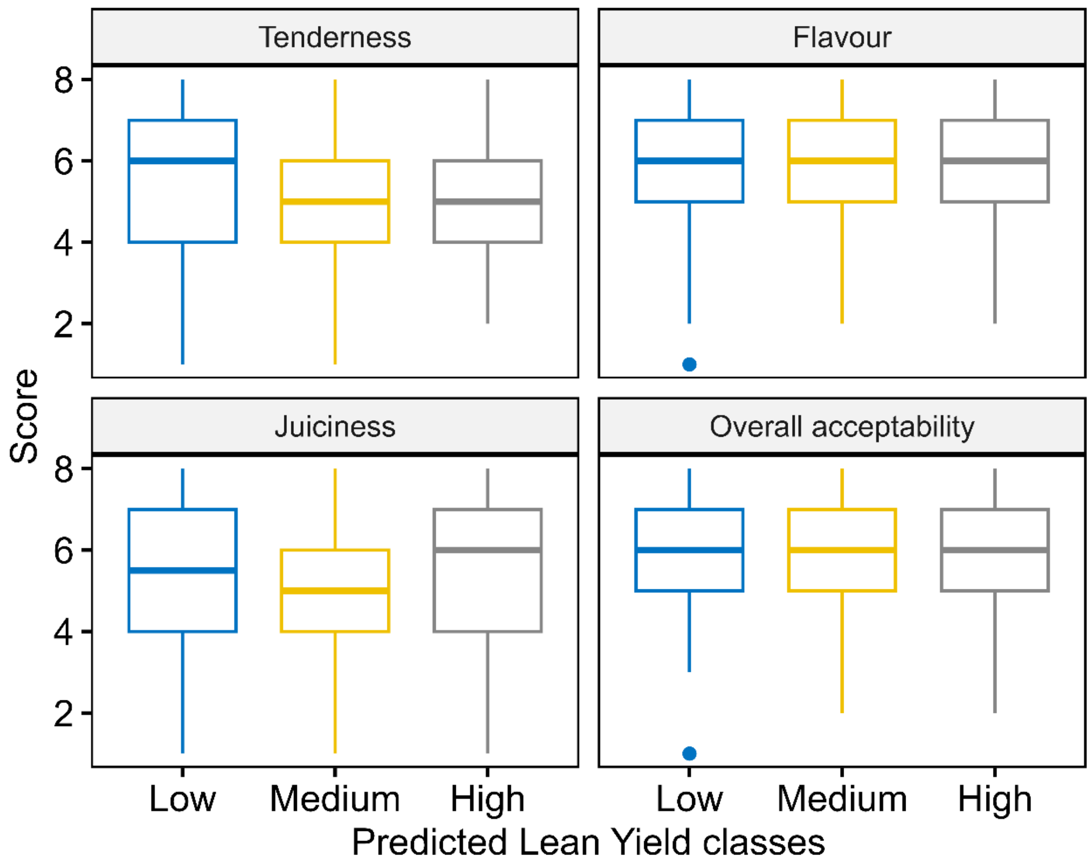

Fig. 5. Descriptive results for the influence predicted lean yield classes on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for predicted lean yield (%) classes: low (59.6 ± 1.19 %); medium (62.1 ± 0.51 %) and high (64.0 ± 0.82 %).

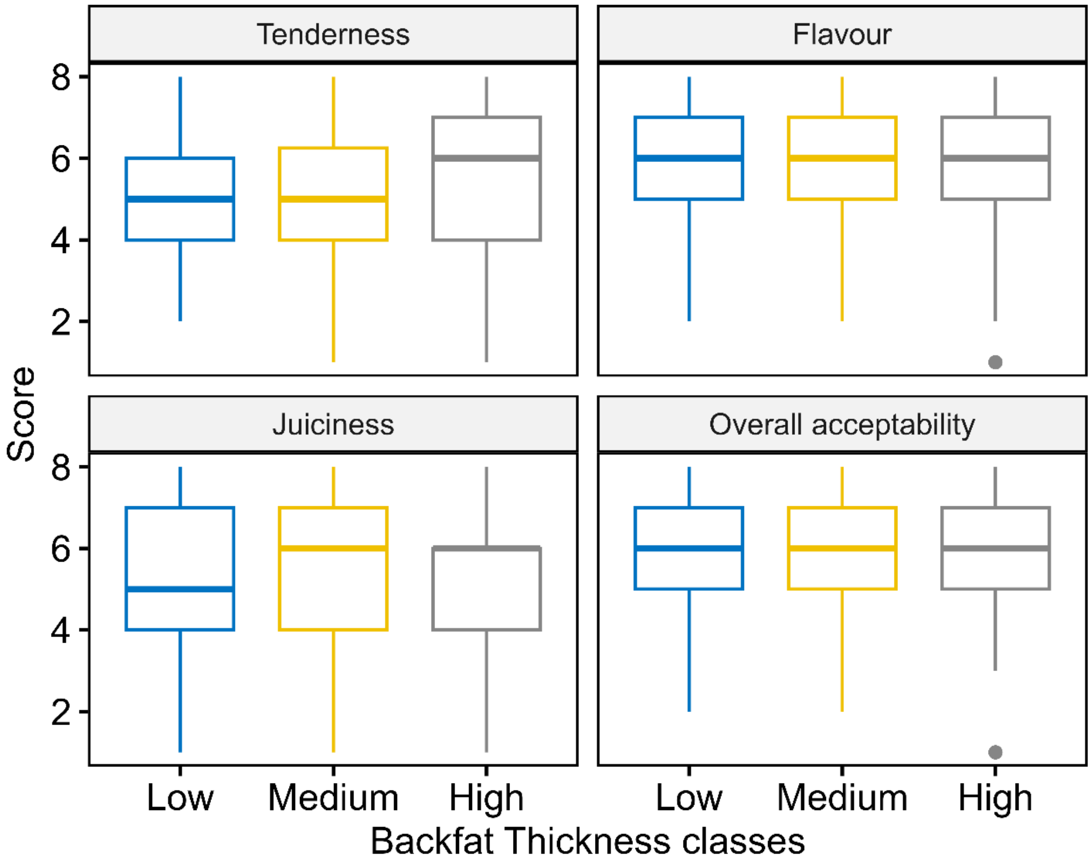

Fig. 6. Descriptive results for the influence backfat thickness classes on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for backfat thickness (mm) classes: low (12.8 ± 1.46 mm); medium (16.5 ± 1.10 mm) and high (22.1 ± 3.06 mm).

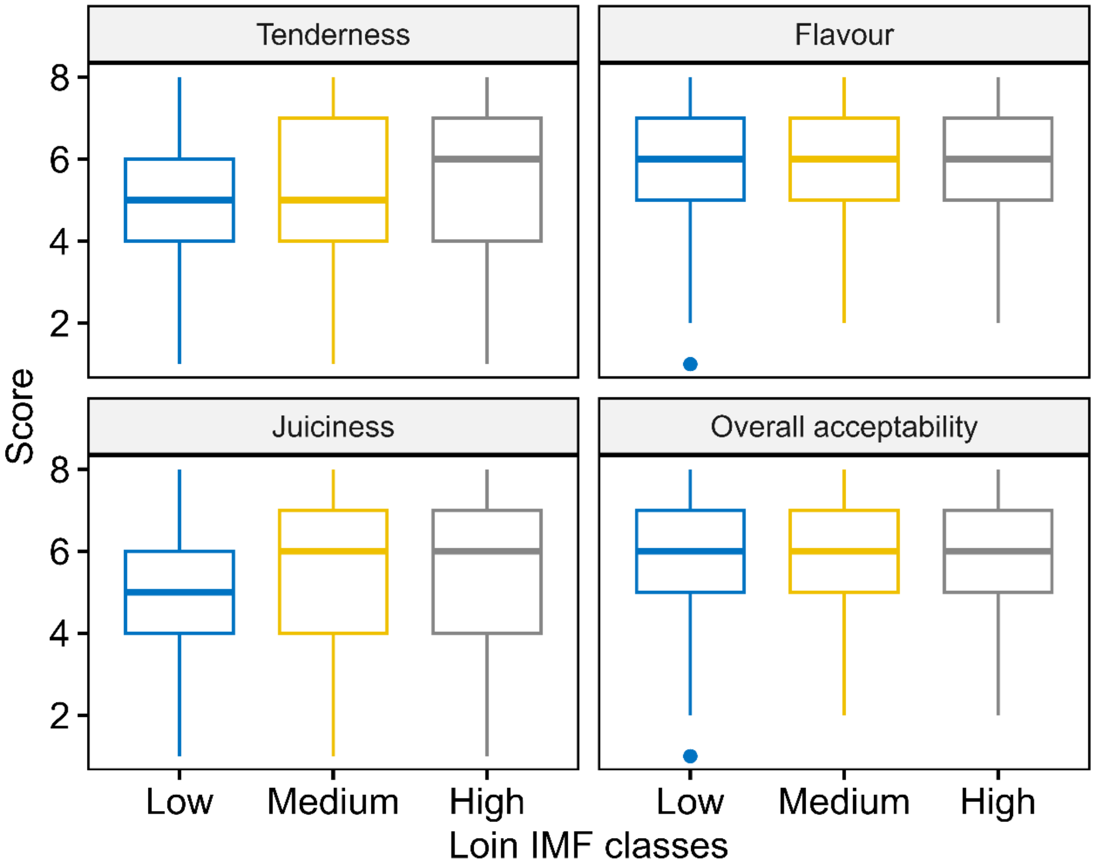

Fig. 7. Descriptive results for the influence of loin intramuscular fat (IMF) content classes on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for loin IMF content (%) classes: low (1.3 ± 0.20 %); medium (2.0 ± 0.22 %) and high (3.2 ± 0.81 %).

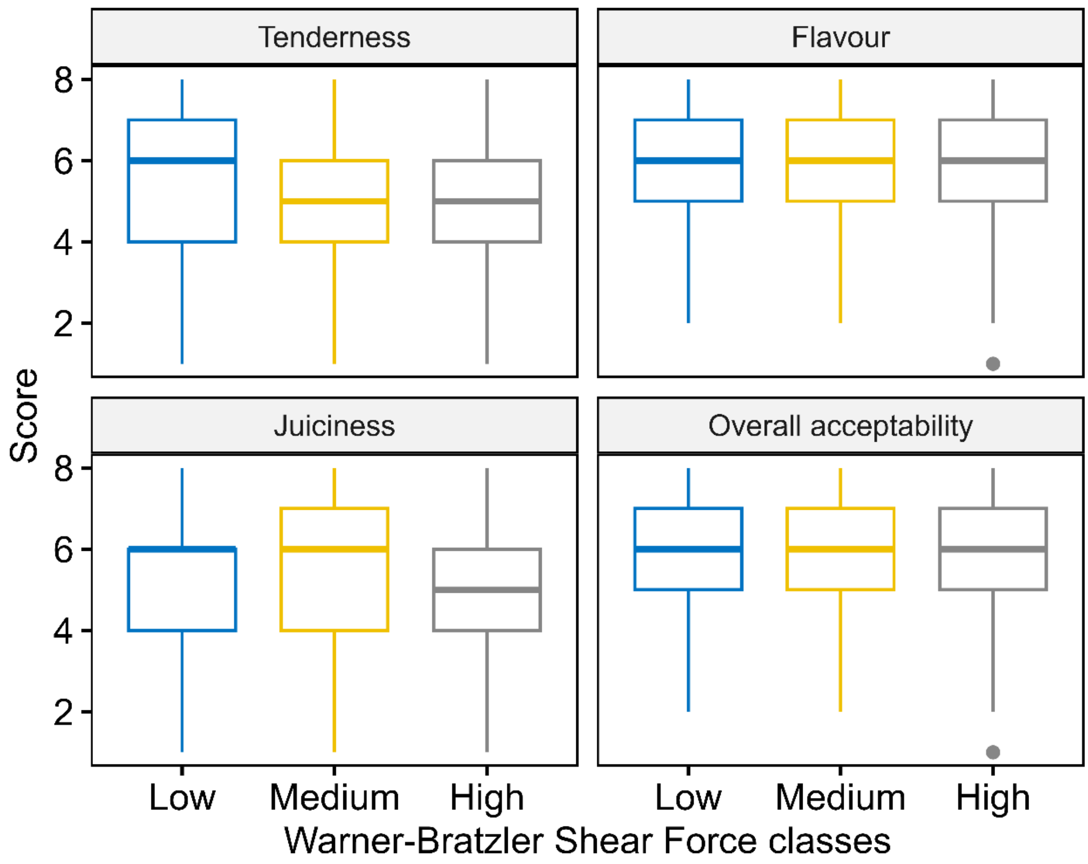

Fig. 8. Descriptive results for the influence Warner-Bratzler shear force classes on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for Warner-Bratzler shear force (N) classes: low (29.8 ± 2.56 N); medium (36.4 ± 1.84 N) and high (44.7 ± 4.68 N).

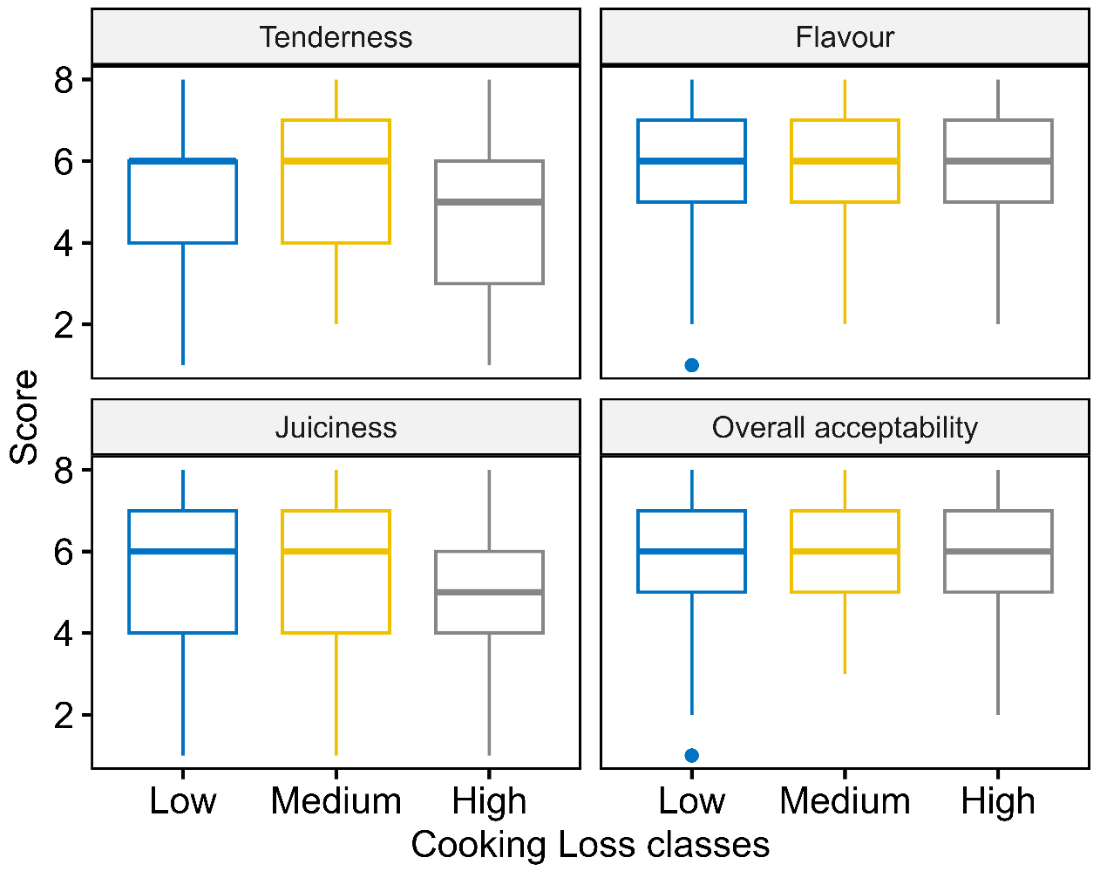

Fig. 9. Descriptive results for the influence cooking loss classes on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for cooking loss (%) classes: low (15.3 ± 2.18 %); medium (20.8 ± 1.14 %) and high (26.2 ± 2.55 %).

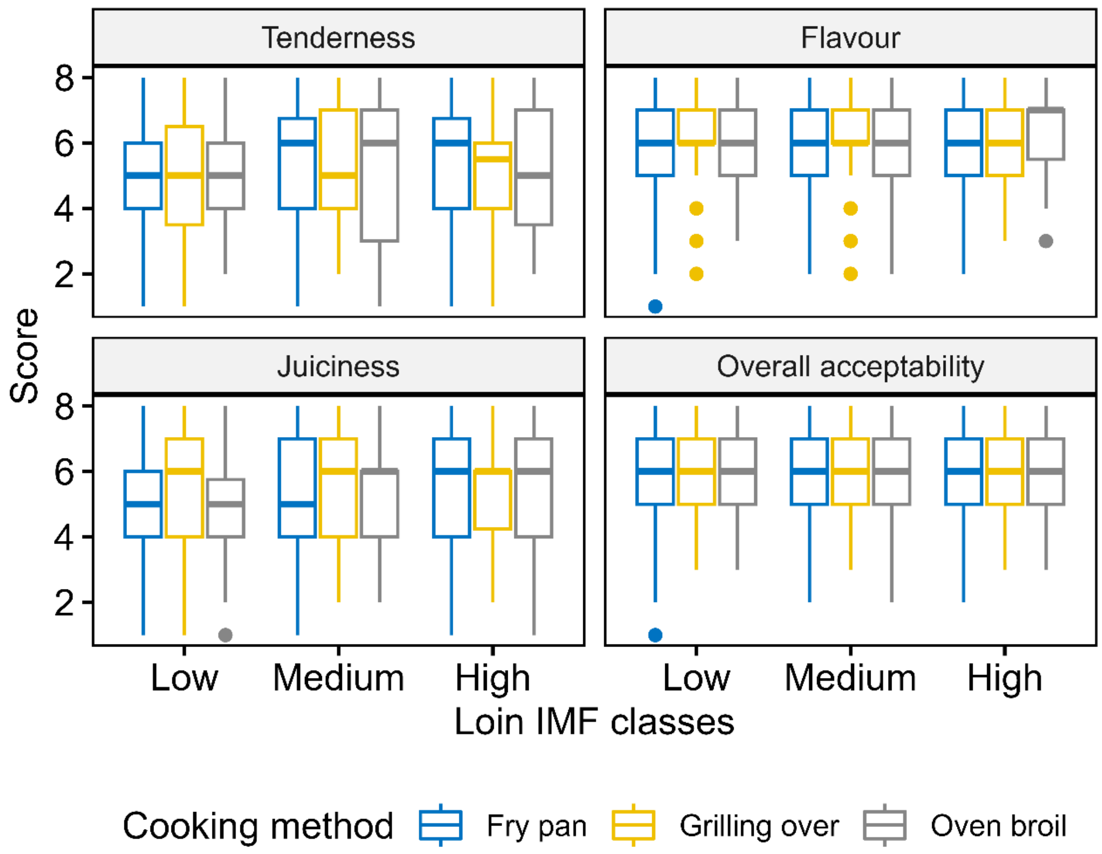

Fig. 10. Descriptive results for the influence loin intramuscular fat (IMF) content classes according to cooking method (fry pan, grilling, oven broil) on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for loin IMF content (%) classes: low (1.3 ± 0.20 %); medium (2.0 ± 0.22 %) and high (3.2 ± 0.81 %).

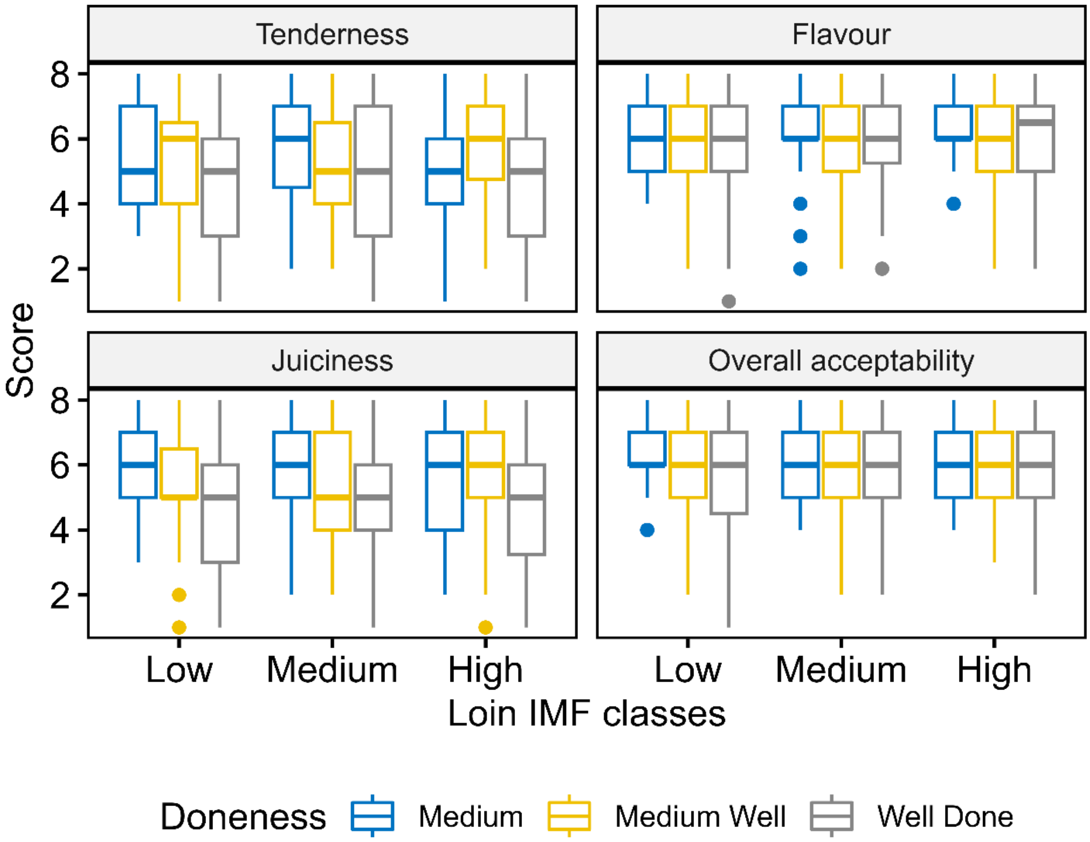

Fig. 11. Descriptive results for the influence loin intramuscular fat (IMF) classes according to degree of doneness (medium, medium well, well done) on consumer response patterns regarding tenderness, juiciness, flavour and overall acceptability. Mean values for loin IMF content (%) classes: low (1.3 ± 0.20 %); medium (2.0 ± 0.22 %) and high (3.2 ± 0.81 %).