Effects of Kalahari Melon *(Citrullus lanatus)* Essential Oil and Butyric Acid Growth Promoters on Gut Health in Growing Pigs

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ABSTRACT

This study was conducted to determine the effect of Kalahari melon essential oil, butyric acid and their blend on intestinal pH, faecal score and incidence of E. coli *and* Salmonella *spp. in the faeces of growing pigs. Forty mixed-sex large white growing pigs were weighed and randomly allocated five dietary treatments: NC – negative control (no growth promoter), PC – positive control growth promoter (zinc bacitracin), EO – Kalahari melon essential oil (0.4%), OA – butyric acid (0.6%), and EOOA – Kalahari melon essential oil (0.4%) + butyric acid (0.6%). Each dietary treatment was allocated eight pigs and each pig was considered an experimental unit. Dietary treatments had no significant effect on intestinal digesta pH, but significantly (P <0.0005) influenced faecal scores in growing pigs. Fluctuation in faecal scores for NC was persistent and characterised by a negative trend. A positive trend in faecal score was noticed for PC, EO and OA, and a fairly constant trend was noticed for EOOA. Dietary treatment OA and EOOA had no traces of* E. coli *and* Salmonella *in the faecal sample compared to NC.* Salmonella *isolates found in NC showed 86% and 98% similarity to* Salmonella enterica *subsp.* enterica *Typhimurium strain CP 11 233.1 16S rRNA and* Salmonella enterica *subsp.* enterica *Typhimurium strain CP 00 6048.1 16S rRNA. Kalahari melon essential oil, butyric acid and their blend have antimicrobial, immunomodulatory properties and have the ability to improve gut health. In the wake of increased incidences of antimicrobial resistance and advocacy for One Health, Kalahari melon essential, butyric acid and their blend can be used as potential growth promoters in growing pigs.*

Keywords: Kalahari melon essential oil, butyric acid, intestinal pH, faecal score, *E. coli*, *Salmonella*

Introduction

Although the use of antibiotic feed additives at non-therapeutic levels to boost growth and production in pigs is of vital importance, it has, however, led to the development of antibiotic resistance and residual contamination of meat (Robinson *et al*., 2019). Antimicrobial resistance is restricted to microbes and their progeny is transmitted to other irrelevant microbial species through the transfer of special genes. The resistant bacteria are then transferred to livestock, humans and the environment (Agyare *et al*., 2022). In 2019, 4.95 million deaths were recorded globally from drug resistant infections, and 1.27 million of those deaths were directly linked to antimicrobial resistance (AMR) (Murray *et al*., 2022). Antibiotic agents frequently used in the treatment and control of enteric infection in pigs have increased the risk of antimicrobial resistance (Burow *et al*., 2014). Luppi *et al.* (2015) reported an increase in the prevalence of resistant *E. coli*, with the infected piglets failing to respond to treatment. Gut health has an important role in the development of the homeostasis of the immune system and provides barriers against pathogens (Clemente *et al*., 2012). *E. coli* infection

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has been identified to be the major challenge in the South African pig production system (Kanengoni *et al*., 2017). It is one of the main factors causing diarrhoea in weaned pigs and is strongly associated with decreased growth performance and increased mortality in pigs (Xu *et al*., 2020). Diarrhoea-causing pathogens involved in pig enteric infections include enterotoxigenic *E. coli* (ETEC), entero-aggregative heat-stable enterotoxin (EASTI) and heat liable (LT) enterotoxins (Gyles & Prescott, 2010. Mohlatlole *et al*. (2013) reported a high prevalence of ETEC, STEC (Shiga Toxin-producing *Escherichia coli*) and EASTI genes in South Africa. Various substitutes to antibiotic growth promoters put to trial have helped improve gut health and pig production (Sethiya, 2016). Herbs and herbal extracts have been proposed as possible replacements, and they are safe for animals, humans and the environment (Hassan *et al*., 2015). Kalahari melon essential oil contains phenolic acids, gallic acid, caffeic acid, protocatechuic acid and furulic acid (Nyam *et al*., 2009) and, in general, phenolic compounds in essential oils have antimicrobial, anti-inflammatory and anti-oxidant properties as well as immunomodulatory action (Nhara *et al*., 2024). Organic acids, including butyric acid in pig diets, are potential alternatives to antibiotic growth promoters. They have antibacterial effects against different pathogenic micro-organisms (Gheisar *et al*., 2015). The blending of organic acids and essential oils may have complementary effects in improving growth as they may elicit selective microbial control against *Clostridium perfringens*, Enterobacteriaceae, enterococci and mesophilic bacteria (Stamilla *et al*., 2020). Organic acids and essential oils blend may also improve gut health, modulate caecal microbiota, and reduce the prevalence of antibiotic-resistant genes in broiler chickens (Bortoluzzi *et al*., 2021). This study, therefore, sought to determine the influence of butyric acid, Kalahari melon essential oil and their blend as potential growth promoters on intestinal pH, faecal score and characterisation of faecal microbiota in pigs.

Materials And Methods

Experimental Site

The study was conducted at the North-West University experimental farm in Molelwane, South Africa, with an average temperature of 3°C and annual rainfall of 300 mm to 500 mm.

Animal Management

A total of 40 mixed-sex large white growing pigs aged eight weeks were used in a 12-week feeding trial. The pigs were given two weeks for adaptation to the diet which was not part of the experimental period. All the pigs were allowed individual *ad libitum* access to feed and water through a self-feeder and nipple drinker throughout the experimental period. On arrival, pigs were placed in pens and were given two weeks to adapt to the diet and environment. Experimental grower diets were offered from 0.5 kgs per day on arrival, and the amount was increased depending on average weights.

Experimental Design and Dietary Treatments

Forty mixed-sex large white piglets were randomly allocated five dietary treatments arranged in a completely randomised design and were fed individually. Each treatment had eight replicates and each pig was considered an experimental unit. Dietary treatments were formulated with the inclusion of the antibiotic growth promoter, zinc bacitracin; organic feed additives, butyric acid (OA); and Kalahari melon essential oil (EOOA) as follows: negative control (NC) no growth promoter, positive control (PC) zinc bacitracin growth promoter, grower diet + EO (0.4%), grower diet + OA (0.6%), and grower diet + OA (0.4%) + EO (0.6%). The diets were analysed for dry matter (DM), crude protein (CP), ether extract (EE), ash, neutral detergent fibre (NDF) and acid detergent fibre (ADF), using the protocol of the Association of Official Analytical Chemicals (AOAC). The diets and compositional analysis are shown in Table 1. The antibiotic growth promoter, zinc bacitracin, was purchased from Animate Animal Health, Edenvale, Gauteng, South Africa. The essential oil and butyric acids were purchased from Nutrica Organics (Pvt Ltd), South Africa. The essential oil was extracted from *C. Lanatus* seed using the cold pressing method. All other dietary components were purchased from Nutri Feeds, Pretoria (SA). The experimental procedure and designs of the study were approved by the North-West University Animal Production Research Ethics Committee (NWU-AnimProdREC), and was granted ethics number: NWU-02010-20-A5.

Table 1: Feed ingredients for dietary treatments

	Dietary Treatments				
Ingredient (%)	**NC**	**PC**	**EO**	**OA**	**EOOA**
Maize	68.245	68.245	68.245	68.245	68.245
Soya bean meal (Local)	25	25	25	25	25
Soya bean crude oil	1	1	1	1	1
Salt – Fine	0.5	0.5	0.5	0.5	0.5
Monocalcium phosphate	0.8	0.8	0.8	0.8	0.8
Calcium carbonate	0.9	0.9	0.9	0.9	0.9
L-Lysine HCl	0.32	0.32	0.32	0.32	0.32
Dl-methionine	0.13	0.13	0.13	0.13	0.13
L-tryptophan	0.01	0.01	0.01	0.01	0.01
L-threonine	0.13	0.13	0.13	0.13	0.13
Vitamin premix	0.1	0.1	0.1	0.1	0.1
Trace mineral premix	0.1	0.1	0.1	0.1	0.1
Copper sulphate, 25%	0.05	0.05	0.05	0.05	0.05
Antioxidant	0.015	0.015	0.015	0.015	0.015
Additives other	0	0	0.1	0.1	0.1
Zinc bacitracin	0	0.1	0	0	0

NC – negative control (no antibiotic growth promoter); PC – positive control (zinc bacitracin growth promoter); EO (Kalahari melon essential oil); OA (butyric acid); EOOA (Kalahari melon essential oil butyric acid blend)

Slaughter of Pigs

At the end of the feeding trial, pigs were deprived of feed to allow for emptying of the gastro intestinal tract (GIT). All pigs were taken to the Roogroid commercial abattoir for slaughter, 15 kms from the farm. The pigs were slaughtered by electrical stunning following the standard procedures for slaughter (Chambers & Grandin, 2001).

Data Collection

Faecal Score

The occurrence and severity of diarrhoea were monitored and assessed for each pig on day three every week using a faecal consistency scoring system (1 = normal faeces, 2 = soft faeces, 3 = mild diarrhoea, 4 = severe diarrhoea (Pedersen & Toft, 2011).

Molecular Characterisation of Salmonella Serovars and Escherichia Coli Isolates

At week four of the feeding trial, faecal samples were collected from the piggery and analysed for *Salmonella* serovars, *E. coli* strains and other bacterial strains colonising pigs. The samples were collected using rectal gloves directly from the rectum, and aseptically gathered in plastic screw-top cylinders. The samples were transported to the Microbiology Diagnostic Laboratory at the North-West University (Mahikeng Campus) for analysis. Total bacterial counts were enumerated, *Salmonella* and *E. coli spp* were isolated from the faecal samples, using Rapaport-Vassiliadis (RV) broth and peptone buffered saline as enrichment media (Zouboulis *et al*., 2004). Dilutions were plated on XLD agar (*Salmonella*) and Eosin Methylene blue agar (*E. coli*) plates and incubated at 42°C for 48 hours. Bacterial identification tests were performed as described by Ahmed and Shimamoto (2012).

A commercial Zymo-Fungal/Bacterial DNA kit was used for DNA extraction, and the purified genomic DNA was quantified using a NanoDrop Lite spectrophotometer. The eluted DNA was purified and stored at -20°C for further analysis. Colonies were suspended in one mL sterile distilled water in Eppendorf tubes and centrifuged at 10.000 rpm for five minutes. Pellets were then suspended in 750 µL lysis solution and vortexed at 14.000 rpm for five minutes. The concentration of purification of DNA was assessed using a nanodrop (Bio-Rad, California, USA). The DNA fragments' concentration for the bacterial isolates was measured using the NanoDrop ND-1000 UV spectrophotometer.

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µL of the upper aqueous phase was aliquoted into a new Eppendorf tube and centrifuged at 7.000 rpm for one minute. The volume of 1 200 µL of the buffer was added to the filtrate, and 800 µL of the mixture was transferred to the new collection tube and centrifuged at 10.000 rpm for one minute. The filtered DNA was pre-washed by adding 200 µL DNA pre-wash buffer and centrifuged at 10.000 rpm for one minute. A total of 500 µL of the DNA wash buffer was added to the new collection tube, centrifuged at 10.000 rpm for one minute and 100 µL of the DNA elution buffer was added to elute the DNA in a clean 1.5 mL micro-centrifuge tube. DNA purity was assessed using a nanodrop (Bio-Rad, California, USA). The DNA fragments' concentration for the bacterial isolates was measured using the NanoDrop ND-1000 UV spectrophotometer (Thermo-Fisher Scientific Inc., USA). A 260/280 ratio between 1.8 and 2.0 indicates pure DNA, a ratio above 2.0 indicates phenol and contamination, and a ratio below 1.8 indicates protein contamination (Psifidi *et al*., 2015). The 16S ribosomal gene amplification was employed to identify *Salmonella* serovars and *E. coli* strains (Muyzer *et al*., 1995; Njie Ateba & Mochaiwa, 2014). The primers used, obtained from Inqaba Biotechnical Industries (Pty) Ltd, South Africa, are shown in Table 2.

Table 2: Primers for amplifying *S.* serovars and *E. coli* strains

Target Gene	Primers Sequence	Predicted Amplicon Size (bp)	References
16S rRNA genes (Salmonella) spp.	27F: (5'-AGAGTTTGATCCTGGCTCAG-'3) 1492R: (5'-GGTTACCTTGTTACGACTT-'3)	1450 bp	(Muyzer *et al*., 1995)
16S rRNA genes (*E. coli*)	GM5F: (5'-TACGGGAGGCAGCAG-'3) 907R: (5'-CCGTCAATTCCTTTGAGTTT-'3)	550 bp	(Muyzer *et al*., 1995)

The polymerase chain reaction (PCR) amplification was done using a Bio-Rad C1000 Touch Thermal Cycler. The thermocycling conditions for *Salmonella* serovars consisted of an initial denaturation step at 95°C for five minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for five minutes and, finally, a single and final extension step at 72°C for seven minutes (Ngoma *et al*., 2013). *Salmonella* Typhimurium ATCC 14028 was used as a positive control, while *E. coli* ATCC 25922 was used as a negative control. The thermocycling conditions for *E. coli* were performed at 95°C for five minutes, 45 cycles of 95°C for 30 seconds, 62°C for 60 seconds, 72°C for 60 seconds, and a final elongation step at 72°C for 5 minutes (Ateba *et al*., 2008). *E. coli* O157: H7 (ATCC 43889) was used as a positive control, while *Salmonella* Typhimurium *ATCC* 14028 was used as a negative control.

After amplification, the molecular weight of samples was determined by gel electrophoresis as described by Sambrook and Russell (2001). About 10 µL of

amplicons were loaded into 1% agarose gel wells containing 0.25 µg ethidium bromide per mL. A DNA marker (Fermentas Life Science, Lithuania) was used. The gel was viewed and photographed under UV light. *Salmonella* serotyping was performed according to the White-Kauffmann-Le Minor scheme by slide (O antigen) and tube (H antigen) agglutination, using specific antisera. At the same time, in *E. coli,* it was done following conventional methods. The amplified product was sequenced using an automated DNA sequencer (SpectruMedix model SCE 2410) at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria. The resulting sequences were compared with those in the National Centre for Biotechnology Information (NCBI) database. The isolates were identified based on the highest percentage of similarity. The search for amino acid homology of the resulting sequence was done using the NCBI Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

Intestinal pH

During slaughter, the intestines were removed and divided into three small intestinal segments: small intestines (duodenum, jejunum and ileum); caecum; and colon (proximal and distal). Intestinal pH was measured in the duodenum, the ileum, caecum and colon (pH values of digesta). The pH values were determined using a portable pH metre. The electrode was rinsed with distilled water and recalibrated between measurements.

Statistical Analysis

All data was subjected to appropriate statistical analysis (SAS, 2010) and comparisons particular to the GLM procedures for once-off data. The PDIFF option of SAS was used for separation of means, while contrasts were used to assess the specific effects of organic acids and essential oils on the different parameters. For all tests, the level of significance was set at $P < 0.05$. The following model was used for data analysis of the once-off data, with diet as the main factor:

$$Yij = \mu + Ti + \varepsilon ij$$

where Yij = observation (intestinal digesta pH and incidence of *E. coli* and *Salmonella* isolates in faeces), µ = population mean constant common to all observations, Ti = effect of diet, and εij = random error term.

Results

Intestinal pH

Table 3 presents the effect of Kalahari melon essential oil, butyric acid and their blend on intestinal digesta pH in growing pigs. Dietary treatments did not influence intestinal digesta pH ($P < 0.05$), although PC (5.67 ±0.23) numerically had the lowest duodenal pH, and EO (6.16 ±0.23) had the highest pH. Ileal pH was also not affected by diet ($P > 0.05$). Colon pH was highest in NC (6.17±0.16) and was significantly different from all other dietary treatments ($P < 0.05$). Caecal pH was highest in NC (6.28±0.13) and lowest in PC (5.71±0.13).

Table 3: Effect of Kalahari melon essential oil, butyric acid and their blend on intestinal digesta pH in growing pigs

Parameter	NC	PC	EO	OA	EOOA	SE
Duodenum pH	6.06	5.67	6.16	6.06	5.92	0.23
Ileum pH	6.38	6.49	6.46	6.23	6.20	0.15
Colon	6.17^{b}	5.66^{a}	5.78^{a}	5.61^{a}	5.77^{a}	0.16
Caecum	6.28^{b}	5.71^{a}	5.90^{a}	5.87^{a}	6.01^{a}	0.13

^{abc} Means in the same row with different superscripts are significantly different (P <0.05). ***(P <0.0005),** (P <0.005),*(P <0.05) and NS – no significant difference, NC – negative control (without growth promoter), PC – positive control (with growth promoter), EO – Kalahari melon essential oil, OA – butyric acid, EOOA – Kalahari melon essential oil and butyric acid blend, SE - standard error

Faecal Score

Figure 1 presents the effect of Kalahari essential oil, butyric acid and their blend on faecal score and diarrhoea incidence on growing pigs. Dietary treatments influenced faecal scores and diarrhoea incidence (P <0.05) during the 12-week feeding trial in growing pigs. High diarrhoea incidence was noticed in the first three weeks of the feeding trial for dietary treatments NC, PC, EO and OA. Noticeably, high values of faecal scores for NC treatment persisted throughout the whole feeding trial. In contrast, a negative trend showing a decline in faecal score was noticed for dietary treatments PC, EO and OA over the feeding period. High faecal scores were recorded in the first three weeks of the feeding trial, and they declined as the feed trial reached termination. A fairly constant trend was noticed for dietary treatment EOOA throughout the feeding trial, and was characterised by a low faecal score (1±0.73).

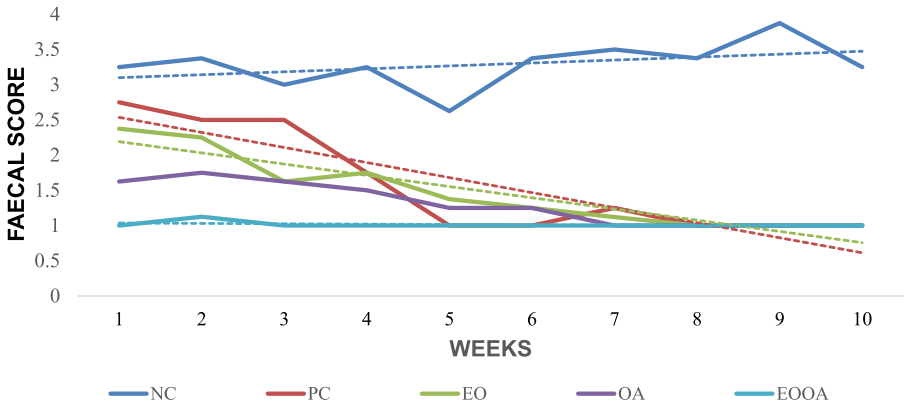


Figure 1: Effect of Kalahari melon essential oil, butyric acid and their blend on faecal scores in growing pigs

Molecular Characterisation of Salmonella Serovars and Escherichia Coli Isolates

Table 4 presents results on presumptive *E. coli* and *Salmonella* strains isolated from faecal samples of infected growing pigs fed Kalahari melon essential oil, butyric acid and their blend. Dietary treatment influenced the presence or absence of *E. coli* and *Salmonella* strains in faecal samples. Dietary treatment OA and EOOA had no traces of *E. coli* and *Salmonella* in the faecal sample. Dietary treatment NC contained three strains of *E. coli* and two strains of *Salmonella*. *E. coli* strain G388-aco-37 had 98% similarity with the cultured isolate from faecal samples. Dietary treatment PC contained isolate strain similar to *E. coli* strain IS 03 165 rRNA, with 90% similarity. *Salmonella* isolates found in NC showed 86% and 98% similarity to *Salmonella enterica* subsp. *enterica* Typhimurium strain CP 11 233.1 16S rRNA, and *Salmonella enterica* subsp. e*nterica* Typhimurium strain CP 00 6048.1 16S rRNA.

Table 4: Identity of presumptive *E. coli* and *Salmonella* strains isolated from faeces of infected growing pigs fed Kalahari melon essential oil, butyric acid and their blend

Treatments/ Isolates	Reference from NCBI	% Similarity	Account number in GenBank (PCR)
NC	*Escherichia coli* strainG388-aco-37 16S ribosomal RNA gene	98.00	KY962911.1
NC	*Escherichia coli* strain S-P-C-026.01 chromosomal	80.58	CP092816.1
NC	*Escherichia coli* strain S-P-N-038.01	80.66	CP092701.1
PC	*Escherichia coli* strain 1503165 ribosomal RNA	90.00	KY971288.1
EO	*Escherichia coli* strain 1AUK 8730165 16S ribosomal RNA	99.00	MK560211.1
NC	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain CP11233.1 16S ribosomal RNA	86.00	MH356714.1
NC	*Salmonella enterica* subsp. *enterica* serovar Typhimurium strain CP006048.1 16S ribosomal RNA	98.00	MH356714.1

NC – negative control (without growth promoter), PC – positive control (with growth promoter), EO – Kalahari melon essential oil, OA – butyric acid, EOOA – Kalahari melon essential oil and butyric acid blend

Discussion

Intestinal Digesta pH

Overall, intestinal digesta pH was slightly high for dietary treatment with no growth promoter. Organic acids reduce pH in the GIT and inhibit the invasion of

pathogenic bacteria (Kil *et al*., 2011). Upadhaya *et al*. (2018) reported an increase in the *Lactobacillus* population in pigs fed a diet supplemented with an organic acid. Survival of pathogenic bacteria like *E. coli* is influenced by low pH, and *E. coli* is less tolerant of acidic conditions (Warnecke & Gill, 2005). Low pH in the GIT increases the activity of enzymes and alters villus height and depth in the small intestines (Montoro-Huguet *et al*., 2021). Low pH in the colon for dietary treatment butyric acid is in line with the findings of Merchant *et al.* (2011) on the presence of bacteria, and their fermentation products such as VFA lower colonic pH.

Faecal Score

Dietary treatments with the antibiotic growth promoter, Kalahari melon essential oil and butyric acid showed a positive response to faecal scoring due to their ability to suppress pathogenic microbes in the GIT. Essential oils and organic acids have shown effectiveness in modulating intestinal health and decreasing the incidence of diarrhoea. Studies on essential oils have confirmed antimicrobial activity on pathogenic microbes. In the current study, Kalahari melon essential oil could have initiated damage to bacterial cell membranes by compromising the pH homeostasis of the bacterial cell membrane, leading to a collapse of the proton motive force and deletion of the ATP pool in the microbe. Essential oils have a certain degree of selectivity toward gram-negative bacteria than toward gram-positive bacteria (Lange *et al*., 2010). Organic acids lower pH in the GIT, maintaining the balance of microflora. Organic acids, when included in diets, prevent the growth of gut microbial pathogens without damaging the beneficial bacteria *(Long et al*., 2018; Suiryanrayna & Ramana, 2015). Butyric acid reduced coliform bacteria in the ileum and reduced post-weaning diarrhoea in pigs (Ferronato & Prandini, 2020). Essential oils and organic acid blends promote beneficial microbes in the GIT (Han *et al*., 2018), and they have antibacterial and antifungal properties (Allagui *et al*., 2024) which inhibit pathogens, reduce the amount of diarrhoeal incidence and improve animal health (Cho *et al*., 2018).

Salmonella Serovars and Escherichia Coli *Isolates*

In the current study, dietary treatments, Kalahari melon essential oil butyric acid blend, and butyric acid showed no traces of *Salmonella* and *E. coli* isolates. Inclusion of the antibiotic growth promoter and butyric acid alone in the dietary treatments resulted in the isolation of different strains of *E. coli* and *Salmonella*. The inclusion of antibiotics in pig diets cannot be that efficient as some pathogenic bacteria have the potential to mutate and develop antibiotic-resistant genes (Monger *et al*., 2021). The absence of antibiotics and/ or antibiotic alternatives gives pathogenic bacteria an ideal environment to multiply and colonise the GIT as shown in the dietary treatment with no growth promoter. Acidifying feed in weaned pigs has the potential to reduce haemolytic *E. coli* count in the small intestines (Suiryanrayna & Ramana, 2015). The organic acid, lactic acid, reduces GIT pH and delays the multiplication of enterotoxigenic *E. coli.* Organic acids balance GIT bacteria by reducing acid-intolerant bacterial species like *E. coli* and *Salmonella* (Ferronato & Prandini, 2020). Butyric acid has the potential to

reduce faecal shedding of *S.* Typhimurium (Boyen *et al*., 2008). Short-chain fatty acids and medium-chain fatty acids have antimicrobial properties and the ability to decrease intestinal colonisation by *Salmonella*, influence virulence gene expression and epithelial invasion (Boyen *et al*., 2008).

Conclusion

It can be concluded from this study that Kalahari melon essential oil, butyric acid and their blend can be used in place of non-therapeutic antibiotics in pig diets and production. Butyric acid, Kalahari melon essential oil and the complementary effect of their blend help promote gut health and reduce diarrhoea prevalence without affecting pig health and general performance. While this points to an improvement in the general performance of pigs, it may be interesting to assess the influence of the two products and their blend on incidence of resistant *E. coli* and *Salmonella* genes and pork quality, the last being the ultimate product consumed by humans.

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