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Performance and Microbiological Profiles of Piglets Fed with Diets Enriched with Bio-flavonoids and Ascorbic Acid

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ABSTRACT

The objective of this study was to evaluate the performance and microbiological profile of 40 piglets (females and males) in the nursery phase. The experimental design was completely randomized, with four treatments, five replicates and sex as a blocking factor. The treatments were distributed in: T1 (control); T2 (Plant Extract as PE, 500 ppm); T3 (Amoxicillin as A, 20 mg kg^{-1}) and T4 (PE+A, 500 ppm + 20 mg kg^{-1}). There was no influence (P>0.01), between treatments for both the initial and the final weight and average daily gain, but the control group males had an average daily feed intake of 1.8% or higher (P<0.01) compared to other treatments. The total count control bacterial colonies were 35.9%, 70.9 % and 63.8 % higher (P<0.01) to treatment with A, PE+A and PE, respectively. For MacConkey test, the treated group A was 88.44 %, 91.78 % and 56.50 % higher (P<0.01) compared to PE+A, PE and control, respectively. The antibiogram of 48 stool samples had shown that Amoxicillin disk were at 85.7 %, 72.7 %, 44.5 % and 100 % resistant in the control treatments, PE, A and PE+A respectively. The bioflavonoids and ascorbic acid and the interaction with amoxicillin did not alter the performance of pigs in the nursery phase but had reduced the presence of bacterial colonies.

Key words: Amoxicillin, Bacterial colonies, *E. coli,* Nursery, Plant extract

INTRODUCTION

The continuous overcoming of the technical difficulties is a challenge that marks the lives of modern pig farming. There were numerous contributions ranging from genetic improvement, the improvement of knowledge about nutrition and health, ambience, facilities and reproduction. Nevertheless, there are several challenges to be overcome in all sectors of pork production, because even with the modern technologies available, the piglets are still suffering from the enteropathies (Anami et al., 2008).

Diarrhea, and other diseases that affect the digestive tract of pigs have many factors and the major contributors involved are the *Clostridium perfringens* type A or type C, *Escherichia coli* enterotoxigenic, *Isospora suis, rotavirus* and transmissible gastroenteritis virus of pigs (Yaeger et al., 2007; Hur and Lee, 2012). In a brief consideration of the occurrence of diarrhea in pigs, which determines the importance of these episodes are factors such as the number of patients, the course of the disease, the degree of dehydration of the affected piglets, the specific mortality due to a problem, the repetition of episodes in different lots and the quantities and efficiency of drugs and vaccinations in course (Barcellos et al., 2011). Diarrhea occurs with clinical signs such as loss of solutes and water, electrolyte depletion, acid-base imbalance and dehydration, which can be fatal if not treated properly (Zlotowski et al., 2008). The treatments are difficult, facing high and often inefficient costs, but prevention and proper management are effective ways to reduce the incidences of diarrhea. The need to ensure the zootechnical and economic results of pig production encouraged the

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routine incorporation of antibacterial (growth promoters) in feed intended for stages of the production process. Accordingly, new alternatives to ensure animal performance, quality of final product and reduce undesired waste to consumer health are being scientifically considered.

Research has been conducted with prebiotics, enzymes, organic acids and plant extracts (PE). Organic acids (OAs) have been widely used in pig diets, acting as a promising substitute for antibiotics and antibiotic growth promoters (AGP) (Lei et al., 2017; Long et al., 2018). Previous studies showed that both free and encapsulated organic acids supplementation improved the performance of weaned piglets and also intestinal morphology and health (Diao et al., 2016). The PE have been represented by phenolic compounds (flavonoids or bioflavonoids), and ascorbic acid. The bioflavonoids are natural antioxidants with anti-inflammatory action, anti-microbial, antiallergics and immune-stimulating (Cushnie & Lamb, 2005). Ascorbic acid takes part in several metabolic processes, such as the formation of collagen, synthesis of epinephrine, corticosteroids and bile steroids (Pion et al., 2004). Besides enzyme cofactor, ascorbic acid participates in the redox processes, enhancing iron absorption and inactivation of free radicals (Padayatty et al., 2003). The benefits of therapeutic use of ascorbic acid in pigs are observed in the performance, pre-slaughter stress and meat quality (Pion et al., 2004).

The individual properties and synergistic action of its active ingredients, the use of PE can enhance the immune response of piglets in nursery phase. Although there is positive information related to the synergy of the constituents of PE, its use in the control of clinical signs of diarrhea in piglets are weak and inconclusive. The objective of this work was to evaluate the performance and the microbiological profile of piglets in the nursery phase fed with diets containing bio-flavonoids and ascorbic acid.

MATERIALS AND METHODS

The animals were housed in Sector Swine Department of Animal Science UFSM, Brazil. The experiment was performed in the period 2 to 23 December 2016. The experimental units had an average weight of ± 5.89 kg. The nursery shed had 48 raised bays at 0.40m from the ground, with leaked plastic floor with $2m^2$ of area per bay 48 raised bays. The stalls were equipped with semi- automatic feeders and drinker's pacifier type, with height adjustment. The shed had automatic drive curtains, a set of four exhaust fans and air conditioning.

The animals upon arrival were weighed, identified and distributed in the treatments. Forty animals housed were used in 40 stalls, 20 females and 20 males weaned, arranged in a completely randomized design with four treatments, five replicates and used sex as blocking factor.

Treatments

The treatments were as follows, respectively: T1 (control); T2 (PE, 500 ppm); T3 (Amoxicillin, 20 mg kg^{-1}) and T4 (PE+A, 500 ppm + 20 mg kg^{-1}). The PE consists of lactic acid (180 g kg^{-1}), vitamin C (5.200 g kg^{-1}), flavonoids (344 mg kg^{-1}), citric acid (400 g kg^{-1}), phosphoric acid (15 g kg^{-1}), fumaric acid (20 g kg^{-1}). The Amoxicillin (50 g) was used at a dose of 20 mg per kilogram of weight. The animals were subjected to constant clinical evaluations regarding the degree of hydration and diarrhea symptoms. The minimum and maximum temperature was recorded twice daily. The animals had consumed a commercial iso-nutritive diet, following the nutritional requirements of NRC (2012) (Table 1), the animals were fed at will and had ad libitum access to water. The weight gain data were obtained weekly and weights of individual animals. The daily feed intake was obtained by according to the weighing the ration provided, that were remaining as daily leftovers present in the feeders. Feed conversion was estimated from the previous variables.

Ethical approval

This work was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil.

Microbiological profile

To evaluate the microbiological profile, animal feces were collected, identified and sent to the Bacteriology Laboratory (LABAC) of the Federal University of Santa Maria (UFSM) for counting bacterial colonies. The fecal samples were taken on days the 7th, 14th and 21st of the experiment. PCA was used to count colonies (Plate Count Agar) and MacConkey. Further biochemical tests were performed (oxidation and fermentation of sugars (GOF), Indol production (SIM), use of sugar (TSI) and urease) for the identification of *E. coli*. The colonies were stored in glycerol for subsequent culture and sensitivity of 48 preserved specimens. The samples passed through subculture in VBR and then taken to an oven for 24 hours at 37ºC. After this period, with platinum loop, some colonies were added to Muller Hilton broth (MH) and thereafter on MH agar and added to the antibiotic disks to be routed to incubator for 24 hours at 37°C. After 24 hours the reading made with a transparent millimeter ruler to determine the diameter in mm for each inhibition zone. The following discs were used for susceptibility testing: Polymyxin (30 ug), Cefepine (30 µg), Amoxicillin (10 mg), Neomycin (30 µg), Norfloxacin (10 mg), Ceftriaxone (30 µg), Meropenem (10 mg) and Ampicillin (10 mg).

Table 1. Proximate analysis of diets supplemented piglets in nursery phase diets containing bioflavonoids and ascorbic acid

Ingredients	Pre-initial I*	Initial I*
Crude Protein (g kg^{-1})	180	177.99
Humidity (g kg^{-1})	120	120
Formic Acid (mg kg^{-1})	325	-
Folic Acid (mg kg^{-1})	-	0.60
Pantatenic Acid (mg kg^{-1})	-	13.65
Biotin (mg kg^{-1})	-	75
Cobalt (mg kg^{-1})	-	19.95
Lactic Acid (mg kg^{-1})	760	-
Calcio Pantothenate (mg kg^{-1})	11	-
Fruit Aroma (mg kg^{-1})	30	-
BHT (mg kg^{-1})	97	-
Calcio (mg kg^{-1})	8,000	8
Calcio (mg kg^{-1})	5,000	6.25
Cuprum (mg kg^{-1})	8	0.6
Colina (mg kg^{-1})	-	349
Etoxiquin (mg kg^{-1})	-	25
Ethereal extract (mg kg^{-1})	25	27.6
Iron (mg kg^{-1})	79	112
Gross fiber (g kg^{-1})	35	22.6
Phosphorus mg kg^{-1})	3,500	5.4
Halquinol (mg kg^{-1})	120	-
Iodine (mg kg^{-1})	0.49	1.95
Lisina (g kg^{-1})	12	6,175
Manganes (mg kg^{-1})	30	62.25
Mineral Material (g kg^{-1})	60	33.38
Metionina (mg kg^{-1})	5,000	3,394
Niacina (mg kg^{-1})	18	33
Selenio (mg kg^{-1})	0.25	0.3
Sodio (mg kg^{-1})	1,800	0.24
Treonina (mg kg^{-1})	1,800	-
Triptofano (mg kg^{-1})	790	-
Vitamin A (UI kg^{-1})	6,400	15,900
Vitamin B12 (mcg kg^{-1})	30	30
Vitamin B1 (mg kg^{-1})	-	1.2
Vitamin B2 (mg kg^{-1})	4	8.4
Vitamin B6 (mg kg^{-1})	2	3.39
Vitamin D3 (UI kg^{-1})	1,200	32,400
Vitamin E (UI kg^{-1})	30	39
Vitamin K3 (mg kg^{-1})	1	1.65
Zinco (mg kg^{-1})	2,500	2,250

Basic product composition: folic acid, nicotinic acid, calcium carbonate, sodium chloride, choline chloride, etoxiquin, transgenic soybean meal, ground corn, phytase, dicalcium phosphate, halquinol, potassium iodate, zinc oxide, calcium pantothenate, sodium selenite, cobalt sulfate, copper sulfate, iron sulfate, manganese sulfate, vitamin A, vitamin B1, B2, B6, B12, D3, E and K.

Data Analysis

The data were submitted to variance analysis by the GLM procedure in the 5% level of significance. The effects included in the analytical model were treatments (T) and week (S). Any differences between the means were compared by Tukey test (P<0.05). Statistical analyzes were performed using the statistical software Minitab version 15 (Mckenzie and Goldman, 2010). The antibiotic susceptibility data were assessed through sensitivity percentage, intermediate susceptibility and resistance to antibiotics.

RESULTS

Animal performance did not differ (P>0.01) between treatments for both the initial and the final weight and average daily gain. However, the control group males had an average daily feed intake of (CDMR) 1.8% higher (P<0.01) to other treatments and feed conversion (FC) 2.11% higher (P<0.01) compared to treatment with PE (Table 2). The total count of bacterial colonies in the control group were at 35.9%, 70.9% and 63.8% higher (P<0.01) to treatment with A, PE+A and PE, respectively. The count of small colonies in the control group was 72.3%, 75.99% and 75.35% higher (P<0.01) to treatment with A, PE+A and PE, respectively. The average colony count of treatment with A was 58.27%, 75.37% and

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99.82% higher (P<0.01) to treatment with PE, PE+A and the control group, respectively. Regarding the large colonies there were no differences (P>0.01) between treatments. Therefore, in the count MacConkey way, the treated group A was 88.44%, 91.78% and 56.50% higher (P<0.01) to treatment with PE+A, PE and control, respectively (Table 3).

In assessing the susceptibility testing (percentage data), it was observed that antibiotics (Cefepine, CPM; Ceftriaxone, CRO and Merepenem, MPM) showed 100% sensitivity independent of the treatments. The polymyxin showed 100% resistance in the control treatments, PE, and PE+A, but 66.6% of resistance in the treatment A. The antibiotic susceptibility to the amoxicillin disc had showed 85.7%, 72.7%, 44.5% and 100% resistance in the control treatments, PEA and PE+A, respectively (Table 4). The disks with ampicillin and Norfloxacin showed 88.8% and 88.8% strength, respectively in treatment A. In summary, we can see that the antibiogram with Amoxicillin discs, Norfloxacin and Ampicillin showed higher percentage of resistance in dependent of treatments. Still, we can see that the antibiogram with neomycin discs showed 100% of intermediate sensibility in treatment with PE+A and0% resistance in the other treatments.

Table 2. Performance of piglets in nursery phase fed diets containing bioflavonoids and ascorbic acid

Treatments (T)	Weight In		Weight Fi		ADFI		DWG		CF	
	M	F	M	F	M	F	M	F	M	F
Control	5.69	5.55	9.01	8.90	0.53[a]	0.53	0.36	0.36	1.42[a]	1.41
Plant Extract (PE)	5.78	6.61	9.16	9.98	0.52[b]	0.52	0.36	0.36	1.39[b]	1.39
Amoxicillin (A)	5.88	6.61	9.22	9.92	0.52[b]	0.52	0.36	0.36	1.39[ab]	1.41
PE+A	5.41	5.67	8.76	8.79	0.52[b]	0.52	0.36	0.36	1.40[ab]	1.41
RSD	0.56	0.54	0.89	0.83	0.01	0.01	0.01	0.01	0.01	0.03
Probability										
T	0.87	0.91	0.23	0.17	0.01	0.08	0.85	0.94	0.01	0.54
R	0.01[1]	0.01[2]	0.69	0.70	0.01[3]	0.07	0.11	0.54	0.01[4]	0.21

Weight In= initial weight; Weight Fi= final weight; ADFI= average daily feed intake; DWG= daily weight gain; CF= conversion food; RSD = residual standard deviation; R= repeat; M= male; F= female; Regression equations for [1]Weight In males: (Weight In=4.65+0.59R) =; [2]Weight In females: (Weight In=6.03+0.10R); [3]ADFI males: (ADFI=0.52+0.0008R); [4]CF machos: (CF=1.38+0.012R).

Table 3. Count of bacterial colonies of fecal content in initial phase of piglets nursery fed diets enriched with bioflavonoids and ascorbic acid

Treatments	Count (UFC mL^{-1})				
	Total Count	Small Colony	Medium Colony	Big Colony	mC
Control	684797437.5[a]	507353125[a]	515000[c]	24855375	24855375[b]
Plant Extract (PE)	247453000[c]	125062500[b]	121118125[b]	1334875	4693963[b]
Amoxicillin (A)	438725000[b]	140493750[b]	290243750[a]	250000	57148625[a]
PE+A	198959562.5[c]	121786875[b]	71472688[c]	2668750	6604125[b]
RSD	179	129	142	288	239
Probability					
T	0.01	0.01	0.01	0.14	0.01
W	0.01[1]	0.01[2]	0.01[3]	0.01[4]	0.01[5]

Atb= antibiotic; RSD=residual standard deviation; W=Week; mC= MacConkey Agar; Regression equation for week evaluation: [1]W=2.80 - 0.000000 The total colony count; [2]W=2.84 - 0.023000 Count small colonies;[3]W=2.44 + 0.031000 Count medium colonies; [4]W=2.72 - 0.0420000 Count large colonies; [5]W=2.62 - 0.063000 mC.

Table 4. Antibiogram (sensitivity in %) of piglets from stool samples in nursery phase fed diets containing bioflavonoids and ascorbic acid

Treatments	Sensitivity	Polym	CPM	AMO	NEO	NOR	CRO	MPM	AMP
	S	100	100	14.3	85.7	100	100	100	14.3
Control	IS	0	0	0	14.3	0	0	0	0
	R	0	0	85.7	0	0	0	0	85.7
	S	100	100	27.3	54.5	36.4	100	100	27.3
Plant Extract (PE)	IS	0	0	0	45.5	9.1	0	0	0
	R	0	0	72.7	0	54.5	0	0	72.7
	S	66.6	100	33.3	55.5	11.2	100	100	11.2
Amoxicillin (A)	IS	22.2	0	22.2	44.5	0	0	0	0
	R	11.2	0	44.5	0	88.8	0	0	88.8
	S	100	100	0	0	0	100	100	33.3
PE+A	IS	0	0	0	100	0	0	0	0
	R	0	0	100	0	100	0	0	66.6

Polym= polymyxin; CPM= Cefepine; AMO= Amoxicillin; NEO= Neomycin; NOR= Norfloxacin; CRO= Ceftriaxone; MPM= Merepenem; AMP= Ampicillin; S= Sensitive; IS= Intermediate sensitivity; R= Resistant

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DISCUSSION

Currently, the nutritional aspects involving the plant extracts have been measured by the antimicrobial activity they have on the enzyme systems, cell structures and biological molecules. However, it is interesting to note that the biological effects of natural antioxidants are enhanced by interactions between the constituents of the formula (Middleton et al., 2000). For example, bioavailability and efficiency of vitamin C and bioflavonoids are lower than if they were administered alone (Navarro et al., 2008). Among the various actions, antioxidants protect the immune system. The bioflavonoids modulate inflammatory responses, such as inhibition of PGE2 inhibition of IgE and membrane myelin phagocytosis in multiple sclerosis process (Flórez, 2002). Currently, the acidifying is used in the diets of pigs in the early stages of growth, aiding in performance after weaning (Miguel, 2008).

In our study, we observed a higher average daily feed intake of males in the control group and consequently lower feed efficiency, compared to the other treatments. In another study, one of the constituents of the formula for PE, more specifically fumaric acid was used in the diet of piglets in post- weaning, it had improved animal performance, both the weight gains and feed conversion as well as increased feed intake (Teixeira et al., 2003). Already using 1.5 to 3.0 % citric acid in piglet's diet post weaning, showed no improvement in weight gain and feed efficiency (Radecki et al., 1988). Already, Xu et al. (2018) testing the combination of OAs and oils essentials didn't show a larger positive effect on intestinal health than when supplied individually, but it may increase the growth performance according to the complementary effects of OA and oils essentials in weaned piglets. Therefore, the responses of the performance characteristics and the apparent digestibility coefficients of nutrients, acidifying front of supplementation are variable and contradictory (Miguel, 2008).

Several hypotheses are suggested regarding the mechanism of action of acidifying and between the reduction in stomach pH, changes in intestinal microflora (by control bactericide) or bacteriostatic, improved digestibility and nutrient retention (Miguel, 2008). It is important to remember that the secretion of hydrochloric acid in young piglets is limited due to insufficient production of hydrochloric acid. This was observed in another study, which assessed liquid diets fermented or not, and stressed that reducing the pH favor the use of short-chain fatty acids (organic acids) and control of enterobacteria (Canibe et al., 2007). Accordingly, the use of acidifiers in the diets of weaning piglets in the post may serve as an adjuvant to control pH of the stomach and assist in the digestion of food grain-based and vegetable bran (Gallo et al., 2003). However, we note that the most consistent results are relative to antimicrobial power of acidifying.

This power, in most cases occurs when stomach pH has decreased. One of microbial control mechanisms refers to the capacity of that acidifying have to change the pH of the environment due to its potential dissociation (pKa) between the dissociated and non- dissociated (Partanen and Mroz, 1999). The absorption of organic acids takes place faster when the luminal pH value is smaller than pKa of the acids. The pKa of an acid is the pH at which 50% of the acid is in the ionized form, being determined by the negative logarithm of the acid ionization constant, or Ka, which in turn indicates the acid strength, so its tendency to donate protons. For be expressed logarithmically, one pH unit above the pKa of an acid indicates that 90% of the acid is in the non-dissociated form and with two pH units above the pKa, 99% of the acid will not be dissociated and pKa of the most acidic are between 3 and 5 (Bellaver and Scheuermann, 2004; Thompson and Hinton, 1997).

When the acid is in the ionized form can diffuse freely through the semipermeable membrane of the microorganism to its cellular cytoplasm and into the cell in a more alkaline environment releases the proton resulting in a decrease of the intracellular pH (Canibe et al., 2001). This aspect influences the microbial metabolism, inhibiting the release of important enzymes and forcing the bacterial cell to use energy to release protons, leading to intracellular accumulation of anions and consequently reduces their growth rate and this due to energy consumption through the action of pumping ATPase proton pump (H^+) until exhaustion of this bacterium (Gauthier, 2005).

These events reported above may have happened in our study because the total count of bacterial colonies was more favorable to treatment with PE and PE+A. As an example, the total colony count of treatment with PE was 70.9% lower than the control group, which brings us the possibility of PE effectively present antimicrobial properties. In this sense, the result for *Mac Conkey* method way helps to differentiate presumptively, the genera and species of microorganisms by staining or colony morphology. In our work, we found that the group receiving PE, had decreased by 91.78% in colony as compared to the control group. Through these results and the biochemical tests carried out, we can estimate that these colonies are of the genus *E. coli.* is the microorganism present in samples of feces and part of the intestinal saprophytic flora. *E. coli* is facultative anaerobic bacterium rod morphology, gram negative, fermenting lactose and easily grows in culture media such as *Mac Conkey* agar, forming large red colonies (Gyles and Fairbrother, 2004). Under biochemical evaluation, it shows a positive reaction for indole, negative for urease production and hydrogen sulfide and does not use citrate as a carbon source. These tests sallow a distinction between Enterobacteriaceae (Debroy and Maddox, 2001).

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Among the measures to reduce and control *E. coli* is antibiotic therapy (Glattleider, 1993). Sensitivity to antibiotics many work shaves been performed with varying results (Wilson, 1981; Brito et al., 2000). Because of the diversity of *E. coli* front antimicrobial behavior, especially by using sub doses of antibiotics and the easy transfer of resistance by plasmids from bacterial samples in our study was conducted antibiogram of feces samples from all treatments. We have observed that the treatments, actually did not influence the susceptibility testing results. The observed sensitivity can be the direct effect of antibiotic disks ad deed to the samples.

The antibiotics (Cefepine, Ceftriaxone, and Merepenem) were efficient in all samples. These antimicrobial agents are probably more efficient to inhibit microbial wall synthesis, resulting in bacterial death. This is possible because the Gram-negative cells such as *E. coli* have a much smaller amount of peptidoglycan than Gram positive. This makes its cell wall is not as thick and strong as the others above, but its structure is more complex due to the fact of the existence of membrane lipoproteins, polysaccharides and phospholipids, which involves its cell wall (Kinn et al., 2005).

To access the bacterial cell, antibiotics must cross through the cell wall porin protein channels embedded in lipid structure that present the inside with hydrophilic characteristics. So, antibiotics with greater activity against gram-negative are those with ionizable groups in its chemical structure (Guimarães et al., 2010).

Therefore, the characterization of an intestinal imbalance, diarrhea, commonly appears as an important sign of the complexity of the process. It is interesting to remember that bowel momentum is continuing, with immune responses of different intensities on substances or offensive and harmless agents. These tax challenges, often multifactorial actions that are caused by a plethora of causes, nutritional or dietary resources can be interesting to accelerate the recovery of any damage to the digestive system. To this end, further studies are needed to evaluate the optimal levels of inclusion and better combinations of plant extracts, given the possibility of improving the immune response of piglets in nursery phase.

CONCLUSION

The use of bioflavonoids and ascorbic acid did not alter the performance of pigs in the nursery phase. The use of plant extracts and associated with Amoxicillin reduces the count of bacterial colonies. It was observed that the high resistance of the studied samples for amoxicillin, neomycin and Norfloxacin. The antimicrobial Cefepine, Ceftriaxone and Merepenem were more efficient in inhibiting the growth of E. coli strains isolated from pigs supplemented with ascorbic acid and bioflavonoid.

DECLARATIONS

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Author’s contribution

The authors, Magali Fernandes de Oliveira, Carlos Augusto Rigon Rossi, Matheus Shardong Lucca, Marcelo Soares, were responsible for collection and tabulation of data, experimental management of the pigs, as well as the article writing. The authors, Carlos Augusto Rigon Rossi, Vladimir de Oliveira, Julianni Dornelles, Luara Medianeira de Lima Schlösserand Cristian Guilherme Gräf were responsible for reviewing the manuscript.

Consent to publish

The authors, Magali Fernandes de Oliveira, Carlos Augusto Rigon Rossi, Matheus Shardong Lucca, Marcelo Soares, Vladimir de Oliveira, Julianni Dornelles, Luara Medianeira de Lima Schlösserand Cristian Guilherme Gräf are in favor of publishing the article entitled: “Performance and Microbiological Profiles of Piglets Fed with Diets Enriched with Bio-flavonoids and Ascorbic Acid” in the World’s Veterinary Journal.

Competing interests

The authors have declared that there is no competing interest exists.

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