Original Article

Fluctuations in *Lactobacillus* and coliforms population in faeces of piglets and antibiotic susceptibility of *Escherichia coli* strains isolated from stool samples of piglets

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Abstract

The aim of study was to assess the intestinal health status of the weaning piglets by analyzing the *Lactobacillus*: coliforms ratio (L/C ratio), the number of *E. coli*. as the pathogenic indicator, and to determine the antibiotic susceptibility of *E. coli* isolates. A total of 180 weaned piglets (weaning at 25d of age) of two trials were allotted to 3 treatments (n = 90, 30 piglets/treatment): control (CON, = Basal diet (BD); treatment 1 (T1 = BD + Super-Biotic) and treatment 2 (T2= BD + Bergazyme P®). The total *Lactobacillus, coliforms*, and *E. coli* were counted from fecal samples one day before weaning and four times at the post-weaning stage until 55 days old (31, 37, 44, and 54 d of age). Minimum Inhibitory Concentrations (MICs) were performed by micro-broth dilution and interpreted conforming to standard references. The dynamics of the lactobacilli population as influenced by growth stage and diet supplementation were not significant difference between the 3 treatments. *Lactobacillus* count in feces of weaned pigs was up to 10^6 to below 10^9 cfu/g. The average fecal coliform concentration was at above 10^5 cfu/g to below 10^8 cfu/g. The variation of *E. coli* population was below 10^7cfu/g at different stages after weaning. No significant differences between treatments were observed in the proportion of piglets with L/C ratio scoring less than 1.3. The most *E. coli* isolates were MDR (multi-drug resistance) to 12 antimicrobials. The highest prevalence of resistance was to oxytetracycline and amoxicillin with MIC >256 µg/ml accounting respectively for 95.2% and 92.8%, however, 81.9% of isolates were susceptible to cefpodoxime, and 84.3% of isolates were susceptible to ceftiofur. Oxytetracycline belonged to the 32/35 identified resistance patterns. The highest resistance patterns were OXY- STR- AZI- POD- XNL- PEN- FFN- ENRO- DOX- NEO- AMOX- COL with 15.7 % of pan drug resistance (PDR).

Keywords: *coliforms*, *E. coli*, *Lactobacillus*, MIC, resistance patterns

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Introduction

The composition and distribution of intestinal flora are particularly important for piglets' health. The role of gut microbiota focuses on regulating host metabolism, maintaining the host physiological homeostasis (Sommer and Bäckhed, 2013), in promoting immune system development (Maynard *et al*., 2012; Tremaroli *et al.,* 2012; Wang *et al*., 2020) and inhibiting the growth of pathogens known as enterobacteria and/or coliforms (Canibe *et al*., 2022; Cubillos, 2017). It has been shown that the diversity, dynamic, composition, and function of the pig gut microbiota community are influenced by various factors, including physiological stage, diet, different intestinal segments/contents, and environmental factors (Upadhaya and Kim, 2022; Luo *et al*., 2022). *Lactobacillus* has been identified as one of the core genera in the gastrointestinal tract (GIT) of pigs, colonizing soon after birth (Delia *et al*., 2012) and influenced by growth stage. The fecal bacteria indicators are fecal coliforms and *Escherichia coli* (US EPA, 2012). The *Lactobacillus* and coliforms count depends on the nursery or weaning periods and is associated with the status of diarrhea in piglets because the fecal *Lactobacillus* counts were greater than coliform counts in healthy swine and the reverse in scouring animals (Muralidhara *et al*., 1977). In view of the high diversity of intestinal microbiota, the previous authors have tried to apply particular microbial groups that could serve as an index of a gut microbiota balance. The *Lactobacillus*: coliform (L/C) ratio is an index of scientific interest that was considered a very practical tool to analyze intestinal health in a group of piglets. It is used experimentally in efficacy tests of feed additives and acidifiers looking to promote immune defense (Adli *et al*., 2019). The main objective is to achieve a L/C ratio of 1.3 or greater, which means the lactobacillus obtained from the fecal sample is at just over 9 log cfu/g, and coliforms under 7 log cfu/g. If an index below 1.3 is obtained, it may be indicative of diarrhea in piglets. In terms of population, it is very important to know that when the L/C ratio of 15% or more of the samples is lower than 1.3, the population is at high risk of developing diarrhea and preventive measures must be taken as a priority. When the prevalence of animals scoring less than 1.3 points is 5% or lower, the chances of an enteric problem in the population are minimal. Currently, it is a very practical tool to analyze intestinal health in a group of piglets on farm (Cubillos, 2017). After weaning, this ratio is reversed. Depending on the animal's coliform growth and/or immune development, a digestive infection might develop related to pathogenic agents such as *E. coli* and *Salmonella* (Pierper *et al*., 2006). The study in vitro also showed that Enterotoxigenic *E. coli* (ETEC) is strongly inhibited when exposed to live lactobacilli cells (Piper *et al*., 2009). The number of *Lactobacillus* spp. increases evidently in piglet feces of piglets even providing probiotics associated with decreased *E. coli* counts (Nguyen *et al*., 2019).

As is known, digestive disorders are common problems at times of stress (e.g., at weaning) and the highest death loss of post-weaned pigs is caused by enterotoxigenic *Escherichia coli* (ETEC), in which post-weaning diarrhea (PWD) is still serious problems for the pig industry (Rhouma *et al*., 2017; Fairbrother and Nadeau, 2019). As commensal and pathogenic bacteria, *E. coli* is considered a good indicator of antimicrobial resistance due to the selection pressure driven by antimicrobials commonly used to treat enteric colibacillosis in piglets. The subtherapeutic dose is frequent with oral administration in pigs and this condition can favor the selection of resistant bacteria. Further, the resistance can be transferred between commensal (e.g. *E. coli*) and pathogenic bacteria (e.g. *Salmonella* spp.; Burow *et al*., 2019). The main antimicrobials used in swine for the treatment of enteric colibacillosis are enrofloxacin, apramycin, ceftiofur, neomycin, gentamicin, amoxicillin/clavulanic acid, trimethoprim-sulphonamide and colistin (Luppi, 2017). An association between antibiotic resistance in *E. coli* from sows and their piglets was determined for quinolones, aminoglycosides, lincomycin, carbadox, and tetracycline. Isolates from swine showed significantly higher antimicrobial resistance than humans, especially in fluoroquinolone and aminoglycosides (Rhouma *et al*., 2015).

With the purpose of evaluating the gut microbiota balance in a group of piglets, the objective of the research was carried out to determine the impact of probiotic supplements to improve L/C ratio associated with *Lactobacillus* and coliforms fluctuations. On the other hand, the current study was carried out to determine the antibiotic susceptibility of *E. coli* isolates identified from fecal samples collected from weaning piglets.

Materials and Methods

Animals, diets and experimental design: All experimental procedures used in this study were approved by Animal Ethics Committees in Nong Lam University (AEC – NLU) (Approval No: NLU-240708). This research was carried out through an experimental design analysis, including two trials (15 days interval) conducted in an industrial farm. Experimental piglets were reared in an environmentally controlled room at 27^{o}C with a slatted plastic floor. Animals had access ad libitum to feed and water throughout the experimental period. For each trial, a total of 90 twenty-six-day-old (26d) weaning pigs (Yorkshire x Landrace) with an average body weight (BW) of 7.5 ± 1.24 kg were allotted in the group of 30 piglets within three treatments, balanced by BW, sex and litter. Dietary treatments included: control (CON, = Basal diet (BD); treatment 1 (T1 = BD + Super-Biotic) and treatment 2 (T2= BD + Bergazyme P®). The basal diet was a standard commercial feed formulation without antibiotics (Sagrifeed company) Vietnam) and the nutrient composition was provided in Table 1. The probiotic used in this study was provided by a local commercial company as Super - Biotic. This product comprised: *Lactobacillus acidophilus*, *Bacillus subtilis* and *Bifidobacterium* (2 x 10^{7} cfu for each one). The Bergazyme-P® contained mainly β-pentosanase, β-glucanase, α-amylase and protease at 6000 EPU/g, 32,000, 17,600 and 142 EU/g, respectively. The piglets were fed a standard non-medicated ration, and probiotics/enzymes were added according to the

experimental treatment design for post-weaning. The piglets in the untreated control group (control group) did not receive probiotics/enzymes in the feed, and the diets of the treated experimental piglets were supplemented with probiotics at a dose of 2g/kg of diets for the probiotic group (T1) and at a dose of 0.25g/kg of diets for enzyme group (T2) from post-weaning (26d of age until 54d of age).

Fecal sampling and microbiological analysis: The stool samples were collected respectively one day pre-weaning and four times at the post-weaning stage until 54 days old (31, 37, 44, and 54 d of age). At each time, 18 samples were collected and a total of 180 samples were collected from two trials (90 samples/trial) during the experimental period. During experimental design, the stools were collected from the same piglets (an individual identification ear tag was applied to an experimental piglets). The stool samples collected from individual pigs were at least approximately 100 grams and should be placed in a sterile bag, kept refrigerated and analyzed within 6 hours in the laboratory. The type of analysis should be a CFU (colony forming units) count for coliforms and *Lactobacillus*/*E. coli*.

The stool samples were homogenized and prepared for ten-fold serial dilutions in Tryptone Salt Broth (M1500I, Himedia), then three consecutive diluted concentrations (10^{-2}, 10^{-3}, 10^{-4}) were plated on MacConkey Agar (M081B, Himedia) and incubated at 37^{o}C/18-24 hours to count the total coliforms and *E. coli*.

The total *lactobacillus* was counted on MRS agar (CM0361B, Thermo Scientific™) with three diluted concentrations (10^{-4}, 10^{-5}, 10^{-6}) after the ten-fold serial dilutions in Tryptone Salt Broth (M1500I, Himedia). Plates were incubated anaerobically at 37 C for 2 days (48 ± 24 h). Anaerobic conditions (about 5% C02) were accomplished by using Oxoid™ AnaeroGen™ 2.5L Sachet (Oxoid™/ Thermo Fisher Scientific).

Counts were recorded as colony-forming units per gram (cfu/g) and the CFU was changed from cfu/g to log (cfu/g) in statistic results. The main objective is to achieve a L/C ratio of 1.3 or greater, where the amount of lactobacillus in caecum obtained from the rectum is higher than 9 log cfu/g, and coliforms lower than 7 log cfu/g. If an index below 1.3 is obtained, it may be indicative of diarrhea in piglets (Cubillos, 2017).

Antimicrobial Susceptibility of E. coli strain isolated from fecal samples: A total of 83 *E. coli* isolates obtained from stool samples of weaning pigs were used to determine antimicrobial susceptibility. Antimicrobial Susceptibility Testing (AST) was performed by micro-broth dilution using commercially prepared dryform panels (AST Sensititre YT3339, Thermo Fisher). The antimicrobials tested available were oxytetracycline, streptomycin, azithromycin, cefpodoxime, ceftiofur, penicillin, florfenicol, enrofloxacin, neomycin, amoxicillin, doxycycline, colistin and were selected based on consultation with the swine industry. Minimum inhibitory concentrations (MICs) were interpreted using CLSI (Clinical Laboratory Standard Institute, 2020) guidelines or NARMS guidelines (Centers for Disease Control and Prevention (CDC), U.S. Department of Agriculture (USDA), and Food and Drug Administration (FDA), 2015, 2021) (Table 2).

Statistical analysis: Statistical analysis bacterial counts were log10 transformed prior to data analysis to normalize distributions. Total bacterial counts were analyzed with repeated-measures ANOVA. The prevalence of piglets scoring less than 1.3 points (L/C ratio) was analyzed with the Chi-Square Test. Statistical analyses were carried out with Minitab 17. The level of statistical significance was set at $P < 0.05$ for all analyses.

Table 1 Nutrient composition of basal diet for experimental piglets

Nutrient composition	Calculated composition
Humidity (max)	13,0
Protein (%, min)	19,5
ME (Kcal/kg)	3.200
Crude Fiber (%, max)	5,0
Calcium (%, min-max)	0,8 – 1,0
Total phosphorus (%, min-max)	0,6 – 1,0
Lysine (%, min)	1,2
Methionine and cystine (%, min)	0,6
Antibiotic and hormone	no

Table 2 Breakpoints used for AST testing of *E. coli* isolates

Antibiotics	Abbreviation	Concentration (µg/ml)	Breakpoints for Enterobacterales (µg/ml)
Oxytetracycline	OXY	4 - 512	<=4; 8; >=16
Streptomycin	STR	4 - 512	<=16; >=32
Azithromycin	AZI	0.5 - 64	<=16; >=32
Cefpodoxime	POD	0.25 - 32	<=2; 4; >=8
Ceftiofur	XNL	0.25 - 32	<=2; 4; >=8
Penicillin	PEN	0.06 - 8	<=8; >8
Florfenicol	FFN	2 - 256	<=8; 16; >=32
Enrofloxacin	ENRO	0.25 - 32	<=0.25, >=2
Doxycycline	DOX	1 - 128	<=4; 8; >=16
Neomycin	NEO	0.5 - 64	<=4; 8; >=16
Amoxicillin	AMOX	4 - 256	<=8; 16; >=32
Colistin	COL	0.25 - 16	<=2; >=4

Results

Total bacteria count: The results showed no significant difference among three treatments at each time of post-weaning for all two trials. The average number of *lactobacillus* was lower at different stages post-weaning in trial 2 (6 < *lactobacillus* (log cfu/g) < 7.43) compared to trial 1 (7 < *lactobacillus* (log cfu/g) < 8.24) ($P < 0.05$). In trial 1, *lactobacillus* counts increased from d25 (1 day before weaning) to different stages after weaning and peaked either at d37 in CON or at d44 in T1 and T2 ($P < 0.05$); however, the higher *Lactobacillus* number was observed only at d37 compared to other times post-weaning in trail 2 ($P<0.05$) (Fig.1).

In trial 1, the total coliform count increased from d25 (1 day before weaning) (6,04 ± 0,18 log cfu/g) and remained at a high level at d37 until the end of the experiment (d54) (approximatively 7,37 log cfu/g) ($P < 0.05$), the point of note the average number of coliforms tended to increase (7,68 log cfu/g) after d54 in CON. In trial 2, the coliform counts decreased nevertheless quickly from d25 to d44 in all three treatment groups ($P < 0.05$) (Fig.2).

The fluctuation of *E. coli* numbers occurred like the variation of coliforms number at different times post-weaning in 3 treatments for each trial. The bacterial population increased from d25 to d31 in trial 1, while the number of *E. coli* decreased progressively until d44 in trial 2. The variation of *E. coli* population was compatible with the coliform population at different stages after weaning and approximatively lower 6,79 log cfu/g ($<10^7$cfu/g). It is clear that *E. coli* counts of all treatments excluding the probiotic group of trial 1, increased after d54 of the experiment ($P < 0.05$) (Fig. 3).

Lactobacillus: coliforms ratio: Looking at the chart of trial 1 (Fig. 4A), the proportion of piglets ranged from at least 50% to 100% with an index of L/C less than 1.3 in all three treatments of the trial; however, there were no significant differences in CON treatment compared to T1 and T2 ($P > 0.05$). In trial 2 (Fig. 4B), 100% of fecal samples scored less than 1.3 at d25 (before weaning) and it remained stable until d31 in all three treatments ($P > 0.05$). Decreasing the proportion of piglets with L/C ratio below 1.3 at d44 was a significant difference between other times of experiment in 3 treatments and was related to the low number of coliforms at this time ($P < 0.05$).

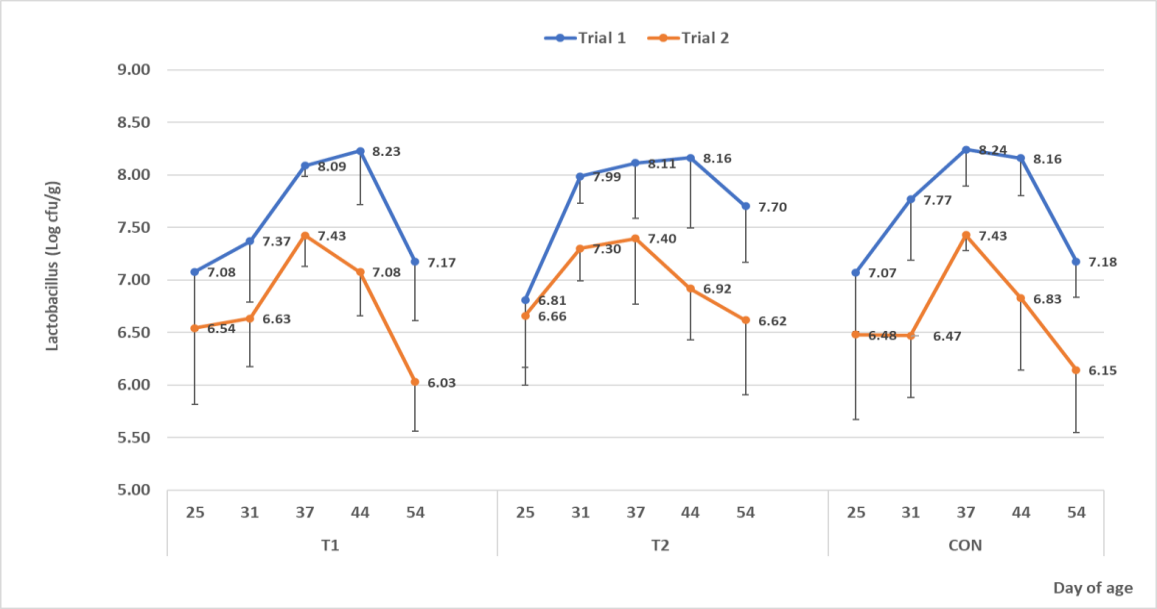

Figure 1 Average number of *Lactobacillus* contained in fecal samples of piglets at different stages after weaning

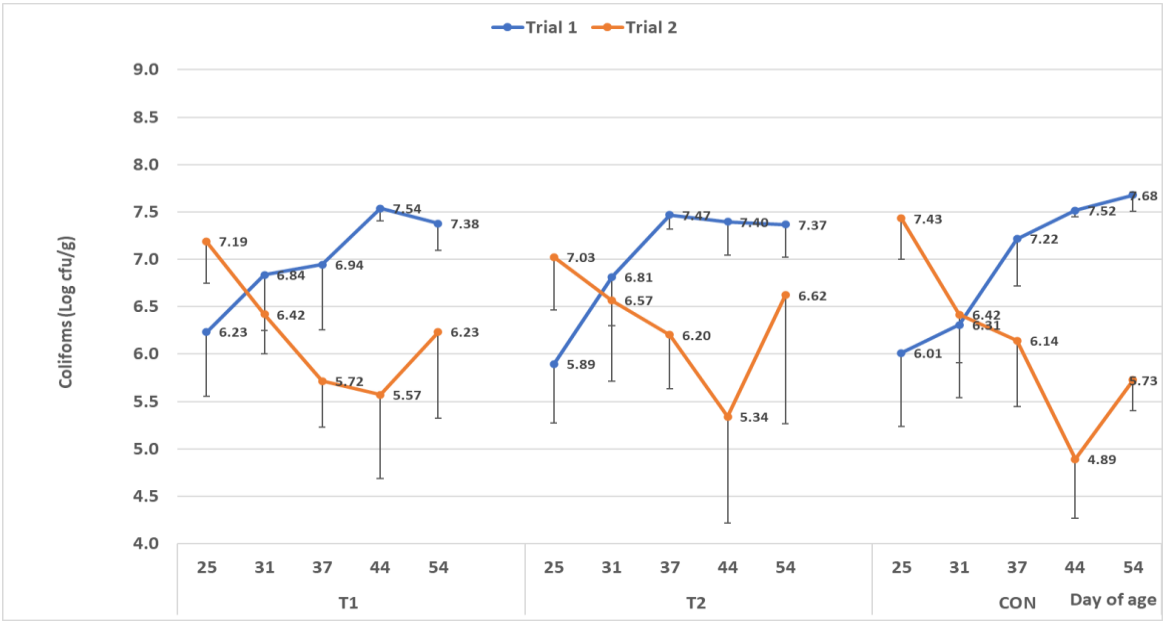

Figure 2 Average number of coliforms contained in fecal samples of piglets at different stages after weaning

Figure 3 Average number of *E. coli* contained in fecal samples of piglets at different stages after weaning

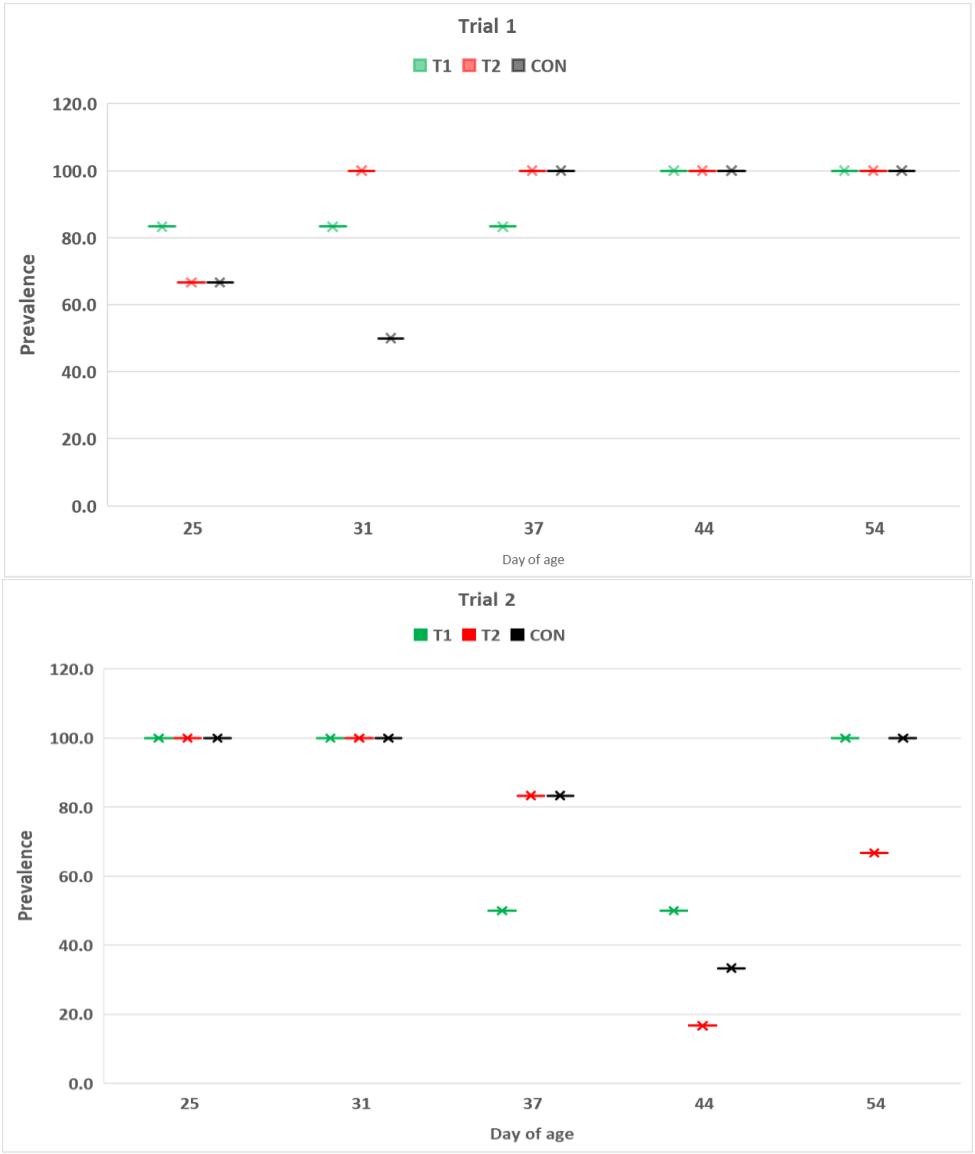

Figure 4A & 4B The proportion of piglets with an index of lactobacillus:coliforms < 1.3

Antimicrobial susceptibility of E. coli strain isolated from fecal samples: The highest prevalence of resistance was oxytetracycline and amoxicillin with MIC >256 µg/ml accounting respectively for 95.2% and 92.8%. *E. coli* isolates were sensitive to cefpodoxime, ceftiofur, enrofloxacin, and colistin with MICs at 0.25 µg/ml, accounting for 12%, 19.3%, 10.9%, and 1.2% of *E. coli* isolates respectively. However, 81.9% of isolates were susceptible to cefpodoxime, and 84.3% of isolates were susceptible to ceftiofur (Table 3).

The MIC50 and MIC90 values describe the MICs at which at least 50% or 90%, respectively, of a given bacterial population are inhibited in their growth or killed by a defend antimicrobial agent or combination of antimicrobial agents. MIC50 and MIC90 values of oxytetracycline were the highest registered (together with those of 4 other antibiotics including azithromycin, penicillin, doxycycline, and amoxicillin). Oxytetracycline had the highest MIC and widest range (4 to > 512 µg/ml) for *E. coli*, so the resistance of *E. coli* isolates to oxytetracycline was highest in this study (95.2%). This study showed that the MIC50 of cefpodoxime at a concentration of 1 µg/ml (74.8%), and ceftiofur at a concentration of 0.5 µg/ml (63.9%) is within the sensitivity range to the growth of *E. coli* isolates.

Phenotypic Antimicrobial Resistance: Based on the Fig. 5, none of the isolates were resistant to all antibiotics; however, 96.4% of *E. coli* isolates were classified as MDRs (Multi-drug resistance) due to their resistance to at least 3 antibiotics. The highest proportion of isolates was antimicrobial resistance to 10 antibiotics (19.3%) and 12 antibiotics at 15.7% classified as PDR (Pan Drug Resistance).

The resistance patterns of *E. coli* strains are presented in Table 4. A total of 35 resistance patterns were observed. The OXY- STR- AZI -POD-XNL- FFN-ENRO- NEO- AMOX- COL and OXY -STR- AZI- FFN-ENRO- DOX- NEO- AMOX- COL resistance patterns exhibited the highest of multi-resistant strains, followed by the OXY- STR- AZI- POD- XNL- PEN-FFN- ENRO- DOX- NEO- AMOX- COL with 15.7 % of pan drug resistance (PDR). Oxytetracycline belonged to the 32 identified resistance.

Table 3 Breakpoints used for AST testing of E. coli isolates

Antibiotic agent	Resistance (%)	MIC50* (µg/ml)	MIC90* (µg/ml)	Frequency distribution of MIC (µg /mL)														
				0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
OXY	95,2	>512	>512							4** (4.8)	0	0	0	0	0	(3) (3.6)	(4) (4.9)	72 (86.7)
STR	84,3	256	>512							0	5 (6)	8 (9.7)	8 (9.7)	10 (12)	10 (12)	3 (3.6)	7 (8.4)	32 (38.6)
AZI	65,1	>64	>64				0	0	0	10 (12)	12 (14.5)	7 (8.4)	9 (10.9)	3 (3.6)	42 (50.6)			
POD	18,1	1	>32			10 (12)	31 (37.4)	21 (25.3)	2 (2.4)	4 (4.8)	0	0	0	15 (18.1)				
XNL	15,7	0.5	>32			16 (19.3)	37 (44.6)	15 (18.1)	1 (1.2)	1 (1.2)	1 (1.2)	1 (1.2)	0	11 (13.2)				
PEN	92,8	>8	>8	0	0	0	0	0	0	0	5 (7.2)	78 (92.8)						
FFN	73,5	256	>256						4 (4.8)	8 (9.6)	6 (7.3)	4 (4.8)	3 (3.6)	4 (4.8)	7 (8.4)	6 (7.3)	41 (49.4)	
ENRO	57,8	2	>32			9 (10.9)	16 (19.3)	10 (12)	9 (10.9)	4 (4.8)	5 (6)	3 (3.6)	2 (2.4)	25 (30.1)				
DOX	91,6	>128	>128					1 (1.2)	4 (4.8)	0	2 (2.4)	3 (3.6)	11 (13.3)	6 (7.2)	0	56 (67.5)		
NEO	68,7	> 64	>64				3 (3.6)	2 (15.7)	2 (2.4)	6 (7.3)	2 (2.4)	3 (3.6)	4 (3.6)	7 (8.4)	54 (49.4)			
AMOX	92,8	>256	>256							3 (3.6)	2 (2.4)	1 (1.2)	0	0	0	4 (4.8)	73 (88)	
COL	59	8	>16			1 (1,2)	27 (32.5)	4 (4.8)	2 (2.4)	6 (7.3)	22 (26.5)	7 (8.4)	14 (16.8)					

**Concentrations that inhibited the growth of 50% (MIC50) and 90% (MIC90) of isolates, respectively; MIC50 and MIC90 that were above the dilution range are marked with the sign ">" ** The data in the table is the number of samples (percentage of samples) at the respective MIC level.*

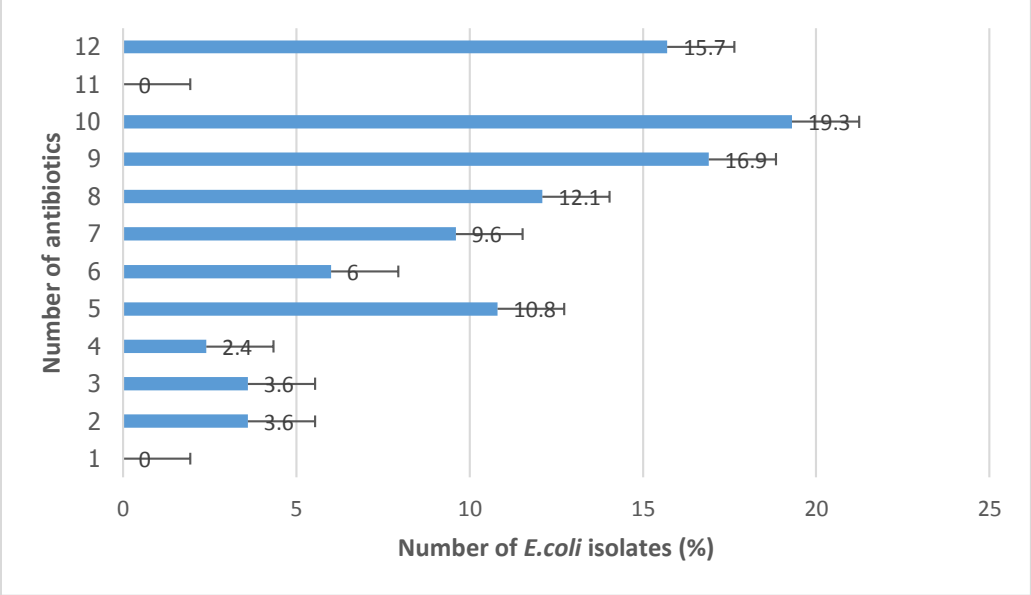

Figure 5 Antibiotic resistance profiles with the highest frequency in *E. coli* (n = 83)

Table 4 Resistance patterns of all *E. coli* isolates (n = 83) from experimental piglets

Pattern of Phenotypic Resistance	No. resistance antimicrobials	No.Classes	Isolate (s)
AZI NEO	2	2	1
DOX AMOX	2	2	2
OXY STR AMOX	3	3	1
OXY STR AZI	3	3	1
STR POD ENRO	3	3	1
OXY STR DOX AMOX	4	3	2
OXY STR FFN DOX AMOX	5	4	2
OXY STR FFN NEO AMOX	5	4	3
OXY FFN DOX NEO AMOX	5	3	1
OXY DOX NEO AMOX COL	5	4	1
OXY STR DOX NEO AMOX	5	3	2
OXY STR AZI DOX NEO AMOX	6	4	2
OXY STR FFN ENRO DOX AMOX	6	5	3
OXY STR AZI FFN DOX NEO AMOX	7	5	2
OXY FFN ENRO DOX NEO AMOX COL	7	6	1
OXY STR FFN DOX NEO AMOX COL	7	5	1
OXY STR AZI FFN ENRO DOX AMOX	7	6	1
OXY STR AZI FFN DOX AMOX COL	7	5	1
OXY AZI PEN FFN DOX AMOX COL	7	6	1
OXY STR AZI FFN ENRO DOX AMOX	7	6	1
OXY STR FFN ENRO DOX NEO AMOX COL	8	6	1
OXY STR AZI FFN DOX NEO AMOX COL	8	6	2
OXY AZI FFN ENRO DOX NEO AMOX COL	8	6	1
OXY STR AZI FFN ENRO DOX NEO AMOX	8	6	1
OXY STR AZI ENRO DOX NEO AMOX COL	8	6	1
OXY STR FFN ENRO DOX NEO AMOX COL	8	6	2
OXY STR AZI FFN ENRO DOX AMOX COL	8	7	1
OXY STR AZI POD FFN ENRO DOX AMOX	8	7	1
OXY STR AZI FFN ENRO DOX NEO AMOX COL	9	7	11
OXY AZI POD XNL FFN ENRO DOX AMOX COL	9	7	1
OXY STR AZI POD FFN ENRO DOX NEO AMOX	9	7	2
OXY STR AZI POD XNL FFN ENRO NEO AMOX COL	10	8	11
OXY STR AZI POD PEN FFN ENRO NEO AMOX COL	10	9	3
OXY STR AZI POD FFN ENRO DOX NEO AMOX COL	10	8	2
OXY STR AZI POD XNL PEN FFN ENRO DOX NEO AMOX COL	12	9	13

Discussion

Due to the *Lactobacillus* population being the most dominant genera in pig GITs from birth until 56 days of age (König *et al.*,2021), the present study investigated only the total *Lactobacillus* species in the intestinal environment of weaning pigs. The benefits of the administration of probiotic lactobacilli to pigs include ultimately improving the population of *Lactobacillus* (Nguyen *et al.*, 2018; Kenny *et al*., 2011; Yang *et al.,* 2015). The results showed that the dynamic distribution of *lactobacillus* increased progressively post-weaning with a more pronounced abundance at d44 (>8 logcfu/g) and d37 (>7 log cfu/g), respectively, in trial 1 and trial 2 ($P< 0.05$). These results consistent with observation that the gut microbiota's maturing development depends obviously on the growth stage (Luo *et al.,* 2022). Age is one of the determinant factors affecting the succession of gut microbiota in neonatal piglets (Bian *et al*., 2016), and the bacterial abundance and diversity increase with age (Wang *et al*., 2019). The weaning period offers a special window for modifying the gut microbiota even without the addition of any factors in dietary affecting the gut microbiota. Furthermore, previous studies have detected *Lactobacillaceae* members across all growth stages with a more pronounced abundance post-weaning (Valeriano *et al*., 2016) without diet supplementation. There has been a steady decrease in the number of *lactobacillus* at d54 (approximately eight weeks of age) in this study ($P<0.05$). This result is according to the previous report where the lactobacilli averaged 11% of the total bacterial population in 10-week-old pigs, and lactobacilli comprised only 3,2% of 22-week-old pigs (Kim *et al.,* 2011), although the investigation of *Lactobacillus* population was observed in the earlier stage of swine production. However, the dynamics of the lactobacilli population as influenced by growth stage and diet supplementation were not significantly different between CON and T1/T2 at each time of experimental study in all two trials ($P>0.05$).

Lactobacillus counts were no significant differences between all treatments. It suggested that the supplementation of probiotic did not affect the population of *lactobacillus* in this experimental framework. It is important to perceive that the effects of probiotics depending on the variety of conditions under which probiotics have been tested: the use of different strains, diverse doses, and the effect of uncontrolled variables such as the age of pigs at the time of challenge, variations in treatment dose, as well as the duration of the adaptation period or duration of the treatment period. These factors limit the power to retrieve robust and reproducible results (Canible *et al*., 2022). In this study, dietary supplementation of a mixture of *Lactobacillus acidophilus*, *Bacillus subtilis* and *Bifidobacterium* (2×10^7 cfu/g for each one) does not affect fecal *lactobacillus* counts in weaned piglets. This agrees with Dell'Anno *et al*. (2021), who reported that dietary supplementation with *L. plantarum* (2×10^8 cfu/g) and *L. reuteri* (2×10^8 cfu/g) individually or in combination (1×10^8 cfu/g *L. plantarum* plus 1×10^8 cfu/g *L. reuteri*) did not influence fecal lactobacilli and coliform counts. Meanwhile, our results did not consist with a study where feeding up to 0.3% probiotic mixture of *B. coagulans* (1×10^{12} cfu/g), *B. licheniformis* (5×10^{11} cfu/g), *B. subtilis* (1×10^{12} cfu/g) and *C. butyricum* (1×10^{11} cfu/g) to weaning pigs significantly increased *Lactobacillus* counts and decreased *E. coli* counts (Nguyen *et al.,* 2016). It is clearly shown that the bacteria strains of mixed probiotic and the low concentration of bacteria (2 x 107 cfu/g for each one) compared to other applications influencing the results of the current study. This is consistent with Lactobacillus number in faeces of weaned pigs, which was lower (up to 10^6 to 10^8 cfu/g) than in the normal pigs of the recent studies (10^7 -10^9 cfu/g).

Coliforms are among the earliest groups of bacteria to colonize the gut after birth. The coliforms play an important role in the establishment of the normal flora in the gut (Zoric *et al*., 2002) and the most tested fecal bacteria indicators are fecal coliforms and *Escherichia coli* (US EPA, 2012). It is noticeable that the population of coliforms was opposite between 1st trial and 2nd trial post-weaning, so the decline of coliforms number was considered as a good trend in gut health, which was observed the 1st trial. The average fecal coliform concentration observed in the present study was above 105 cfu/g to below 108 cfu/g, reflecting the variation in different stage was at high level compared to previous studies. However, the report of Barth *et al.* (2018) showed that the coliforms number was at 1,4 x 10^9 cfu/g of feces (approximately 9 log cfu/g) in a normal pig at 30 days post-weaning (Barth *et al*., 2017). It suggests that gut health depends not only on the total bacteria population but also on the diversity of coliform populations associated with the intestinal microbiota in pigs. Therefore, analyzing intestinal health based on the proportion of bacteria in feces sampling post-weaning is important.

Escherichia coli is by far the dominant type among the coliform bacteria in the gastrointestinal tract (Overland *et al*., 2000), approximatively, the total represents 96.2% of coliforms isolated from livestock feces (Lavoie, 1983). Most *E. coli* strains are harmless commensals of the intestinal microbiome, but some pathogenic serotypes dominate severe intestinal infections (Stromberg *et al*., 2018). The enterotoxigenic *E. coli* (ETEC) strains were associated with post-weaning diarrhea (PWD) which always the potential impacts on gut health in the post-weaning period (Pluske *et al.,* 2018). Amongst the physiological and GIT factors impacted by the weaning transition, microbiota disruption in the GIT is likely a key influence leading to PWD. The diarrhea due to *E. coli* is frequently observed 2–3 weeks after weaning and although not exceptionally, it can be recorded at 6–8 weeks after weaning (Luppi, 2017). This is according with the increased trend of *E. coli* number observed post weaning about 3-4 weeks in this experiment. The mean number of *E. coli* phenotypes in piglets increased as animals aged (Katouli *et al*., 1995) and *E. coli* populations in the pig fecal microbiota and the farm environment are dynamic and show high levels of diversity. Nevertheless, it is suggested only pathogenic *E. coli* are associated with diarrheic post-weaning so when more than 10^7 CFU/g of *E. coli* F4 and/or E. coli F18 were detected, this was correlated with the cultivation of a high number of potentially pathogenic *E. coli* (Luppi, 2017). In the same way with

the distribution of fecal coliforms, the *E. coli* number was at a low level in this study. It seems to improve the swine gut microbiota, but that is not certain enough to minimize the risk of developing intestinal diseases. However, the *Lactobacillus* spp. group decreased during the weaning transition as in previous works (Pollock *et al.*, 2018).

Based on the L/C ratio, it is currently a practical tool in farms. In our study, L/C ratio was applied in the experimental framework (Cubillos, 2017). It is obvious that most of the piglets were at high risk of developing diarrhea in different stages post-weaning due to the L/C ratio of 50 – 100% of the fecal samples was lower than 1.3. In the weaning period, the use of probiotics is related to the competitive exclusion of pathogenic bacteria. This effect could be a result of their positive influence on gut microbiota balance, intestinal epithelium integrity, appropriate gut-associated tissue maturation and neuro-endocrine system function (Valeriano, 2016). However, the response of piglets to probiotic supplementation is variable due to the additional dosage, living environment, health status, strain differences, etc. (Wang *et al.*, 2023). The most promising effect of probiotics depends on the predominant endogenous probiotics in the GIT of pigs. Various species of *lactobacillus* exert effects on the expression and secretion of mucins. Intestinal mucins are the mucus's main protein component that covers the gastrointestinal tract's epithelium (Aleman *et al.*, 2023). The previous study suggested five Lactobacillus spp. with higher percentages in normal piglets compared to diarrheal ones such as L*. reuteri, L. amylovorus, L. acidophilus, L. johnsonii,* and *L. crispatus*. In our current study, probiotic supplementation containing viable spores of *Lactobacillus acidophilus* (2 x 10^7 cfu) did not show significant effects compared to the enzyme supplementation group even the control diet group on the L/C ratio. Due to the population of coliforms was inverse from d25 (before weaning) until different stage post weaning between trial 1 and trial 2, so the risk of diarrhea of piglets seems impoverished significantly at d44 in T2 compared to T1 and CON in trial 2 *(P*< 0.05). However, there were sporadic slight diarrhea cases as well as the diarrhea prevalence was no significant difference in piglets in T2 compared to T1/CON throughout the experiment (data not shown). Actuellement, it is very difficult to explain the contradictory results in the two trials for coliform and *E. coli* counts because the experimental condition was not different for 2 trials with 15 days intervals. It is not excluded that there were some factors affecting the experiment that we cannot detect in the experimental framework.

With the limitation of fecal numbers, the L/C ratio seems not to be a reliable index to assess the risk of diarrhea in pig herds. Cubillos (2017) suggested that the collection of representative samples of the pig population is a priority to an analysis of a group of piglets to determine the intestinal health status of the farm. Further, because of the coliform bacteria which belong to the enterobacterial family, suggests that L/C could be replaced by the lactobacilli: enterobacteria ratio (L/E). L/E ratio has been used conventionally as a simple index in some studies. An increase in this ratio is related to a higher resistance to intestinal disorders (Castillo *et al.*,2006) also, the L/E ratio could be applied to evaluate the protein modifies the effect of plant extracts in the intestinal ecosystem of the pig at weaning (Manzanilla, 2009).

The development of resistance to a wide range of antimicrobial drugs, as well as the demonstrated trend of resistance in ETEC strains to the antibiotics used for the treatment of colibacillosis in pigs, is nowadays a reason for concern (Luppi, 2017). The MIC results showed that the main antimicrobials used in swine for the treatment of enteric like enrofloxacin and colistin remained the antibacterial potential against inferior 50% *E. coli* strains isolated. It is concerned with the resistance of *E. coli* isolates to colistin (59%). Colistin has been classified as a critically important antimicrobial for human health. The World Health Organization (WHO) has classified colistin as a 'highest priority critically important antimicrobial' to be used for the treatment of human infections due to multidrug-resistant (MDR) Gram-negative bacteria (Ahmed *et al.*, 2021). In Vietnam, colistin is widely used in pigs for the oral treatment of intestinal infections caused by *E. coli*, and particularly of PWD, as well as being used as a growth promoter (ICARS, 2023). Excessive utilization of colistin in animal production has created selective pressure, which has contributed to the increased resistance to colistin. Consequently, it is proposed internationally that the use of colistin in animal production should be terminated or at least as a last resort treatment only. Resistance to enrofloxacin was described in *E. coli* strains isolated in Brazil, where nearly 30% of the isolates from cases of neonatal colibacillosis were resistant to this antibiotic colistin sulphate is the only approved product in some countries for oral use in pig production to control intestinal infections caused by *Enterobacteriaceae*. The use of colistin in Europe varies widely between countries. Countries with intensive livestock production can have a level of usage below 1 mg/PCU (e.g. Denmark and the UK) or much higher, up to 20 to 25 mg/PCU (Italy and Spain) (EMA, 2016). In the last few years, *E. coli* strains resistant to colistin have become more common. Strains of *E. coli* with acquired resistance are encountered among pathogenic isolates, commonly in pigs suffering from diarrhea. However, resistance to amoxicillin was noted in *E. coli* strains isolated from experimental piglets, where nearly 92,8% of the isolates were resistant to this antibiotic (Luppi, 2017). Amoxicillin is usually an antimicrobial used in the treatment of enteric colibacillosis. A study performed in Korea showed how *E. coli* strains isolated from diarrhea pigs were multi-resistant (resistant to more than four antibiotics) with high levels of resistance to several antibiotics: gentamicin (77%), trimethoprim-sulfamethoxazole (75.7%), amoxicillin (75.7%), ampicillin (73%) and enrofloxacin (64.9%). Ceftiofur sodium is available for use on swine in the treatment of neonatal colibacillosis which was until effected in superior 80% *E. coli* isolates in this study. The present study was in accordance with the previous report that the resistance rates of *E. coli* strains isolated from healthy and diseased pigs in different countries to ceftiofur was at a low level (from 1 to 20%) (Luppi, 2017).

Since the significance of MIC_{50} and MIC_{90} increases with the number of strains tested, sufficiently large test populations should be used for the most meaningful statements on MIC_{50} and MIC_{90} values (Schwarz et al., 2010). The association of high MIC to tetracycline class was observed also in commensal *E. coli* strains (75.2%) in four Bulgarian swine farms (Urumova *et al*., 2016). Due to the *E. coli* strains was only sensitive at high rate to cefpodoxime and ceftiofur in this study, using ceftiofur should base on MIC50 and MIC90 in clinical cases. The recommendation of ceftiofur for the treatment of enteric colibacillosis in swine was at 3mg/kg/IM. The administration of ceftiofur at 0.5 µg/ml could be used to control at least 50% of *E. coli* pathogen isolates but the dose needs to be increased 64-fold to inhibit 90% of *E. coli* isolates (> 32 µg/ml) (Luppi, 2017).

Escherichia coli isolates were phenotypically resistant at different rates because MIC values above the breakpoint occurred for all antibiotics tested. No isolates were resistant to one antibiotic. The results of the present study supported that MDR *E. coli* was found in a high proportion of fecal samples (96.4%) isolated from non-diarrheic piglets (no clinical signs). This issue is very worrying because the same prevalence of MDR *E. coli* (97.3%) was reported from fecal samples isolated from diarrheic piglets) (Nguyet *et al*., 2022). *E. coli* is intrinsically susceptible to almost all clinically relevant antibiotic agents, but this bacterial species has a great capacity to accumulate resistance genes, mostly through horizontal gene transfer (Poirel *et al*., 2018). A previous study in Chongming Island, Shanghai, reported that all swine isolates collected prior to 2017 were MDRs (LV *et al*., 2022). Another study in Indonesia showed that *E. coli* strains were non-susceptible to approximatively 15% to one antibiotic, while 82% of *E. coli* isolates were MDR profile and 23% of which were resistant to 3 antibiotics being the highest followed by four antibiotics (22%) (Kallau *et al*., 2018). Likewise, the MDR was observed in all *E. coli* strains isolated from pigs in Korea at a high percentage (93.8%), and approximately 23.4% of pig isolates were resistant to seven subclasses (15 isolates) in Korea between 2008 and 2016 (Do *et al*., 2022). In this study, we found high rates of multi-drug resistance to 10 and 12 antimicrobial subclasses (19.3% and 15.7%), the values obtained were higher than those obtained in other studies in Korean (6.3% to 10 subclasses) (Do *et al*., 2022). This shows that the situation of multi-antibiotic resistance of *E. coli* bacteria isolated in pigs is very interesting. The high level of antimicrobial resistance is directly linked to challenges in the treatment of diseases; therefore, it is important to manage antimicrobial resistance.

In Thai Lan, the highest MDR of isolated *E. coli* was found in the piglets rather than the fattening and sows and AMP-AMX-AMC-PIP-CEX-CEV-CPD-XNL GM-IMP-ENR-MBR-TE was the most common pattern at 41.0% (9/22) found in the piglets (Dawangpa *et al*., 2022). While the core multi-resistance pattern of porcine *E. coli* in Europe was ―AMP-AMC-PIP-DOX―which had only four antibiotics comparable to the core of the present study (9-10 antibiotics) (Bassitta *et al*., 2022). In terms of disease control and the use of antimicrobials, most operate under the supervision of veterinarians concentrating on preventing and controlling diseases. The highest MDR of isolated *E. coli* was found in the non-diarrheic experimental piglets associated with the extensive use of antimicrobials in pig production in Vietnam. This may increase the risk of selecting resistant bacteria compared to individual antibiotic applications to sick pigs only.

In conclusion, the current study, the low dose treatment of probiotics could be the consequence of not showing the significant effects of probiotic supplementation on *Lactobacillus* number. The next studies should re-evaluate using L/C ratio with the collection of representative samples (a large number of fecal samples) and associate other parameters (e.g fecal score, intestinal integration, villus status) to access the GUT health. *E. coli* number was at a low level, it seems to improve the swine gut microbiota with probiotic and digestive enzymes but that is not certain to minimize the risk of developing PWD.

The highest MDR of isolated *E. coli* was found in this study and the highest antimicrobial class resistance in *E. coli* was tetracycline (OXY, DOX) and beta-lactam (AMOX, PEN). The core PDR pattern of *E. coli* isolates was OXY- STR- AZI- POD- XNL- PEN- FFN- ENRO- DOX- NEO- AMOX- COL.

Conflicts of interest: There were no conflicts of interest that may have biased the work reported in this study.

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