Effects of copper and zinc sources and inclusion levels of copper on weanling pig performance and intestinal microbiota[1]

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[1]The authors gratefully acknowledge the support of the SENESCYT (Secretary for Higher Education, Science, Technology and Innovation of Ecuador) for the provision of a pre-doctoral scholarship. The authors are also grateful to the Servei d´Anàlisi Química at the Universitat Autònoma de Barcelona for the chemical service.

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ABSTRACT

A 42 day experiment was conducted to evaluate the effect of Cu and Zn source and Cu level on pig performance, mineral status, bacterial modulation, and the presence of antimicrobial resistant genes in isolates of *Enteroccocus* spp. At weaning, 528 pigs (5.9 ± 0.50 kg) were allotted to 48 pens of a randomized complete block design in a 2×2 factorial arrangement with two Cu and Zn sources (SF: sulfate and HCl: hydroxychloride) and two Cu levels (15 mg/kg and 160 mg/kg). As a challenge, the pigs were reared in dirty pens used by a previous commercial batch. Two-phase diets were offered: the pre-starter (PS) phase from d 1 to 14 and the starter phase (ST) from d 14 to 42. At d 14 and 42, pigs were individually weighed and blood samples from 1 pig/pen were taken. At the end of the experiment, 1 pig/pen was euthanized to collect samples. Feeding high levels of Cu increased BW from 16.6 to 17.7 kg ($P < 0.001$). Furthermore, ADG, G:F, ADFI and mineral status was enhanced with Cu at 160 mg/kg ($P < 0.05$) compared with Cu at 15 mg/kg. There was no effect of the interaction between source × level on any of the growth performance responses except for ADFI ($P = 0.004$) and G:F ($P = 0.029$) at the end of the ST period and for G:F ($P = 0.006$) for entire nursery period (d 0-42). At the end of the ST period, pigs fed Cu at 160 mg/kg as HCl had higher ADFI but also lower G:F than those fed Cu as SF at 160 mg/kg. Meanwhile, for the entire nursery period, G:F did not differ between pigs fed Cu at 160 mg/kg as HCl or SF. In colonic digesta, the relative abundance of *Streptococcus*, *Enterobacter, Escherichia,* among others, decreased (*P*-adjust < 0.05), while *Lachnospira* and *Roseburia* tended (*P*-adjust < 0.10) to increase in pigs fed Cu at 160 mg/kg as HCl compared to those fed Cu SF at 160 mg/kg. An increase (*P*-adjust < 0.05) in *Methanosphaera* and *Roseburia* was observed in pigs fed Cu at 160 mg/kg. From colon digesta, *Enterococcus* spp. was

isolated in 40 samples, being *E*. *faecalis* the most dominating (65%) regardless of the experimental diet. Genes of ermB (7.5%) and tetM (5%) were identified. No genes for Cu (tcrB) or vancomycin (vanA, vanB, vanC1, vanC2) were detected. In conclusion, EU permissible levels of Cu (160 mg/kg), of both sources, are able to increase performance, mineral status and bacterial modulation compared to nutritional level. Different effects on growth performance, mineral tissue content and microbial modulation were observed between Cu and Zn sources.

Key words: antimicrobial resistance genes, copper, European levels, microbiota, weaned pigs, zinc

Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
BW	Body weight
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EU	European Union
FDR	False discovery rate
FEEDAP	European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed
G: F	Gain to feed
GIT	Gastrointestinal tract
GPX	Glutathione peroxidase
HCl	Hydroxychloride
ICP-OES	Inductively coupled, plasma-optical emission spectroscopy
MIC	Minimum inhibitory concentration
NMDS	Non-metric dimensional scaling
NRC	National Research Council
PCR	Polymerase chain reaction

PRRS	Porcine reproductive and respiratory syndrome virus
PS	Pre-starter period
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
SF	Sulfate
SOD	Superoxide dismutase
ST	Starter period
TBCC	Tribasic copper chloride
US	United States

INTRODUCTION

Copper and zinc are essential trace minerals involved in many physiological processes (Olivares and Uauy, 1996). The National Research Council (NRC) established a minimum requirement of Cu (6 mg/kg) and Zn (100 mg/kg) for weanling pigs (NRC, 2012). However, stressing factors at weaning frequently result in low feed intake, gastrointestinal disorders and, consequently, impaired gut integrity and growth (Lallès et al., 2007). In the European Union (EU) until 2003, and today in other regions of the world (including the United States, US), commercial practice generally used high doses of Cu (200-250 mg/kg) and Zn (2,000-3,000 mg/kg) as therapeutic additives in post-weaning diets. The main positive effects attributed to these therapeutic doses are growth promotion (Cromwell et al., 1998) and antimicrobial activity (Højberg et al., 2005; Namkung et al., 2006). However, at high dietary levels, Cu and Zn are barely absorbed in the intestine, affecting the availability of other nutrients (Pang and Applegate, 2006, 2007) and generating a major environmental concern (EFSA FEEDAP, 2016) as well as a public health risk due to microbial tolerance and resistance to other antimicrobial agents (Hasman and Aarestrup, 2002; Van Noten et al., 2016). Based on these considerations, the EU approved new maximum levels of Cu for pigs, being 150 mg/kg up to 4 weeks after weaning, followed by a reduction to 100 mg/kg until 8 weeks after weaning (European Commission, 2018). The previous regulation allowed the inclusion of Cu at 170 mg/kg in diets up to 12 weeks age, when this experiment was performed. The current total feed content of Zn for pigs until 11 kg body weight (BW) is 150 mg/kg (European Commission, 2016); however, a further reduction to 110 mg/kg is not excluded (EFSA FEEDAP, 2017).

Sulfate is the most used mineral source in swine diets. It is characterized by a labile molecular bond which allows high solubility in water and acid solutions and is commonly used as a reference to compare the bioavailability of different mineral sources (Park and Kim, 2016). Alternatively, hydroxychloride mineral sources have a crystalline structure formed by covalent bonds, with slow solubility in the gastrointestinal tract (GIT) and a high amount of biologically active ions (Cohen and Steward, 2014). Thus, in the present study we hypothesize that due to differences in chemical properties of trace mineral sources, growth performance of early weaned pigs fed diets supplemented with hydroxychloride mineral sources will be higher or similar than those fed sulfate, hence offering an alternative to the use of high levels of sulfate and antimicrobials as growth promoters. The low solubility of hydroxychloride trace minerals, which are different to sulfates, makes them less prone to antagonistic nutrient interactions at the proximal section of GIT, hence increasing mineral and nutrients availability as well as probably promoting a greater impact on intestinal microbiota. Thus, the objective of this study was to compare the effect of two sources of Cu and Zn (sulfate and hydroxychloride) as well as the effect of two Cu dietary levels (15 mg/kg as nutritional or 160 mg/kg as high level) on growth performance, mineral status, microbial modulation and the possible presence of antimicrobial resistant genes in newly weaned pigs. Pigs were allocated to dirty pens used by a previous nursery batch in order to provide a more challenging scenario due to early contact with a dirty and non-disinfected environment.

MATERIALS AND METHODS

All animal experimentation procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

Animals and Housing

The experiment was performed on a commercial farm of Catalonia, Spain. At weaning (21 d), 528 pigs ([Large White × Landrace] × Pietrain) obtained from the same commercial farm and with an initial average BW of 5.9 ± 0.50 kg were used in a 42 day study in a commercial nursery unit. Pigs were ear tag identified, blocked according to the initial BW and distributed into four experimental diets in 48 pens (12 pens/treatment, 11 pigs/pen). Entire males and females were randomly assigned to the same pen. The pigs were housed in dirty pens used by a previous commercial batch in order to increase the environmental challenge. Each pen (3.12 m^2) had fully slatted floor and was equipped with a commercial non-lidded hopper (TR5, Rotecna, Spain) and a nipple drinker to provide *ad libitum* access to feed and water. The facility was environmentally controlled (temperature and ventilation rate) by use of thermostatically controlled heaters and exhaust fans depending on the age of the pigs (28-22ºC). Pigs were allotted to two identical rooms. Each room had 28 pens divided by a central feeding corridor but only 24 pens were used in the experiment (the two at the far ends of the room close to the doors were discarded and used as refusal/hospital pens). In order for the weaned pigs to be kept in poor sanitary conditions, the pens were not cleaned or disinfected after use by a previous commercial batch. Since the pens had a fully slatted floor, an excessive amount of feces was not accumulated. Ventilation and temperature were adjusted prior to the housing of the newly weaned pigs. The commercial swine farm is stable but positive for the porcine reproductive and respiratory syndrome (PRRS) virus. The standard farm practices include the vaccination of pigs at 20 d of age against porcine circovirus type 2 and mycoplasma hyopneumoniae (Suvaxyn Circo + MH RTU, Pfizer, Spain) and the vaccination of sows against PRRS (MSD, Spain) every 4 months. Pigs are weaned at 21 d and with an average BW of 5.8 kg. Nursery period is 6 weeks with a daily weight gain ranging between 280-290 g and 2-3% mortality. Zinc oxide is added to feed at pharmacological levels (2,500 mg/kg) for one week. The usual inclusion of Cu to weaned pig diets is 9 mg/kg. Antibiotics are administered after veterinary prescription if required to treat specific diseases.

Experimental design and dietary treatments

Two-phase diets (Table 1) were formulated to meet or exceed nutrient requirements (NRC, 2012): the pre-starter (PS) phase from d 1 to 14 and the starter (ST) from d 14 to 42.

Four experimental diets were prepared in a 2 × 2 factorial arrangement, with two Cu and Zn sources (sulfate and hydroxychloride) and two Cu inclusion levels (nutritional: 15 mg/kg, and high: 160 mg/kg). Supplementation of Zn was fixed for all diets at 110 mg/kg. Cu sulfate pentahydrate (25%) and Zn sulfate monohydrate (35%) were obtained from Pintaluba, Reus, Spain. The hydroxychloride Cu (54%, IntelliBond C) and Zn (55%, IntelliBond Z) were obtained from Trouw Nutrition, the Netherlands. A vitamin-mineral premix without Cu and Zn was prepared. For each dietary treatment, Cu and Zn products were pre-mixed with 25 kg of basal diet before being put directly in the mixer during the feed preparation process. In order to avoid cross contamination with elements from previous production, feed was prepared in an appropriate rank order starting with the lower concentrations to be included in the diet. The first and last 100 kg of the final pellet diet from each batch (experimental treatment) were discarded to reduce cross contamination. All diets were offered *ad libitum* in pellet form. Composite samples (1 kg) were collected during the bagging process in representation of each experimental treatment. Each sample was therefore proportionally split into four 250 g samples that were stored for further analysis. Zinc oxide was not added at pharmacological levels in the diets and no antibiotics or feed additives with antimicrobial properties were used.

Experimental procedures and sampling

The BW of each pig and feed left in the feeders were recorded on d 14 and 42. From these data, average daily gain (ADG), average daily feed intake (ADFI), and gain to feed (G:F) were calculated. At the end of the PS phase, one pig per pen was selected based on the mean BW within the pen (median) to take samples of blood by jugular puncture. Samples from the same animal were taken at

the end of the ST phase. For antioxidant enzyme determination, blood was collected into 4 mL vacutainer tubes containing lithium heparin (BD Vacutainer, LH, BD-Plymouth, UK) and centrifuged at 3,000 × g for 15 min. The acquired plasma was stored at -80°C for further analysis. Meanwhile, blood samples for Cu and Zn determination were collected into 5 mL vacutainer tubes (BD Vacutainer, Z, BD-Plymouth, UK) free of detectable Zn. Serum was obtained after centrifugation (3,000 × g for 15 min) and immediately frozen at -20ºC. At the end of the experimental period, the selected pig per pen (n=12) was euthanized with an overdose of sodium pentobarbital (Dolethal, Vetoquinol, S.A., Madrid, Spain); and organ samples (liver and left tibia) were collected to determine Cu and Zn concentration. Finally, digesta from the proximal colon (1 m from the ileocecal junction) were collected for microbiota analyses and detection of antimicrobial resistant genes. Samples were immediately stored at -20°C until processing and analysis.

Chemical Analysis

Analytical determinations of diets were performed according to the AOAC International (2005) methods for dry matter (Method 934.01), crude protein with the Dumas Method (Method 968.06), ether extract was determined using traditional Soxhlet extraction (Method 920.39), and ash (Method 942.05). Neutral detergent fiber was analyzed using the Ankom nylon bag technique (Ankom 200 fiber Analyzer, Ankom Technology, Macedon, NY).

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) in plasma were determined by spectrometry and following the instructions of Ransod and Ransel kits, respectively (Randox, County Antrim, UK). Liver was dried in a forced air oven at 102ºC per 12 h and then milled at 0.5 mm. Tibia was autoclaved to remove all the adjacent muscle and tissue (121ºC for 30 min). Subsequently, tibia was oven-dried for 12 h at 102°C and soaked in acetone under a chemical hood for 48 h to extract fat. After this period, tibia was again oven-dried for 12 h at 102ºC and then broken in the middle before being ashed overnight at 550ºC in a muffle furnace. Samples of feed were milled at 0.5 mm before mineral analysis. All samples were solubilized in nitric acid prior to

mineral analysis by inductively coupled, plasma-optical emission spectroscopy (ICP-OES, model Optima 4300DV, Perkin-Elmer Inc.; Massachusetts, US).

Microbial molecular analysis

Bacterial DNA was extracted from 200 mg of colonic digesta by using the commercial MagMAX CORE Nucleic Acid Purification 500RXN Kit (Thermo Fisher, Texas, US) and following the manufacturer's instructions. For 16S rRNA gene sequence-based analysis, the V3-V4 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and 72°C for 5 min) using primers F5′-barcode-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and R5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. A negative control of the DNA extraction was included as well as a positive Mock Community control to ensure quality control. After 25 cycles of amplifications, 550 pb amplicons were obtained. The Illumina Miseq sequencing 300 × 2 approach was used. Raw sequencing reads were quality clipped, assembled, and compared with available genomic sequences using a Microomics Systems S.L (Barcelona, Spain) software and were validated and subsequently completed with the Kraken Metagenomics (Wood and Salzberg, 2014) and QIIME (Caporaso et al., 2010) software. Taxonomic assignment of phylotypes was performed using a Bayesian classifier trained with Silva database version 132 (99% OTUs full-length sequences) (Wang et al., 2007).

Enterococcus spp. isolation, detection of resistance genes and phenotypical antimicrobial resistance tests

Colon digesta samples were plated and incubated on Slanetz-Bartley agar (Oxoid, UK) for 48 h at 37°C. Colonies morphologically compatible with *Enterococcus* spp. were confirmed by PCR (Dutka-Malen et al., 1995). In parallel, using a boiling method (Queipo-Ortuño et al., 2008), bacterial DNA was extracted from Slanetz-Bartley agar plates to detect the following antimicrobial resistance genes: Cu (tcrB) (Hasman et al., 2006), vancomycin (vanA, vanB, vanC1, vanC2) (Dutka-Malen et al., 1995; Kariyama et al., 2000), tetracycline (tetM), and erythromycin (ermB) (Jacob et al., 2008). *In vitro* susceptibility of *Enterococcus* spp. isolates was determined by the disk diffusion method using 13 antimicrobial agents: vancomycin (30 µg, BD, US), penicillin G (10 µg, Oxoid, UK), ampicillin (25 µg, BD, US), imipenem (10 µg, BD, US), erythromycin (15 µg, BD, US), tetracycline (30 µg, BD, US), ciprofloxacin (5 µg, BD, US), enrofloxacin (5 µg, BD, US), clindamycin (2 µg, BD, US), gentamicin (10 µg, BD, US), kanamycin (30 µg, BD, US), streptomycin (10 µg, BD, US) and chloramphenicol (30 µg, BD, US). The CLSI cut-off values were used. Additionally, a minimum inhibitory concentration (MIC) test was performed to assess the susceptibility of *Enterococcus* spp. to Cu sulfate pentahydrate using the broth-microdilution method. Isolates were cultured for 24 h in wells plates with brain heart infusion broth supplemented with 0.5, 1, 2.5, 5, 10, 15 and 20 mM of Cu sulfate pentahydrate. The ATCC 29212 *Enterococcus faecalis* strain was used as a quality control.

Statistical analysis

Data was analyzed as a randomized complete block design using the MIXED procedure of SAS (version 9.4, SAS Institute; Cary, US). The model included the fixed effects of source, level source × level interaction, and the random effects of block. Pen was the experimental unit for performance response. Mineral concentration in organs, antioxidant measurements and microbiota community were analyzed with an individual pig as the experimental unit. The normality and homogeneity of the data were examined using the Shapiro-Wilk and Hovtest statistical tests by SAS®. The

concentration of Cu in liver exhibited heterogeneity, therefore it was log transformed before analysis. Serum mineral content was analyzed as repeated measures. Biostatistical analyses were performed in open source software R Studio v.3.5.1. Diversity was analyzed at specie level using a vegan package (Oksanen et al., 2017). Richness and alpha diversity were calculated with raw counts based on Shannon and Inverse Simpson estimators. Betadiversity was evaluated by multivariate anova based on dissimilarities with the adonis function. To compare any differential effects from treatments, an ANOVA was performed for richness and diversity. Finally, differential abundance analysis was performed with taxa relative abundances under a zero-inflated log normal mixture model, *P*-values were corrected by false-discovery rate (FDR) with the metagenomeseq package (Paulson et al., 2019).

Due to factorial arrangement, the main effects are discussed for responses in which the interaction was not significant. Significantly different means were separated using Tukey adjust. Significance was declared at a probability $P \leq 0.05$ and tendencies were considered when the *P*-value was between > 0.05 and < 0.10.

RESULTS

Analyzed mineral concentrations in feed were according to those planned. In sulfate diets, Cu level was 9.5 and 107.7 mg/kg for nutritional and high Cu level diets, respectively. Whereas, Zn content was 79.8 and 162.9 mg/kg for nutritional and high Cu sulfate diets, respectively. Likewise, in hydroxychloride diets the Cu content for nutritional (9.9 mg/kg) and high (133.4 mg/kg) diets was according to that expected. The Zn content was 110.1 and 176.1 mg/kg for nutritional and high Cu hydroxychloride diets, respectively. The difference between low and high levels of Cu in the diets were achieved with both sulfate and hydroxychloride Cu sources.

Growth performance

Growth performance response for the interaction between two sources of Cu and Zn and two Cu levels is shown in Table 2. Feeding diets with the higher Cu level increased the ADFI, BW and ADG during the PS and ST periods, being the final BW increased from 16.6 to 17.7 kg (P = 0.0002). Likewise, G:F increased as Cu inclusion in the diet increased (P = 0.038), but no effects of Cu and Zn source were observed on growth performance (P > 0.10). There was no effect of the interaction between source × level on any of the growth performance responses except for ADFI (P = 0.004) and G:F (P = 0.029) at the end of the ST period and for G:F (P = 0.006) for the entire nursery period (d 0-42). At the end of the ST period, pigs fed Cu at 160 mg/kg as hydroxychloride had a higher ADFI but also lower G:F than those fed Cu sulfate at 160 mg/kg. For the entire nursery period, G:F did not differ between pigs fed Cu at 160 mg/kg as hydroxychloride or sulfate. Mortality was 2.46% and was not related to any dietary treatment (P > 0.10).

Antioxidant activity

The activity of GPX on pigs fed Cu at nutritional level was greater than that for those fed Cu at a high level (3,389 U/L vs 3,004 U/L; P = 0.013) at the end of the ST period. The GPX activity tended to be higher in animals fed Cu and Zn hydroxychloride than those fed Cu and Zn sulfate (3,437 U/L vs 3,144 U/L; P = 0.057) at the end of the PS period. No interactions between source × level on the activity of SOD or GPX in plasma was observed (P > 0.10; Table 3).

Mineral content in organs and tissues

Feeding diets with higher levels of Cu increased liver and serum content of Cu and Zn (P < 0.05; Table 4). Pigs fed Cu and Zn hydroxychloride had a greater Cu content in the liver (P = 0.036) and serum (P = 0.037) than those fed Cu and Zn sulfate. No effect of mineral source on liver and serum Zn concentrations was observed (P > 0.10). There was no effect of the interaction between source × level on Cu and Zn content on liver and serum except for Zn content on tibia (P = 0.044). Pigs fed Cu

hydroxychloride at high level had a greater accumulation of Zn (277.3 mg/kg) than those fed high Cu level as sulfate (256.1 mg/kg). All treatments had low levels (< 0.02 mg/g) of Cu storage in bone, below the ICP-OES detection limit.

Microbial molecular analysis

A two-way interaction between source and level was observed for alpha estimators (Table 5). The Shannon and Inverse Simpson index were lower in pigs fed a high Cu level as hydroxychloride compared to those fed Cu sulfate (P < 0.05). Beta diversity analysis revealed distances between clustered samples of nutritional and high Cu level group (P_{ADONIS} = 0.001) and a tendency for the two-way interaction between source and level (P_{ADONIS} = 0.054; Fig. 1a-b).

At family level, 224 different families were identified. From those, the families most frequently reported to change are presented in Fig.2 and 3. Diets with high Cu levels increased the relative abundance of Chrysiogenaceae, Halomonadaceae and Ruminococcaceae and decreased the abundance of the Acetobacteraceae and Brucellae families (P-adjust < 0.05; Fig.2). Regarding Cu and Zn source effect, Vibrionaceae family decreased (P-adjust = 0.027) and Methylobacteriaceae tended to increase more in pigs fed hydroxychloride minerals than in those fed sulfate minerals (P-adjust < 0.10; Fig.3).

At the genus level, 554 different genera were detected. From those, the genera most frequently reported to change are presented in Fig. 2 and 3. Pigs fed Cu at 160 mg/kg had higher relative abundance of *Methanosphaera* and *Roseburia* genera compared to those fed nutritional levels (P-adjust < 0.05; Fig.2). The effect of Cu and Zn source was observed in the relative abundance of *Vibrio, Enterobacter, Propionibacterium* and *Halomonas*, being lower for hydroxychloride than sulfate diets (P-adjust < 0.05; Fig.3). Meanwhile, the supplementation of Cu and Zn as hydroxychloride increased the *Methanobacterium*, *Acidaminococcus, Gallibacterium, Anaerovibrio* and *Actinobacillus* abundance compared to Cu and Zn sulfate (P-adjust < 0.05).

Additionally, the increase in Cu as hydroxychloride decreased the abundance of *Blautia*, *Streptococcus*, *Enterobacter*, *Fusobacterium*, *Escherichia* and *Vibrio* whereas sulfate did not (*P*-adjust < 0.05; Fig.4). *Lachnospira* and *Roseburia* tended to increase in pigs fed Cu at 160 mg/kg as hydroxychloride (*P*-adjust < 0.10; Fig.4). At nutritional level Cu as hydroxychloride decreased the abundance of *Enterobacter, Pasteurella, Leptospira, Erysipelothrix, Vibrio, Actinopolyspora* and *Clostridium*, while increasing *Lactobacillus* abundance compared to sulfate at the same Cu level (*P*-adjust < 0.05; Fig.5).

Enterococcus spp. isolation, detection of antimicrobial resistance genes and phenotypical antimicrobial resistance tests

Enterococcus species were isolated in a total of 40 samples with *E. faecalis* being the most dominating (26 samples; 65%). *E. faecium* was isolated from one sample and the remaining samples were identified as *Enterococcus* spp. (32.5%). A similar proportion of the different *Enterococcus* species were isolated in both hydroxychloride and sulfate diets (Table 6). In general, the presence of antimicrobial resistance genes in *Enterococcus* isolates was low. Only, ermB and tetM genes were detected in three and two samples, respectively (Table 6). No resistance genes were identified for Cu (tcrB) and vancomycin (VanA, VanB, VanC1, and VanC2). Meanwhile, using a disk diffusion test, all isolates were resistant to erythromycin, tetracycline, clindamycin, kanamycin and streptomycin and sensitive to vancomycin (Fig. 6). The highest percentages of resistant isolates were observed for gentamicin (98%), ciprofloxacin (95%), chloramphenicol (85%) and enrofloxacin (83%). Whereas, the lower resistances were observed for imipenem (8%), penicillin G (22%) and ampicillin (35%). The rates of antimicrobial resistance did not differ between treatments (Fig. 6). Regarding MIC test results, 65% of the isolates showed MIC values between 5 mM and 10 mM. The mean MIC value of Cu for all isolates was 6.74 mM. No differences were observed among treatments (Table 6).

DISCUSSION

Effect of Cu level supplementation

Higher dietary Cu level increased growth performance, resulting in a difference of 1 kg BW at the end of the nursery period under challenging conditions. Suggested mechanisms for high dietary Cu level effects on performance include their effects on microbiota (Pang et al., 2009), lipase and phospholipase activity and fat digestibility (Luo and Dove, 1996; Gonzales-Eguia et al., 2009), on hormone production in the intermediary metabolism (Li et al., 2008) and ghrelin synthesis in the stomach (Yang et al., 2012). In fact, previous studies reported that high dietary Cu levels (160 mg/kg) in pigs' diets increased feed intake and growth performance (Bikker et al., 2015). It must be noted that in our study, weaned pigs were allotted to previously used pens that were not disinfected or cleaned in order to provide poor sanitary conditions through contact with a wide range of fecal microorganisms from older pigs. In this sense, ADG (280 g) of pigs fed the high dietary levels of Cu diets were lower than the common ADG recorded in the farm (290 g) in standard commercial conditions when pens were properly clean but also with the inclusion of therapeutic doses of Zn in the feed.

Different dietary levels of Cu were also associated with changes in mineral tissue concentration. High Cu levels in the diet increased the Cu and Zn content in the liver and serum. The liver is the primary storage organ and is responsible for regulating the amount of Cu and Zn in the body through bile excretion to the intestinal tract, or distributing it through the blood to other organs. Therefore, the complementary evaluation of trace minerals in serum or plasma could indicate the amount of trace minerals that is circulating in the body (López-Alonso, 2012). Usually, the main antagonistic interaction between Cu and Zn has been observed when high levels of Zn in the diet (> 2,000 mg/kg) are supplemented, resulting in a Cu deficiency (Gaudré, 2016). Meanwhile, dietary Cu has little or no effect on Zn metabolism (Keen et al., 1985).

One of the primary functions of Cu is to be part of a large number of cuproenzymes in the catalysis of superoxide radicals (Suttle, 2010). Nevertheless, in excess or free unbound in the bloodstream, Cu is potentially toxic resulting in oxidation, and catalyzing the formation of hydroxyl radicals (Bremner, 1998; Gaetke et al., 2014). In the present study, feeding pigs high Cu levels resulted in lower GPX activity compared to pigs fed nutritional Cu levels, suggesting that more Cu ions lead to more oxidation in plasma.

High dietary levels of Cu were also able to modify the main variable of microbiome composition. In fact, one of the growth-promoting actions of Cu has been attributed to its antimicrobial effect. Diets supplemented with Cu at 160 mg/kg decreased Brucellaceae, *Streptococcus*, *Pseudomonas*, which may contain opportunistic pathogens, and increased Ruminococcaceae, *Actinobacillus* and *Roseburia* compared to Cu at 15 mg/kg. The bacterial modulation, towards the reduction of opportunistic pathogens together with the development of saprophytic bacteria, could lead to a significant improvement in intestinal nutrient absorption and, therefore, pig feed efficiency. Furthermore, it is known that many members of the family and genera, which increased as a result of high Cu supplementation, produce (directly or indirectly) short-chain fatty acids (SCFA; ie, butyrate, propionate and acetate) (Tungland, 2018). For instance, *Roseburia* is known to be a butyrate producer from the fermentation of dietary non-digestible carbohydrates, but *in vitro* studies have also shown that genera such as *Roseburia* and *Eubacterium* can use the lactate and/or acetate produced by *Bifidobacterium* to produce other SCFA as propionate (Duncan et al., 2002; Tungland, 2018). SCFA are essential forms of energy, which are rapidly absorbed by colonic epithelial cells to exert beneficial effects on the host such as protection against colonic diseases, improvement of intestinal barrier function and reduction of inflammation in the gut (Ríos-Covián et al., 2016). Although SCFA were not directly measured in the present study, these bacterial findings could support improved growth performance of pigs when diets are supplemented with Cu at 160 mg/kg in contrast to diets with Cu at 15 mg/kg. The association between intestinal microbiota composition

of pigs and their growth performance and health has been explored in previous studies (Højberg et al., 2005; Mei et al., 2009; Yu et al., 2017).

The effect of high Cu concentration on microbial cells has been related to the induced production of intracellular reactive oxygen radicals inactivating cell components such as nucleic acids, lipids, and proteins resulting in bacterial death (Djoko et al., 2015). However, to protect themselves from this toxic effect, bacteria evolved a range of mechanisms such as extracellular sequestration of Cu ions, relative impermeability of the outer and inner bacterial membranes to Cu ions, metallothionein Cu-scavenging proteins in the cytoplasm and periplasm, and active extrusion of Cu from the cell. The latter appears to be the chief mechanism of Cu tolerance in bacteria and has been extensively studied in Gram-positive and Gram-negative bacteria (Grass et al., 2011). Most of the Cu-scavenging proteins (CPx-type ATPases), are encoded by genes located on the chromosome. Meanwhile, Cu resistance genes are often located on plasmids, being in most cases transferable (Hasman and Aarestrup, 2002). The transferable and plasmid-located Cu resistance gene designated as tcrB has been identified in several *Enterococcus* species including *E. faecium* and *E. faecalis* (Hasman and Aarestrup, 2002; Hasman et al., 2006). Interestingly, the same plasmid was also found to carry genes ermB and vanA, which encode resistance to macrolides and glycopeptides, respectively (Hasman and Aarestrup, 2002; Hasman et al., 2006). Therefore, in the present study we focused on the detection of Cu, vancomycin, tetracycline and erythromycin resistance genes in *Enterococcus* spp. isolates. A total of 40 samples were identified as *Enteroccocus* spp., being *E. faecalis* the most dominating (65%). From these, all isolates were negative for the tcrB gene. Previous studies conducted in Denmark and the US reported the prevalence of the tcrB gene in enterococcal isolates at 76% (Hasman and Aarestrup, 2002), 11.9% (Amachawadi et al., 2011) and 4.9% (Amachawadi et al., 2010), on pigs, 34% on broiler chickens (Hasman and Aarestrup, 2002), 16% on calves (Hasman and Aarestrup, 2002) and 6.9% on heifers (Amachawadi et al., 2013). It must be noted that the highest prevalence (76%) of the tcrB gene was described in pigs before slaughter in Denmark. The authors (Hasman and Aarestrup, 2002) point out that in Denmark, on the date when the study was

performed (1998), high (165 mg/kg) concentrations of Cu sulfate were supplemented in weaned pigs (< 35 kg) decreasing afterwards (25 mg/kg). Whereas lower prevalence (Amachawadi et al., 2010, 2011) were obtained in US studies feeding lower levels of Cu (16.5 and 125 mg/kg) for 35 to 42 days, similar to our study. Differences in prevalence of the tcrB gene reported in previous studies question whether the prevalence of the Cu resistance gene can be determined by the age of the animal, by a long-term effect of the animal's exposure (e.g. from suckling until slaughter) or by prolonged exposure of the farm to high levels of Cu. In a longitudinal study, Amachawadi et al., (2011) did not find a linear increase (d 0, 14, 28 and 42) in the prevalence of tcrB-positve fecal enterococci in weaned pigs fed diets with a continued supplementation of low (16 mg/kg) or high (125 mg/kg) level of Cu for 42 days. Further longitudinal field studies are requiered to elucidate the effect of high levels of Cu in the diet in the presence of the tcrB gene in animals in a farm environment. The absence of the tcrB gene in our isolates agrees with the low MIC Cu results (< 10 mM). From the literature, it can be drawn that tcrB-positive *Enterococcus* are associated with MIC > 20 mM/Cu, while those tcrB-negative isolates had values < 8 mM/Cu (Hasman and Aarestrup, 2002; Amachawadi et al., 2010, 2011, 2013). No resistance genes for vanA, vanB, vanC1 and vanC2 were detected, and all isolates were phenotypically susceptible to vancomycin. Surprisingly, the prevalence of ermB (7.5%) and tetM (5%) genes was low as opposed to the phenotypically resistant results to erythromycin and tetracycline. In *Enterococcus* spp. the most common genes conferring resistance to antibiotics are for erythromycin, tetracycline and vancomycin (Oravcova et al., 2019; Tian et al., 2019). Nevertheless, it is possible that this phenotypical resistance is conferred by other mechanisms or untested genes. The high rates in phenotypical resistance of Enteroccocal isolates could be explained by the fact that the north-east of Spain has one of the densest pig populations in Europe and different antimicrobial agents are still widely used in livestock. Moreover, a long-term effect on the microbial population after antibiotic administration should be considered. In pigs, the effects of a single intramuscular administration of amoxicillin may persist at least after 5 weeks (Janczyk et al., 2007). Although the potential selective pressure that Cu supplementation could exert

on antimicrobial resistance was not evidenced in the present study, it certainly requires more attention.

Effect of Cu and Zn source supplementation

In the present study, differences between mineral sources were observed in growth performance (ADFI and G:F), mineral tissue content and microbial community. Results of studies with broiler chickens indicate that the effect of Cu and Zn on growth performance could depend on chemical differences between trace mineral sources. Olukosi et al., (2018) reported that broiler chickens receiving Zn and Cu hydroxychloride had greater G:F than those fed Zn and Cu sulfate. Likewise, Lu et al., (2010) described that broiler chickens fed 200 mg/kg Cu as Tribasic copper chloride (TBCC) had greater ADG than those fed Cu sulfate. Similarly, supplementation of diets for broiler chickens with Cu sulfate at 300 mg/kg had reduced ADG and reduced G:F ratio than birds fed Cu_2O at the same level (Hamdi et al., 2018). Results of pig studies have demonstrated that other forms of both Cu and Zn, such as lysine complex (Coffey et al., 1994; Apgar et al., 1995; Cheng et al., 1998) and hydroxychloride (Cromwell et al., 1998; Fry et al., 2012; Carpenter et al., 2016) are as effective in improving growth as sulfate minerals. Although different studies on pigs have shown no differences between Cu and Zn sources at high or low levels, the evaluation of intermediate levels might reveal differences that are mainly driven by the higher or lower bioavailability of trace mineral sources. In

this sense, Veum et al., (2004) reported that feeding pigs with intermediate Cu levels (25, 50 and 100 mg/kg) as Cu proteinate had higher ADG and ADFI than those fed a high level of Cu sulfate (250 mg/kg). The possibility of reducing the amounts of Cu and Zn by using higher bioavailable sources could represent an alternative to the inclusion of pharmaceutical doses of trace minerals in diets. Therefore, the negative interactions between nutrients and the environmental impact attributed to high doses could be reduced without affecting pig performance. In the context of stricter regulations, further studies exploring biovailability through increasing doses of different mineral sources should be explored, particularly to suckling and weaned pigs.

Pigs fed with hydroxychloride minerals had higher Cu concentrations in liver and serum compared with sulfate. An important factor for intestinal absorption of Cu and Zn is their availability as free ions in intestinal lumen (Martin et al., 2013). Results from our laboratory confirmed that sulfates are highly soluble in a wide range of pH from 2.5 to 6.5 whereas hydroxychloride minerals are less soluble at pH 6.5 but highly soluble at pH 2.5, as previously reported (Pang and Applegate, 2006). Consequently, less chelated interactions with other components of the diet may have occurred with hydroxychloride minerals, making them more available to be absorbed compared to sulfate. The fact that pigs fed a high level of Cu as sulfate had lower Zn content in the tibia could suggest a likely antagonistic interaction between high Cu level and Zn, and possibly with other minerals, for metal-binding sites that hydroxychloride minerals did not show. Results from earlier studies reported differences and interactions between Cu and Zn sources in absorption and mineral tissue accumulation. For instance, in broiler chickens, Olukosi et al., (2018) reported that Cu liver was influenced by the Cu and Zn source being greater for hydroxychloride than for sulfate minerals. In 2015, Huang et al., reported greater Zn and Cu storage in the liver of pigs fed Cu as TBCC compared to those fed Cu as sulfate. Further studies involving complementary analysis of both protein and mRNA levels of Cu and Zn transporters could help to clarify differences in mineral storage as well as lead to a more comprehensive understanding of metal absorption pathways.

After 14 days of weaning, a tendency for higher serum GPX activity in pigs fed Cu and Zn hydroxychloride was observed. Highly soluble trace mineral sources may result in greater oxidation rates (Miles et al., 1998). Earlier studies in broiler chickens reported that TBCC was less active than sulfate in promoting oxidation of vitamin E in feed and in reducing vitamin E content in plasma and liver (Luo et al., 2005; Lu et al., 2010). Results of studies with pigs demonstrated that Cu sulfate diet at 225 mg Cu/kg may cause greater oxidative stress in the duodenum than Cu as TBCC (Fry et al., 2012; Huang et al., 2015). The covalent bonding of hydroxychloride trace minerals could allow Cu and Zn to gradually become soluble in the small intestine, thus resulting in less oxidative stress than the sulfate counterparts, as suggested by Fry et al., (2012).

Since mineral sources have different solubility, they may affect the intestinal microbiota differently. In our study, an increase in the relative abundance of some beneficial bacteria was observed in pigs fed hydroxychloride minerals, particularly at high Cu level. From the literature, it is known that gut microbiota plays essential roles in amino acid catabolism and energy harvest from the diet. Indeed, genera such as *Lachnospira, Roseburia* and *Coprococcus* produce various metabolites such as SCFA, and biogenic amines (Tungland, 2018). Based on these results, hydroxychloride diets appear to improve intestinal microbiota profile and some mineral content in tissues. High levels of Cu as hydroxychloride increased BW performance; however, the beneficial effects of hydroxychloride were not completely reflected in pig feed efficiency compared to high Cu sulfate. Additional markers such as fecal consistency score and intestinal integrity indicators, which were not measured in the present study, are needed to draw consistent conclusions.

No differences in the presence of antimicrobial resistance genes or phenotypical antimicrobial resistance profile between Cu and Zn sources were observed. Nevertheless, this relationship should be discussed in field studies in greater depth and with a greater number of *Enterococcus* spp. isolated.

In conclusion, the EU permissible levels of Cu (160 mg/kg) increase growth performance and modulate bacterial communities compared to nutritional levels (15 mg/kg) in weaned pigs reared under challenging conditions. Different effects on mineral tissue content and microbial modulation were observed between Cu and Zn sources. The reduction of Cu and Zn contents in pig diets by using higher bioavailable sources should be explored in order to reduce the environmental impact. Longitudinal field studies are necessary to confirm the influence of high levels of Cu supplement on antimicrobial cross-resistance genes.

Conflict of interest statement. None declared.

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Figure 1. Non-metric dimensional scaling (NMDS) plot of dissimilarity matrix based on Bray-curtis distance clustered by Cu dietary level (P_{ADONIS} = 0.001) (A); and by experimental diets SF-N: Sulfate at nutritional level, SF-H: Sulfate at high level, HCl-N: Hydroxychloride at nutritional level, HCl-H: Hydroxychloride at high level (P_{ADONIS} = 0.054) (B). Data are means of 12 replicate pens for the two-way interaction, whereas for the main effect of level are means of 24 replicate pens (1 pig per replicate pen was sampled).

Figure 2. Differentially abundant taxa (ln change and FDR-adjusted $P < 0.20$) between diets supplemented at high and nutritional Cu levels, regardless of the mineral source. Positive values ( ) and negative values ( ) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa is presented. Data are means of 24 replicate pens for the main effect of level (1 pig per replicate pen was sampled).

Figure 3. Differentially abundant taxa (ln change and FDR-adjusted $P < 0.20$) between Zn and Cu hydroxychloride and sulfate diets, regardless of the Cu level. Positive values ( ) and negative values ( ) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa is presented. Data are means of 24 replicate pens for the main effect of source (1 pig per replicate pen was sampled).

Figure 4. Differentially abundant taxa at genus level (ln change and FDR-adjusted $P < 0.20$) at high Cu supplementation between hydroxychloride (HCl) and sulfate source.

Positive values ( ) and negative values ( ) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa is presented. Data are means of 12 replicate pens for the two-way interaction (1 pig per replicate pen was sampled).

Figure 5. Differentially abundant taxa at genus level (ln change and FDR-adjusted $P < 0.20$) at nutritional Cu supplementation between hydroxychloride (HCl) and sulfate source. Positive values ( ) and negative values ( ) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa is presented. Data are means of 12 replicate pens for the two-way interaction (1 pig per replicate pen was sampled).

Figure 6. Percentage of *Enterococcus* spp. isolates resistant to different antimicrobials agents from pigs fed diets with two Cu and Zn sources (sulfate and hydroxychloride) at two Cu levels (15 and 160 mg/kg). VAN, vancomycin; IMI, imipenem; PG, penicillin G; AMP, ampicillin; CLOR, chloramphenicol; ENR, enrofloxacin; CP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; ST, streptomycin; ERY, erythromycin; TET, tetracycline; CLIN, clindamycin

ANNEX

Figure 7. Differentially abundant taxa at genus level (ln change and FDR-adjusted $P < 0.20$) between sulfate diets supplemented at high and nutritional Cu levels. Positive values ( ) and negative values ( ) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa is presented. Data are means of 12 replicate pens for the two-way interaction (1 pig per replicate pen was sampled).

Figure 8. Differentially abundant taxa at genus level (ln change and FDR-adjusted $P < 0.20$) between hydroxychloride (HCl) diets supplemented at high and nutritional Cu levels. Positive values ( ) and negative values ( ) indicate greater and lower abundance, respectively. Taxa are sorted by level of significance (from higher to lower). Only significant taxa is presented. Data are means of 12 replicate pens for the two-way interaction (1 pig per replicate pen was sampled).

Table 1. Composition of the basal diets for the two phases, as-fed basis[1]

Ingredients, %	Pre-starter	Starter
Wheat	26.32	40.60
Maize	6.75	20.00
Barley	12.20	15.38
Soybean meal 47% CP	0.90	12.98
Fishmeal	4.00	5.00
Lard	2.58	2.48
Soybean meal heat treated	3.60	-
Extruded Wheat	13.05	-
Porcine Plasma	3.00	-
Dextrose	4.00	-
Acid milk whey	4.50	-
Sweet milk whey	8.50	-
Extruded soybeans	7.15	-
Di calcium phosphate	1.36	1.40
Calcium carbonate	0.18	0.11
L-Lysine 50	0.80	0.85
L-Threonine	0.22	0.24
DL-Methionine	0.25	0.16
L-Tryptophan	0.02	0.05
Salt	0.22	0.35
Vitamin premix nucleous[2]	0.40	0.40

Calculated composition		
DM	90.0	89.1
NE, kcal/kg	2550	2401
CP	19.5	17.9
NDF	10.8	10.3
Ether extract	6.5	4.8
Ca	0.70	0.60
Total P	0.68	0.68
Dig P	0.40	0.40
Analysed composition		
DM	91.5	90.2
CP	18.7	18.6
Ether Extract	6.3	6.3
NDF	7.8	7.8
Ash	5.1	4.8

[1]Pre-starter phase diets were fed from d 0 to 14 and starter phase diets were fed from d 14 to 42.

[2]Provided per kg of feed: vitamin A (acetate): 12,000 IU; vitamin A (retinol): 2,000 IU; vitamin D3 (cholecalciferol): 1,204 IU; vitamin D (25-hydroxicholecalciferol): 600 IU; vitamin E: 104 IU; vitamin K3: 2 mg; vitamin B1: 3 mg; vitamin B2: 7 mg; vitamin B6: 3.5 mg; vitamin B12: 0,1 mg; D-pantothenic acid: 17 mg; niacin:45 mg; biotin: 0.2 mg; folacin: 1.5 mg; Fe (chelate of amino acid): 15 mg; Mn (oxide): 6.25 mg; Mn (chelate of glycine): 3.75 mg; I (calcium anhydrous): 1.75 mg; Se (organic): 25 mg; Se (sodium): 50 mg. Phytase: 1,500 FYT (Ronozyme® NP (M), DSM, Basel, Switzerland).

Table 2. Growth performance[1] of pigs fed diets with two Cu and Zn sources (sulfate and hydroxychloride) at two Cu levels (15 and 160 mg/kg) [2]

Cu and Zn	Cu level,	BW, kg			ADFI, g			ADG, g			G: F		
Source	mg/kg	d 0	d 14	d 42	d 0-14	d 14-42	d 0-42	d 0-14	d 14-42	d 0-42	d 0-14	d 14-42	d 0-42
Sulfate	15	5.86	6.97	16.62	126.6	492.6 ^{ab}	370.6	86.9	352.4	256.2	0.695	0.718 ^{b}	0.696 ^{b}
	160	5.86	7.37	17.99	150.5	491.4 ^{ab}	376.3	111.9	381.1	288.8	0.757	0.778 ^{a}	0.770 ^{a}
Hydroxychloride	15	5.86	7.11	16.51	134.1	456.3 ^{b}	348.7	89.1	335.9	253.6	0.673	0.740 ^{ab}	0.729 ^{ab}
	160	5.86	7.36	17.45	138.5	529.0 ^{a}	381.0	107.0	375.6	271.2	0.780	0.714 ^{b}	0.718 ^{ab}
SEM[3]		0.496	0.486	0.883	10.48	38.20	21.75	4.69	17.28	11.36	0.048	0.044	0.041
P-value[4]													
Source		0.894	0.398	0.256	0.701	0.957	0.417	0.767	0.383	0.162	0.989	0.259	0.544
Level		0.894	<.0001	0.0002	0.020	0.005	0.075	<.0001	0.009	0.001	0.003	0.374	0.038

Source × Level	0.689	0.311	0.439	0.106	0.004	0.208	0.428	0.660	0.293	0.403	0.029	0.006

[1]Body weight; Average daily feed intake; Average daily gain; Gain: Feed.

[2]Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (11 pigs per replicate pen).

[3]Standard error of the mean.

[4]a-b: Values within the same column with different letters differ significantly ($P < 0.05$).

Table 3. Antioxidant activity of pigs fed diets with two Cu and Zn sources (sulfate and hydroxychloride) at two Cu levels (15 and 160 mg/kg)[1]

Cu and Zn Source	Cu level, mg/kg	Superoxide dismutase, U/mL		Glutathione peroxidase, U/L	
		d 14	d 42	d 14	d 42
Sulfate		173.8	138.4	3,144	3,109
Hydroxychloride		185.1	134.7	3,437	3,284
SEM[2]		6.00	9.72	106.9	101.3
	15	179.9	143.3	3,373	3,389
	160	179.0	129.8	3,208	3,004
SEM[2]		5.92	9.62	106.9	101.3
P-value[3]					
Source		0.181	0.789	0.057	0.226
Level		0.921	0.328	0.277	0.013
Source × Level		0.465	0.792	0.621	0.637

[1]Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (1 pig per replicate pen was sampled).

[2]Standard error of the mean.

[3]a-b: Values within the same column with different letters differ significantly ($P < 0.05$).

Table 4. Serum and tissue Cu and Zn content of pigs fed diets with two Cu and Zn sources (sulfate and hydroxychloride) at two Cu levels (15 and 160 mg/kg)[1]

Cu and Zn Source	Cu level, mg/kg	Serum, mg/L[2]		Liver, mg/kg DM		Bone, mg/kg[4]
		Cu	Zn	Cu[3]	Zn	Zn
Sulfate		1.69	0.71	1.68 (48.7)	213.6	257.2
Hydroxychloride		1.79	0.72	1.80 (71.0)	221.6	268.0
SEM[5]		0.031	0.017	0.042	9.58	3.56
	15	1.65	0.67	1.59 (39.5)	199.9	258.5
	160	1.83	0.76	1.89	235.4	266.7

			(80.2)		
SEM[5]	0.031	0.017	0.041	9.53	3.72
P-value[6]					
Source	0.037	0.438	0.036	0.553	0.041
Level	<.0001	0.0002	<.0001	0.011	0.115
Source × Level	0.130	0.593	0.584	0.991	0.044

[1]Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (1 pig per replicate pen was sampled).

[2]*P*-value of day of sampling at d 14 and d 42 ($P < .0001$). *P*-value of interaction between source × level × day for Cu ($P = 0.299$) and for Zn ($P = 0.010$).

[3]Log10 transformed liver Cu concentration. Values in parentheses show the non-transformed values.

[4]Cu detected values are lower than 0.02 mg/g by ICP-OES.

[5]Standard error of the mean.

[6]a-b: Values within the same column with different letters differ significantly ($P < 0.05$).

Table 5. Evenness and diversity of colon microbiota of pigs fed diets with two Cu and Zn sources (sulfate and hydroxychloride) at two Cu levels (15 and 160 mg/kg)[1]

Cu and Zn Source	Cu level, mg/kg	Shannon	Inverse Simpson
Sulfate	15	2.38 [ab]	4.31 [b]
	160	2.31 [b]	4.07 [bc]
Hydroxychloride	15	2.54 [a]	5.18 [a]
	160	2.12 [c]	3.36 [c]
SEM[2]		0.047	0.220
P-value[3]			
Source		0.761	0.715
Level		<.0001	<.0001
Source × Level		0.0006	0.0009

[1]Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (1 pig per replicate pen was sampled).

[2]Standard error of the mean.

[3]a-b: Values within the same column with different letters differ significantly ($P < 0.05$).

Table 6. Characterisitics of *Enteroccoccus* spp. isolates[1] and prevalence of antimicrobial resistance genes[2] of pigs fed diets with two Cu and Zn sources (sulfate and hydroxychloride) at two Cu levels (15 and 160 mg/kg)

Cu and Zn Source	Cu level, mg/kg	*Enteroccoccus* spp. isolated, n	*E.faecalis*, n	*E.faecium*, n	*Enteroccoccus* spp., n	Mean MIC Cu, mM	AMR genes[3]			
							tcrB, n	ermB, n	tetM, n	vanA, vanB, vanC1, vanC2, n
Sulfate	15	11	8	1	2	5.41	0	0	0	0
	160	11	6	0	5	6.68	0	0	1 (2.5)	0
Hydroxychloride	15	8	6	0	2	7.85	0	1 (2.5)	1 (2.5)	0
	160	10	7	0	3	7.05	0	2 (5)	0	0

[1]Data are means of 12 replicate pens for the two-way interaction, whereas for the main effects of source and level are means of 24 replicate pens (1 pig per replicate pen was sampled).

[2]Antimicrobial resistance genes for: Cu (tcrB), erythromycin (ermB), tetracycline (tetM) and vancomycin (vanA, vanB, vanC1, vanC2).

[3]Values in parenthesis show the prevalence percentage expressed for the total *Enteroccocus* spp. isolates.

Figure 1A

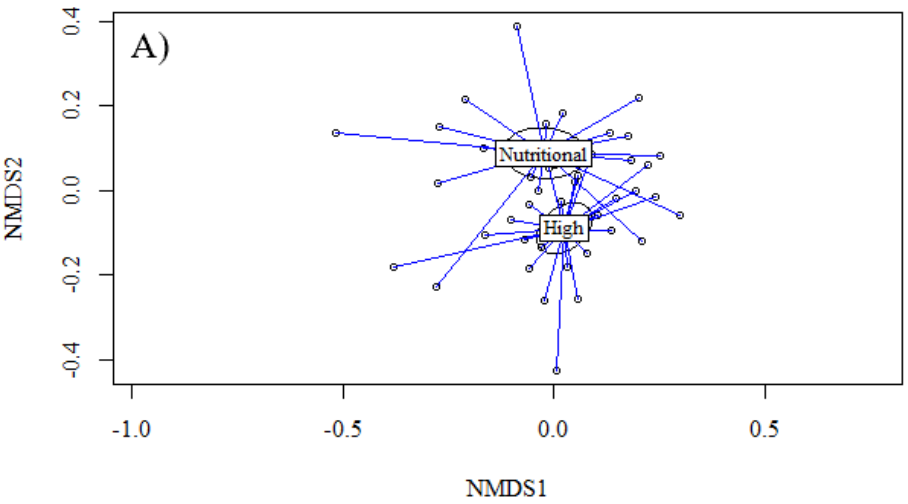

Figure 1B

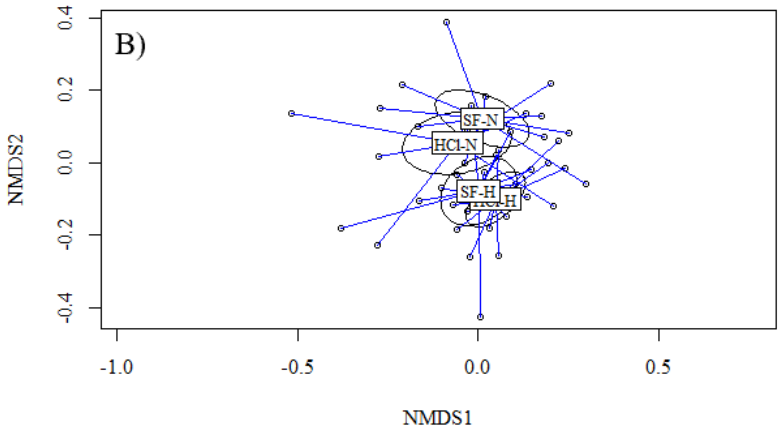

Figure 2

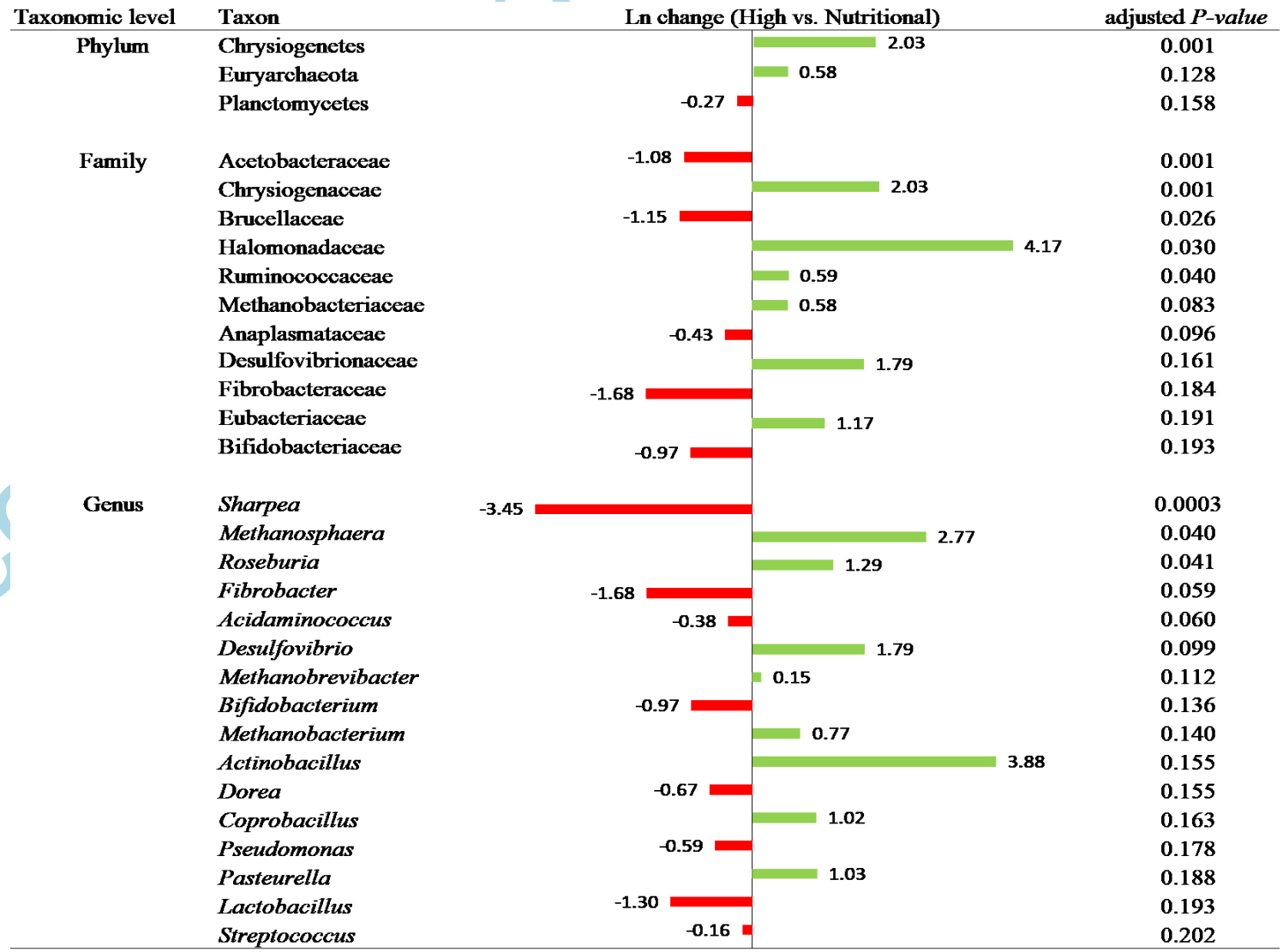

Figure 3

Taxonomic level	Taxon	Ln change (Hydroxychloride vs. Sulfate)	adjusted *P-value*
Family	Vibrionaceae	-1.15	0.027
	Methylobacteriaceae	0.69	0.092
	Rickettsiaceae	0.89	0.098
	Nocardiaceae	-1.45	0.168
	Anaplasmataceae	0.73	0.172
Genus	*Vibrio*	-1.63	0.0002
	Methanobacterium	1.54	0.0002
	Enterobacter	-1.70	0.0003
	Acidaminococcus	2.04	0.0004
	Propionibacterium	-1.46	0.001
	Gallibacterium	1.48	0.006
	Halomonas	-0.79	0.006
	Anaerovibrio	0.75	0.032
	Actinobacillus	3.89	0.045
	Coprobacillus	-1.12	0.091
	Fusobacterium	-0.33	0.145
	Pasteurella	0.44	0.145
	Lachnospira	1.17	0.160
	Chlamydia	1.36	0.183
	Coriobacterium	-0.65	0.200

Figure 4

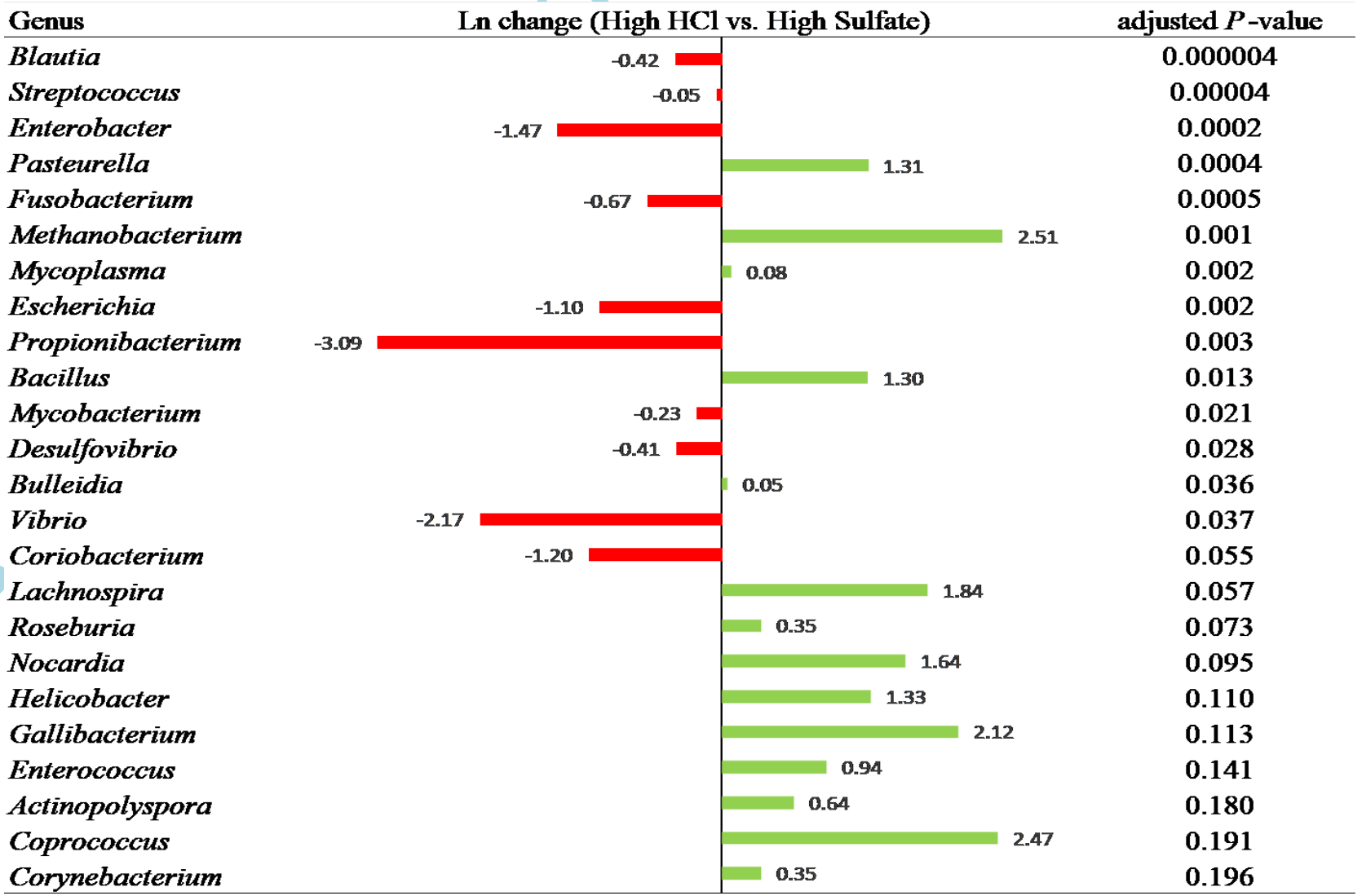

Figure 5

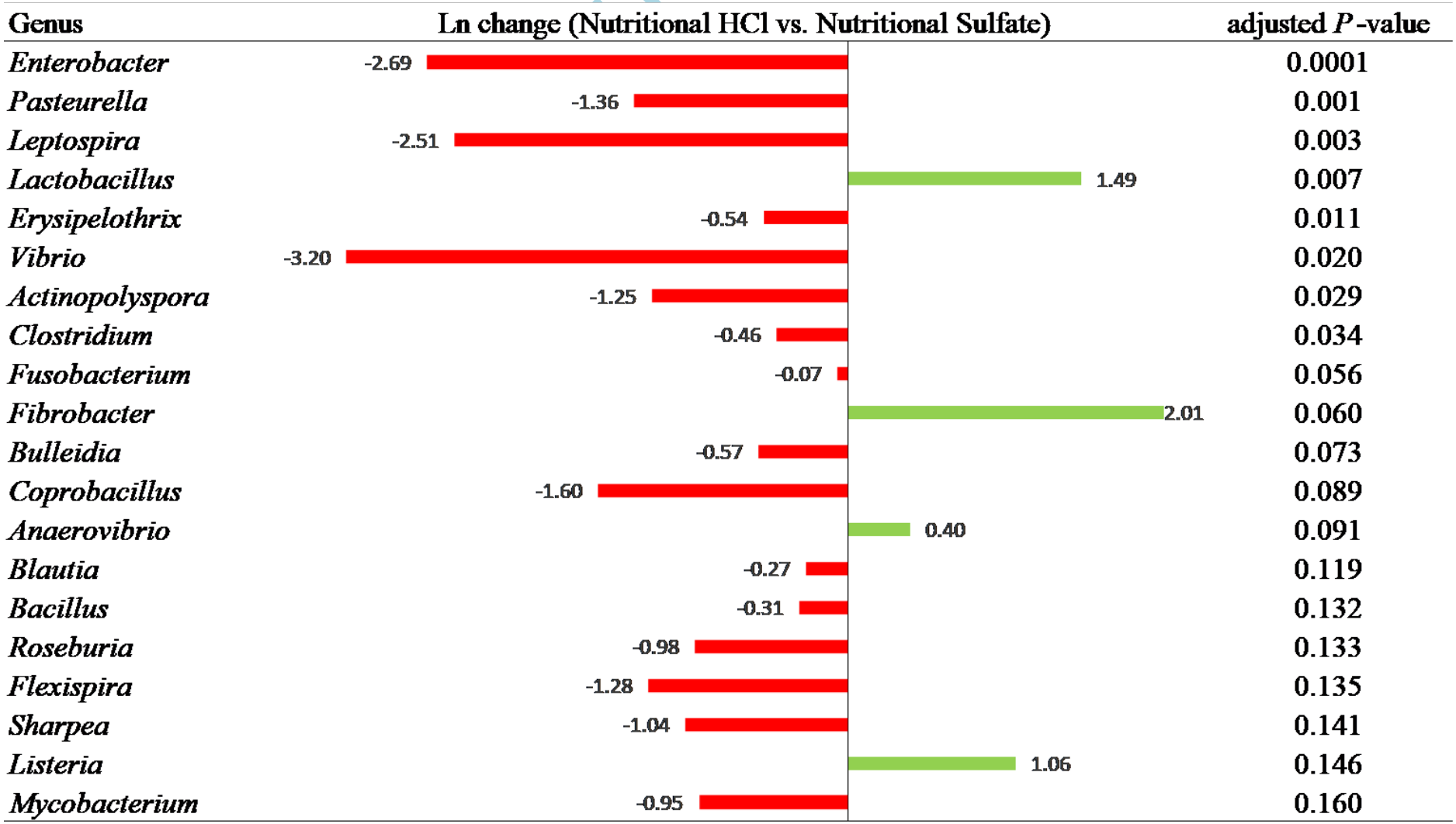

Figure 6

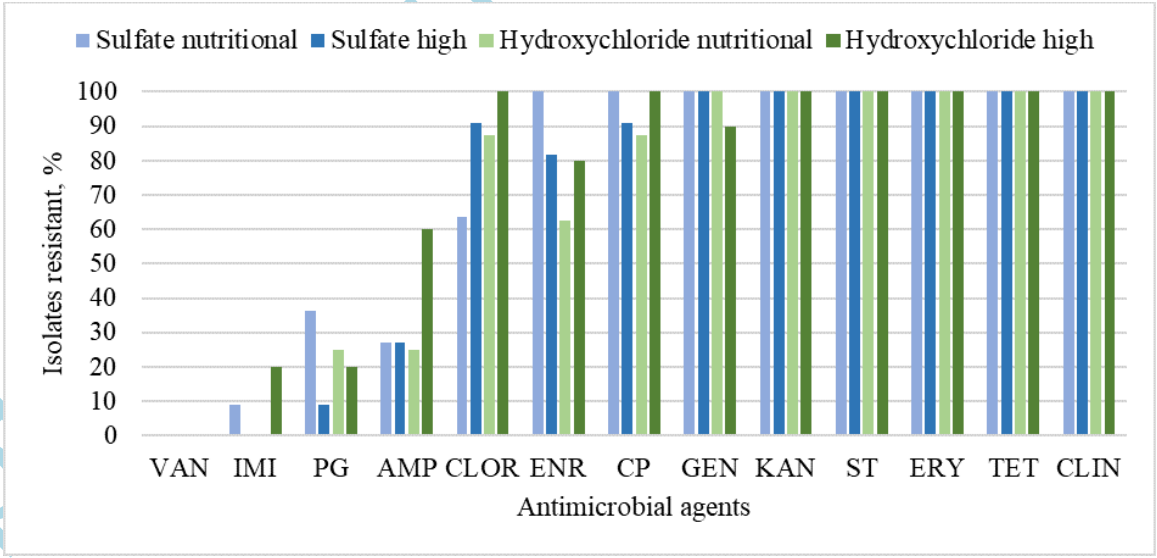

Figure 7

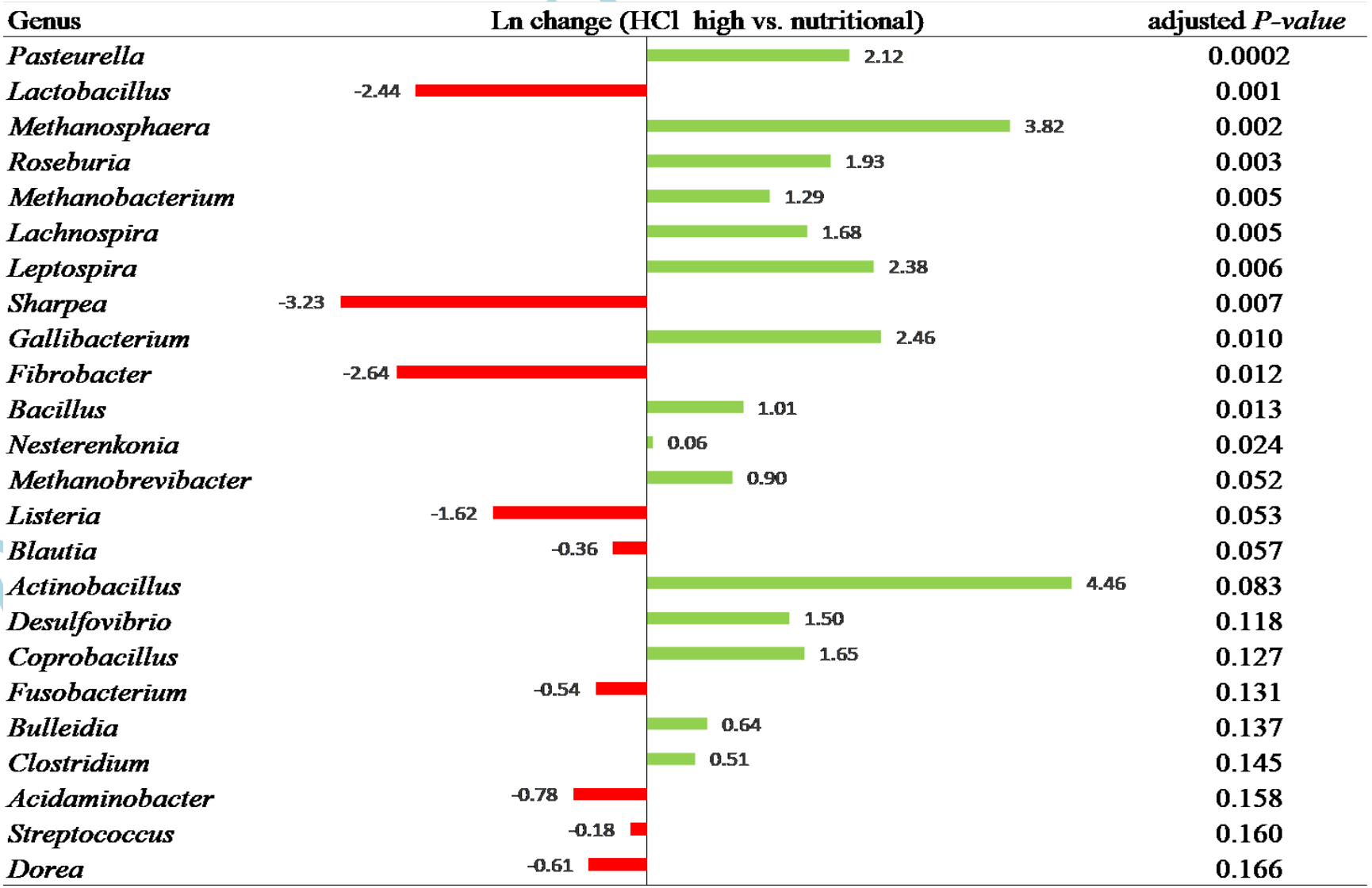

Figure 8