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Effects of exogenous enzymes from invertebrate gut-associated bacteria on volatile organic compound emissions and microbiota in an *in vitro* pig intestine continuous fermentation model

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Abstract

This study aims to assess the efficacies of exogenous enzymes, derived from invertebrate gut-associated microbes, as feed additives, in reducing volatile organic compound (VOC) emissions using an *in vitro* pig intestine continuous fermentation system. An *in vitro* continuous fermentation model was used to simulate a comparable bionic digestion system by co-reacting feed, enzymatic additives (arazyme, mannanase, and xylanase, derived from the gut bacteria of *Nephila clavata*, *Eisenia fetida*, and *Moechotypa diphysis*, respectively), and gastrointestinal microbes, followed by an analysis of their correlations. A significant correlation was observed between exogenous enzyme supplementation and reduced VOC emissions in the fecal phase of continuous fermentation ($p < 0.05$). The concentration of VOCs decreased by 3.75 and 2.75 ppm in the treatment group following arazyme and multi-enzyme supplementation, respectively, compared to that in the control group (7.83 ppm). In addition, supplementation with arazyme and multiple enzymes significantly affected the microbial composition of each fermentation phase ($p < 0.05$). In particular, *Lactiplantibacillus pentosus* and *Pediococcus pentosaceus*, which changed in abundance according to arazyme or multi-enzyme supplementation, exhibited a positive relationship with VOC emissions. These results suggest that exogenous enzymes derived from invertebrate gut-associated bacteria can be efficiently applied as feed additives, leading to a reduction in VOC emissions.

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Introduction

The emission of odors and gaseous pollutants such as greenhouse gases, nitrogen compounds, hydrogen sulfide, and volatile organic compounds (VOCs), from livestock farms, has elevated worldwide caution in the light of sustainable development goals announced by the United Nations in 2015 (Dennehy *et al*., 2017; UN, 2020). Although ammonia and

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hydrogen sulfide are the predominant components of all odor compounds, VOCs, with low olfactory threshold characteristics, can also cause a strong odor (Zhu *et al*., 2016). VOCs are a source of pollution emitted from livestock farms and include volatile fatty acids, sulfur- and nitrogen-containing compounds, phenolic compounds, phenols, esters, ketones, terpenes, and aromatic compounds (Ni *et al*., 2012). Long-term subjection to VOCs is a hazard to the health of livestock and to surrounding human residents, increasing the possibility of nervous prostration, respiratory and central nervous system disorders, and even carcinogenesis (Domingo *et al*., 2015; Nie *et al*., 2020). Moreover, constant VOC emissions can cause environmental problems by generating secondary organic aerosols, which subsequently lead to air pollution and ozone formation (Monks *et al*., 2009; Howard *et al*., 2010). VOCs are formed naturally during complex digestive processes in the stomach and intestines of livestock as well as through the numerous conversions that occur during the composting process of manure (Akdeniz *et al*., 2010). Substances typically responsible for livestock odor are associated with poor nutrient availability which in turn results in an increased excretion of fermentation by-products (O'Shea *et al*., 2014). The microbial community and its activities convert the unutilized nutrients to intermediate or end chemical compounds, leading to an increase odors and gaseous pollutants in livestock facilities (Gaskins, 2000).

Current practices and technologies for reducing VOC emissions include biological treatments that mostly use biomass materials such as biochar, which can remove environmental pollutants (Sánchez-Monedero *et al*., 2019). However, research on reducing VOC production through enzyme supplementation has not been widely performed. Hence, we purified arazyme from *Serratia proteamaculans* HY-3, a gram-negative symbiotic bacterium of the spider, *Nephila clavata* (Bersanetti *et al*., 2005). Arazyme, an exogenous metalloprotease exhibits high relative proteolytic activity and also reduces viscosity, indicating that viscosity, reflecting a marked feed enzyme effect, may lead to odor formation in intestinal contents (Kwak *et al*., 2007; Zi *et al*., 2011). Exogenous carbohydrolases, xylanases, and mannanases from *Cellulosimicrobium* sp. HY-13, a symbiotic bacterium of *Eisenia fetida* (Ham *et al*., 2011), and *Paenibacillus* sp. HY-8, a symbiotic bacterium of *Moechotypa diphysi* (Heo *et al*., 2006). This study aims to assess the efficacy of these invertebrate gut-associated microbial enzymes as additives in reducing VOC emissions through an *in vitro* pig intestine continuous fermentation model that simulated a similar bionic digestion system by co-reacting feed, enzymatic additives, and gastrointestinal microbes. Correlations between these components were then analyzed.

Materials and Methods

Experimental diets and feed additives

A mercantile pig feed (Special One®, TS Corporation, Seoul, Korea) was used in this study. The chemical compositions of the experimental diets are shown in Table 1. The following enzyme additives were procured through Insect Biotech Co., Ltd. (Daejeon, Republic of Korea): arazyme, a 51.5 kDa exogenous protease (Bersanetti *et al*., 2005), and exogenous carbohydrolases, mannanase (ManK, 34.9 kDa) and xylanase (XynA, 19.9 kDa), from *Cellulosimicrobium* sp. HY-13, a symbiotic bacterium of *E. fetida* (Ham *et al*., 2011), and *Paenibacillus* sp. HY-8, a symbiotic bacterium of *M. diphysis* (Heo *et al*., 2006), respectively.

Experimental design and *in vitro* fermentation procedures

The procedure was performed in a completely randomized design, with a 1-L capped bottle as the experimental unit (Fig. 1). The control groups were not treated with enzymes, whereas the treatment groups were individually supplemented with the required units (g^{-1}) of enzyme diet concentration. Gastrointestinal contents were obtained from Apures Co., Ltd. (Pyeongtaek, Korea) and immediately placed into sterile tubes under anaerobic conditions (generated using the GasPak EZ anaerobic pouch system; Becton Dickinson, United States). The gastrointestinal contents were stored at -70 °C until use. Simulated gastric and intestinal media were prepared by the methods of previous study (Thévenot *et al*., 2013; Cordonnier *et al*., 2015) with minor modifications: simulated gastric media was composed of 10 mL of 10 mM HCl (pH 2.0), 0.5 mL of porcine serum, and 0.4 g gastric content; small intestine media was composed of 10 mL of phosphate buffered saline (PBS) (pH 7.4), 0.5 mL of porcine serum, and 0.4 g small intestinal content; and large intestine media was composed of 10 mL of PBS (pH 7.4), 0.5 mL of porcine serum, and 0.4 g small intestinal content. A total of 1.0 g of feed and different enzymes were sequentially digested in four phases as follows: gastric phase (addition of gastric medium and mixing for 2 h at 39 °C, under anaerobic conditions), small intestinal phase (addition of small intestinal medium and mixing for 4 h at

Table 1. Chemical composition of basal diet used in the *in vitro* pig intestine continuous fermentation model

Item	Composition
Ingredients	
Corn, %	70.25
Soybean meal, %	13.48
Wheat bran, %	5.65
Rapeseed meal, %	2.00
Molasses, %	4.00
Animal fat, %	1.68
Limestone, %	1.00
Tricalcium phosphate, %	0.68
L-Lysine-HCl (78%), %	0.39
DL-Methionine (98%), %	0.14
L-Threonine (98%), %	0.21
L-Tryptophan, %	0.11
Salt, %	0.21
Vitamin premix[1], %	0.10
Mineral premix[2], %	0.10
Chemical composition	
Moisture, %	12.66 ± 0.07
Ash, %	4.28 ± 0.08
Crude protein, %	13.00 ± 0.94
Crude fat, %	9.00 ± 0.97
Crude fiber, %	0.27 ± 0.13
Carbohydrate, %	61.06 ± 2.19
NFE, %	60.11 ± 1.67
Total nitrogen, %	1.83 ± 0.15
Lysine, mg/L	1735.69 ± 157.54
Methionine, mg/L	298.56 ± 39.59
Threonine, mg/L	672.33 ± 34.03
Tryptophan, mg/L	211.12 ± 2.96
Isoleucine, mg/L	111.59 ± 0.36
Valine, mg/L	192.43 ± 1.40
Myristic acid (C14:0), %	1.60 ± 0.04
Palmitic acid (C16:0), %	19.34 ± 0.16
Stearic acid (C18:0), %	7.12 ± 0.31
Arachidic acid (C20:0), %	0.16 ± 0.08

Table 1. Continued

Item	Composition
Lauric acid (C12:0), %	1.48 ± 0.22
Behenic acid (C22:0), %	0.13 ± 0.18
Lignoceric acid (C24:0), %	0.16 ± 0.22
Palmitoleic acid (C16:1n-7), %	1.30 ± 0.02
Oleic acid (C18:1n-9), %	32.52 ± 0.82
Linoleic acid (C18:2n-6), %	34.02 ± 0.01
α-linolenic acid (C18:3n-3), %	1.60 ± 0.08
Eicosenoic acid (C20:1n-9), %	0.26 ± 0.37
Eicosadienoic acid (C20:2), %	0.10 ± 0.13
Erucic acid (C22:1n-9), %	0.21 ± 0.30

[1]Vitamin A, 4,000 IU; vitamin D_3, 800 IU; vitamin E, 40 IU; vitamin B_3, 2 mg; vitamin B_{12}, 16 mg; vitamin K, 2 mg; thiamine, 8 mg; riboflavin, 2 mg; pantothenic acid, 11 mg; niacin, 20 mg; biotin, 0.02 mg.
[2]Supplied per kilogram of diet: Cu, 130 mg; Fe, 175 mg; Zn, 100 mg; Mn, 90 mg; I, 0.3 mg; Co, 0.5 mg; Se, 0.2 mg.
Data are presents as means ± SE (n=3)

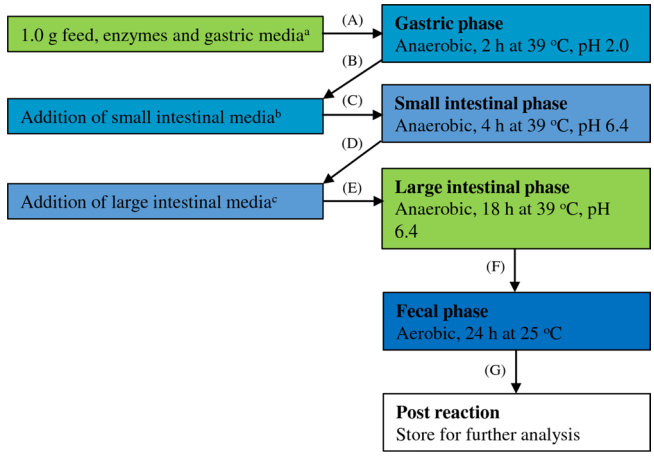

Fig. 1. Step-wise process of the *in vitro* pig intestine continuous fermentation model. (A) The stimulated gastric phase is formulated by mixing feed and enzymes into gastric media. (B) The reaction proceeds for 2 h. (C) The entirety of the contents from the simulated gastric phase (approximately 10 mL) is mixed with the small intestinal media to initialize the simulated small intestinal phase. (D) The simulated small intestinal phase progresses for 4 h. (E) The entirety of the contents from the simulated small intestinal phase (approximately 20 mL) is mixed with the large intestinal media to initialize the simulated large intestinal phase. (F) The reaction proceeds for 18 h. (G) The entirety of the contents from the large intestinal phase (approximately 30 mL) is allowed to proceed continuously into the simulated fecal phase for 24 h. Post reaction, the mixture is stored for further analysis.

39 °C, under anaerobic conditions), large intestinal phase (addition of large intestinal medium and mixing for 18 h at 39 °C, under anaerobic conditions), and fecal phase (addition and mixing of same contents as the large intestinal phase for 24 h at 25 °C, under aerobic conditions). The pH of the samples was determined using a pH meter (Thermo Fisher Scientific, Massachusetts, United States). The experiments were performed in triplicate under the same conditions.

Analysis of odorous gas emissions

For the analysis and quantification of odorous gas emissions, the gas samples from the 1-L capped bottles were collected during each fermentation phase by unsealing an adhesive plaster covering a small hole present in the center on one side of the bottle. The concentration of VOCs, within the range of 0 to 100 ppm was determined by a gas analyzer (Multi-RAE, RAE SYSTEMS, San Jose, CA, USA).

Analysis of microbial communities

To evaluate changes in the microbial population following fermentation, fecal samples were assessed for culturable microbes by the dilution plating technique. Each sample (1 mL) was homogenized, serially diluted to 10^{-8} in a sterile PBS solution and plated onto BL agar plates (MBcell, Seoul, Korea) which were then incubated for 48 h at 37 °C. Following the counting of bacterial colonies, the density of different microbial communities was estimated as colony-forming units (CFUs) per gram of sample. Isolates were identified by nucleotide sequence analysis of the 16S rRNA gene. The 16S rRNA sequences of closely related strains were retrieved from the National Center for Biotechnology Information (http://www.ncbi. nlm.nih.gov/GenBank/index.html) and aligned using CLUSTAL X.

Statistical analysis

Results are expressed as the mean ± standard deviation. One-way ANOVA of variance was performed using SPSS software (version 24, SPSS, Inc., Chicago, IL, USA). Multiple comparisons of the mean values were made by post hoc tests using Scheffé's method, and a p values <0.05 was considered significant.

Results and Discussion

Commercial as well as animal safety issues are limiting factors for conducting field research. Compared with *in vivo* models, *in vitro* models are less costly, prompt, less workforce, and free from the ethical restrictions (Lo *et al*., 2022). *In vitro* systems that simulate the gastrointestinal environment have been applicated to forecast the apparent ileal as well as total tract digestibility of nutrients in diverse feedstuff (Boisen and Ferna, 1995). Recently, a comprehensive understanding has been obtained by using *in vitro* systems to systematically assess the bioactivities of compounds, exogenous enzymes, and microorganisms. However, few studies have reported a correlation between exogenous enzymes and microbial or odor compound production.

Effect of exogenous enzyme on VOC emissions

The effects of exogenous enzyme treatment, under various conditions, on VOC emission during the *in vitro* fermentation procedures in the pig intestine continuous fermentation model are shown in Table 2. The results showed that all of the tested exogenous enzymes displayed a tendency to reduce VOC concentrations in proportion to increasing concentrations (in the fecal phase), demonstrating that the exogenous enzymes' positive correlation with the reduction of VOC emissions occurs in a concentration-dependent manner. Interestingly, arazyme, at a concentration of 2,000 units g^{-1}, exhibited the highest VOC reduction effect in all of the phases. To evaluate the VOCs reducing effects, each exogenous enzyme (arazyme, 2,000 units g^{-1}; XynA, 2,000 units g^{-1}; and ManK, 2,000 units g^{-1}, used individually), and the multi-enzymes (arazyme, 2,000 units g^{-1}; XynA, 200 units g^{-1}; ManK 200 units g^{-1}, used jointly) were supplemented in the *in vitro* fermentation procedures. The results showed a significant correlation between exogenous enzyme treatment and VOC emissions in the fecal phase (p < 0.05) (Fig. 2). Specifically, there was a significant decrease in the concentration of VOCs in the treatment group (by 3.75 and 2.75 ppm following arazyme and multi-enzyme supplementation, respectively) compared to the control group (7.83 ppm).

Insect gut-associated microbes are a relatively untapped source for biotechnological applications and are of increasing importance for industrial applications (Banerjee *et al*., 2021). Various hydrolytic enzymes have been suggested for practical use in industrial fields. To overcome nutritional limitations, insects have improved symbiotic interactions with various gut microbes that produce the vast of the metabolic digestive enzymes (Kannan *et al*., 2019). However, experimental evidence for exogenous enzymes, derived from insect gut-associated microbiota, to reduce VOC emissions in livestock, is limited.

Table 2. Effects of exogenous enzyme supplementation on volatile organic compound (VOC) emissions in the *in vitro* pig intestine continuous fermentation model

Treatments	Concentrations (Unit g^{-1})	Stomach	Small intestine	Large intestine	Feces
Control	-	4.33 ± 0.49^{b}	5.27 ± 1.73^{b}	7.83 ± 0.77^{b}	10.77 ± 0.22^{a}
Arazyme	200	5.12 ± 0.02^{a}	10.50 ± 0.62^{a}	9.39 ± 0.26^{a}	7.97 ± 0.52^{ef}
	500	2.96 ± 0.13^{d}	2.06 ± 0.14^{c}	7.12 ± 0.71^{b}	6.04 ± 0.88^{g}
	1,000	3.18 ± 0.13^{c}	1.46 ± 0.37^{c}	6.49 ± 0.72^{b}	3.50 ± 1.60^{h}
	2,000	2.01 ± 0.21^{d}	2.51 ± 0.61^{c}	4.22 ± 1.28^{c}	3.20 ± 0.33^{h}
XynA	200	4.10 ± 0.04^{b}	5.24 ± 0.04^{c}	7.46 ± 1.00^{b}	9.41 ± 0.26^{c}
	500	4.16 ± 0.06^{b}	4.25 ± 0.00^{c}	7.83 ± 0.50^{b}	8.43 ± 0.30^{e}
	1,000	4.59 ± 0.19^{b}	4.97 ± 0.10^{c}	7.96 ± 0.35^{b}	10.25 ± 0.17^{b}
	2,000	4.59 ± 0.19^{b}	6.43 ± 0.27^{c}	7.91 ± 0.85^{b}	7.57 ± 0.10^{f}
ManK	200	5.25 ± 0.34^{a}	4.62 ± 0.08^{c}	7.40 ± 0.11^{b}	9.80 ± 0.15^{c}
	500	4.34 ± 0.09^{b}	5.83 ± 0.42^{c}	7.42 ± 0.05^{b}	9.22 ± 0.31^{cd}
	1,000	4.53 ± 0.09^{b}	5.98 ± 0.46^{c}	7.78 ± 0.10^{b}	9.15 ± 0.00^{c}
	2,000	4.76 ± 0.10^{b}	5.34 ± 0.27^{c}	7.56 ± 0.11^{b}	8.45 ± 0.17^{e}

Within a row, values with different superscripts differ significantly at $p < 0.05$.
Data are presented as means ± SD (n=3).

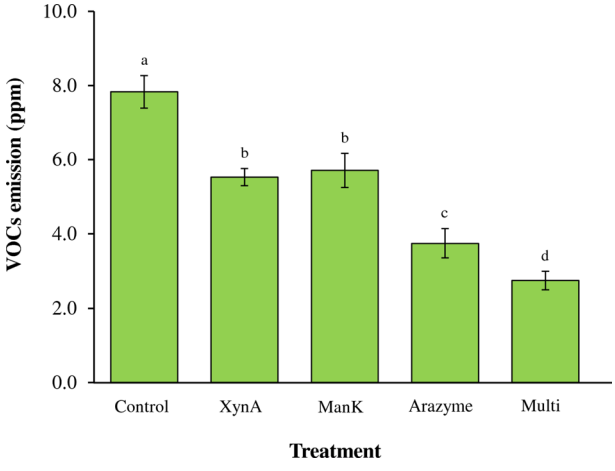

Fig. 2. Effects of exogenous enzyme supplementation on volatile organic compound (VOC) emission in the *in vitro* pig intestine continuous fermentation model. Data are presented as means ± SD (n = 3). Different letters above error bars indicate a significant difference by Scheffé's test ($p < 0.05$).

Exogenous enzymes such as proteases, carbohydrolases, and their enzyme mixtures have recently been used as feed additives in the livestock industry to enhance the efficiency of nutrient utilization as well as reduce noxious gas emissions. Many studies have demonstrated that the addition of exogenous enzymes effectively degrades indigestible components such as non-starch polysaccharides and proteins, thereby neutralizing anti-nutritional factors and improving nutrient utilization (Ojha *et al*., 2019). Recent studies have also shown that exogenous enzyme supplementation, which enhances nutrient digestibility, has a robust correlation with odor emissions. Lei *et al.* (2017) showed that pigs fed a protease (75,000 units g^{-1} Ronozyme® ProAct; DSM Nutritional Products, Ltd., Heerlen, The Netherlands)-supplemented diet had lower fecal ammonia emissions compared to those fed the control diet as well as finishing pigs ($p < 0.05$). However, no differences were observed in fecal ammonia, hydrogen sulfide, or total mercaptans when the diet of finishing pigs was supplemented with 624 units mg^{-1} protease derived from *Pseudoalteromonas arctica* (Liu *et al*., 2019). Jeon *et al.* (2019) reported that *β*-mannanase (CTCZYME®) supplementation reduced fecal ammonia in phase 2 of growing pigs ($p < 0.05$). Further, Lan *et al.* (2017) demonstrated that the consistency of fecal ammonia and hydrogen sulfide was decreased ($p < 0.05$) by supplementing the diet with 9,000 units g^{-1} of endo-1,4-*β*-xylanase (Nytrase Xyla, Nutrex Nv, Lille, Belgium). Similarly, Mc Alpine *et al.* (2012) indicated that ammonia concentrations were significantly decreased by multiple enzymes (75,000 units g^{-1} protease, Ronozyme® ProAct with 1,000 units g^{-1} fungal xylanase, Ronoxzyme® WX) supplementation, as compared to supplementation with protease in isolation which led to

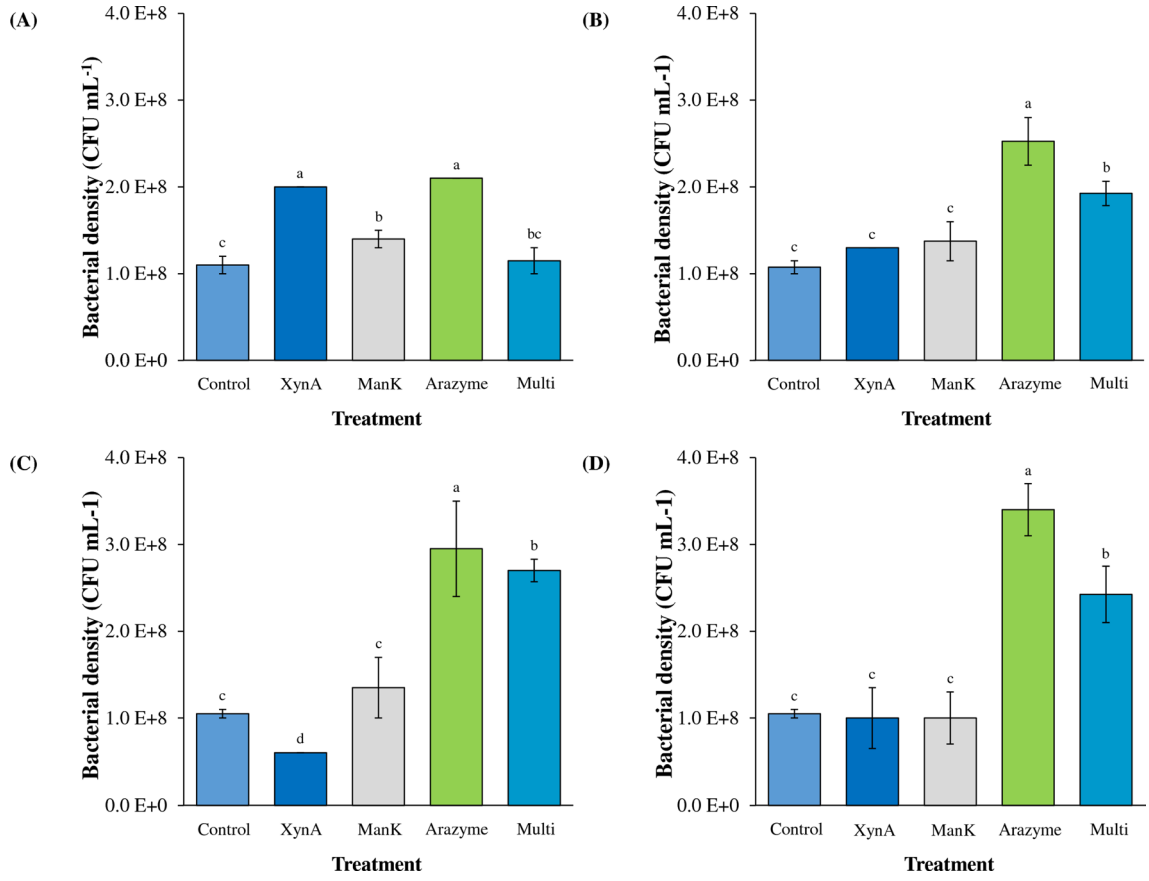

Fig. 3. Effects of exogenous enzymes on bacterial density following the fecal phase of the *in vitro* pig intestine continuous fermentation model. (A) gastric phase; (B) small intestinal phase; (C) large intestinal phase; (D) fecal phase. Data are presented as means ± SD (n = 3). Different letters above error bars indicate a significant difference by Scheffé's test ($p < 0.05$).

significantly higher ammonia emissions in finishing pigs ($p < 0.05$). However, previous studies have demonstrated that the type of feed as well as the animal can affect ammonia concentrations when using the identical enzyme supplement, causing different results (Garry *et al*., 2007). Here, supplementation with invertebrate gut-associated bacterium-derived enzymes, arazyme, XynA, and ManK, as well as their enzyme mixture, led to reduction in VOCs as well as changes in gut microbiota in pig *in vitro* fermentation systems. However, comparisons could not be made with other studies because the influence of exogenous enzymes (protease, xylanase, and mannanase) supplementation on VOC reduction has not been reported to date.

Amongst VOCs, organic nitrogenous compounds were the most abundant group. These are known to originate from undigested proteins and amines, which can be sources of various volatile nitrogenous compounds as well as cause microbial degradation (Sánchez-Monedero *et al*., 2019). Here, VOC emissions were significantly decreased by exogenous enzyme supplementation ($p < 0.05$). This may occur due to the enzyme's effect on nutrient digestibility and microbial fermentation, which contribute to several biological functions, consequently, result in the alteration of odor generation in the host (Ojha *et al*., 2019). It has been reported that alteration of gut microbial communities, caused by exogenous enzyme supplementation, affect manure odor emissions (Xu *et al*., 2021). Therefore, hydrolysis of undigested nutrients that leads to a balanced intestinal microbiota is a key element in odor formation control in pigs.

Effect of exogenous enzyme supplementation on microbial communities

The composition of bacterial communities of each fermentation phase in the pig intestine continuous fermentation model, was analyzed. The density of culturable bacteria (CFU mL^{-1}) of each phase is shown in Fig. 3. The results showed that the bacterial density differed markedly between the treated and control groups of each phase. In addition, the bacterial densities were also

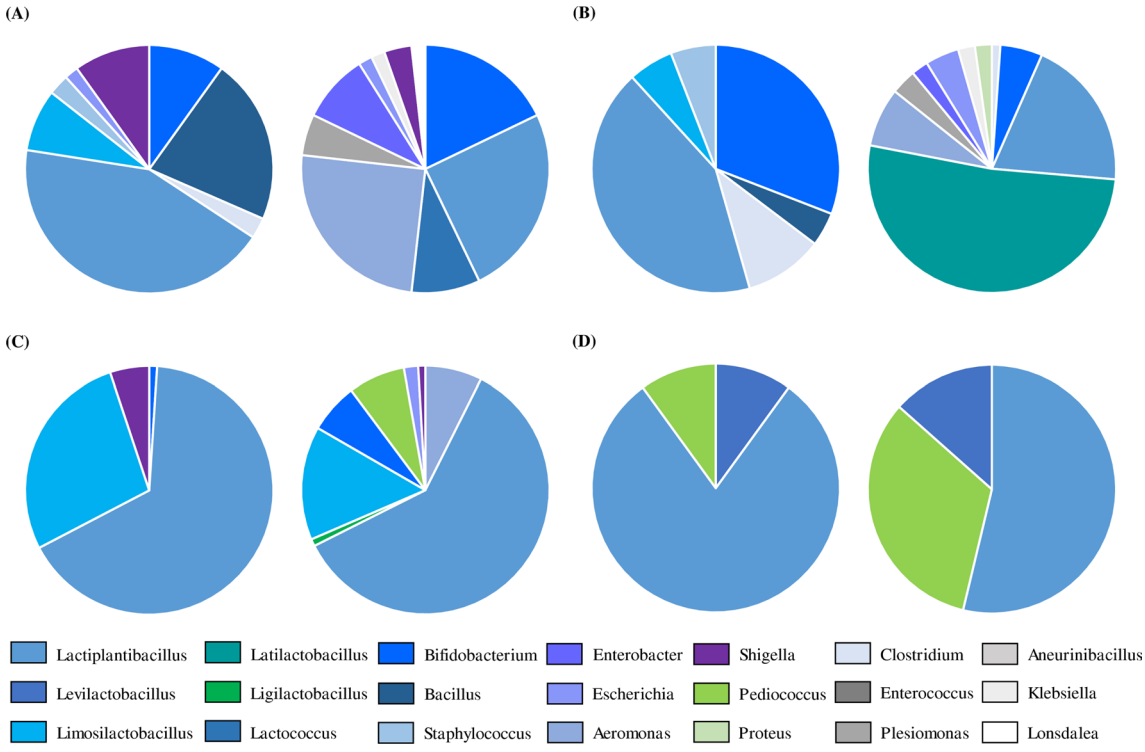

Fig. 4. Comparison of bacterial compositions of control and multi-enzyme supplementation groups at the genus level in each fermentation phase of the *in vitro* pig intestine continuous fermentation model. The pie chart represents the relative abundance as a percentage of the total isolated bacteria, based on the 16S rRNA sequences. The left and right pie chart refer to control and multi-enzyme supplementation groups, respectively. (A) gastric phase; (B) small intestinal phase; (C) large intestinal phase; (D) fecal phase.

dissimilar depending on the fermentation phase. These densities in the arazyme and multi-enzymatic treatment groups were considerably higher than those of the control group, except for the gastric phase, whereas the bacterial densities of the xylanase and mannanase treatment groups were similar to those of the control group. In the fecal phase, the densities of bacteria in the arazyme and multi-enzymatic treatment groups were 3.2- and 2.3-fold higher, respectively, than those in the control group ($p < 0.05$). The bacterial composition of the multi-enzyme treatment group, which showed the highest reduction in VOC emissions, was further analyzed. Although bacterial compositions were clearly different in each phase of multi-enzyme supplementation, the dominant phyla were Firmicutes, Actinobacteria, and Proteobacteria (Fig. 4). In the fecal phase, the dominant family of bacteria was Lactobacillaceae, and the isolated bacterium mainly belonged to four genera: *Lactiplantibacillus* (80.0% and 55.4%, in the control and treatment groups, respectively), *Pediococcus* (10.0% and 32.8%, in the control and treatment groups, respectively), *Bacillus* (4.0% and 6.2%, in the control and treatment groups, respectively), and *Levilactobacillus* (6.0% and 4.2%, in the control and treatment groups, respectively). Bacterial compositions were simpler in the fecal phase than in other phases; *Pediococcus* was notably increased in the treatment group in the fecal phase compared to the control group and other fermentation phases. To understand microbial communities and their correlation with VOC emissions, the cultured bacteria of the control and each treatment group were analyzed in the fecal phase at the species level (Fig. 5). The isolated bacteria belonged to five species of lactic acid bacteria (LAB): *Lactiplantibacillus pentosus*, *L. plantarum*, *Levilactobacillus brevis*, *Pediococcus pentosaceus*, and *P. acidilactici*, which is consistent with previous reports. It has been reported that the supplementation of a single enzyme or similar multi-enzyme could change the proportion of LAB and the gut microbiome in livestock. The application of food-grade acidic aspartic protease, derived from *Aspergillus niger*, resulted in an increase in the number of LAB (Der Bedrosian and kung, 2019). Other studies have shown that supplementation with various types of carbohydrolases increased the population of LAB in livestock (Nguyen *et al*., 2017; Lee *et al*., 2018).

LAB are delegate probiotics that have advantageous influence on the host, such as meliorative immunity and intestinal health,

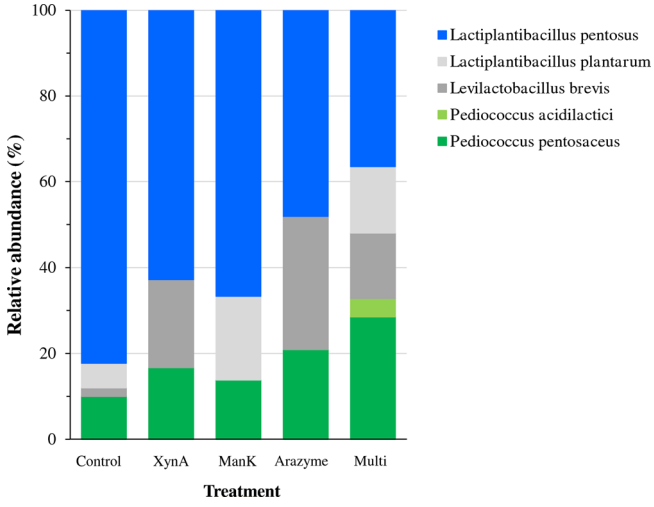

Fig. 5. Relative abundance of the isolated bacterial species in control and treatment groups following the fecal phase of the *in vitro* pig intestine continuous fermentation model.

inhibiting pathogenic microbes, and assimilating nutrients (Duarte and Kim, 2022). It has been reported that the presence of some LAB species is directly related to the occurrence or reduction of odors in livestock. *L. pentosus* has been reported to be responsible for the formation of odors caused by VOCs such as esters and phenols. O'Shea *et al.* (2012) demonstrated that diets supplemented with *L. plantarum* (0.4%) led to a significant decrease in the levels of ammonia, hydrogen sulfide, and volatile fatty acids ($p < 0.05$), whereas fermentation of rice bran as well as wheat bran with the *L. plantarum* 423 strain increased odor intensity, especially of sulfides and aromatics (Wang *et al*., 2020). *Pediococcus*, which increased in the fecal phase following supplementation of multi-enzymes in this study, has also been reported to be associated with odor reduction. Cell-free supernatants of *P. stilesii* SKD11 and *P. pentosaceus* SKD14 effectively reduced trimethylamine in spoiled fish samples by 60% and 59%, respectively (Park *et al*., 2020). *P. acidilactici* and *P. pentosaceus* have also been described as reducing odorous compounds, including trimethylamine nitrogen, total volatile basic nitrogen, peroxides, and free fatty acids (Sudalayandi, 2011). Here, the relative level of *L. pentosus* in each treatment group was significantly different from that in the control group ($p < 0.05$). Its levels in the arazyme and multi-enzymatic treatment groups were 1.7- and 2.3- fold lower, respectively, than that of the control group, which suggests that supplementation with

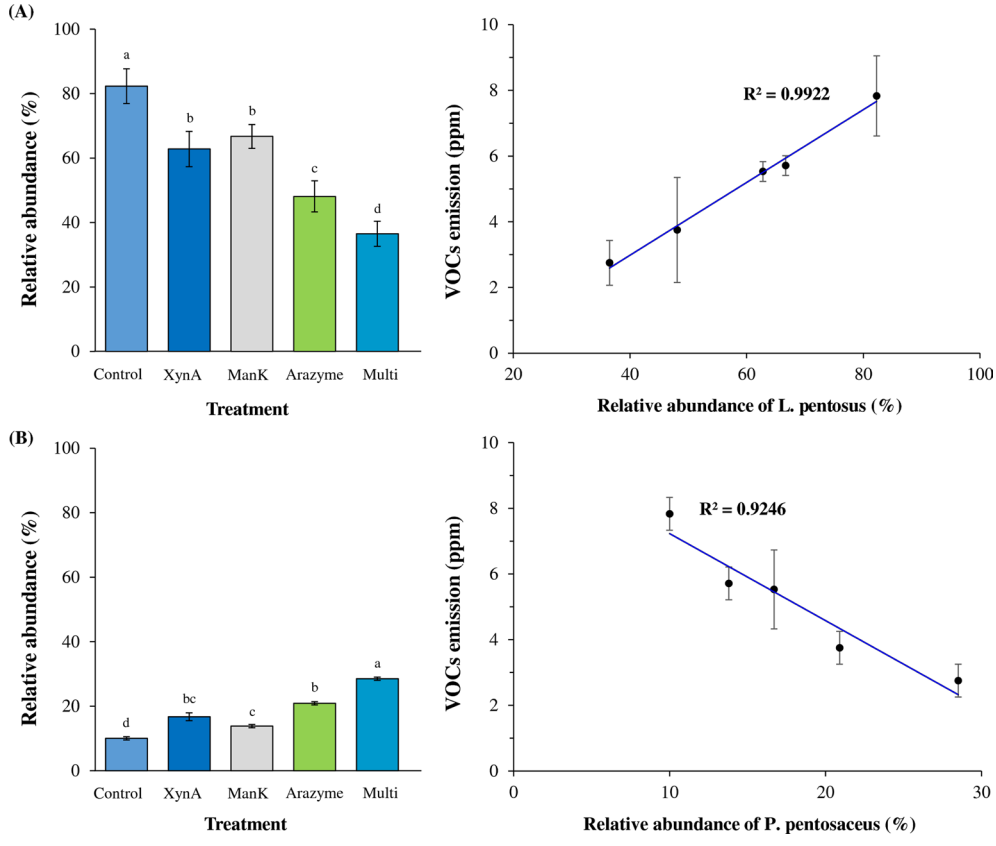

Fig. 6. Linear regression analysis (n = 3) of the relationship between volatile organic compound (VOC) emissions and the isolated bacterial species, (A) *Lactiplantibacillus pentosus* and (B) *Pediococcus pentosaceus*, in the *in vitro* pig intestine continuous fermentation model.

arazyme or multi-enzymes may affect the growth of *L. pentosus*. Interestingly, the best-fit curve following linear regression analysis showed that VOC emissions had a robust correlation with the relative abundance of *L. pentosus* (R^2 = 0.9922) (Figure 6A). The level of *P. pentosaceus* was also higher in each treatment group compared to that in the control group ($p < 0.05$) and showed a strong negative correlation with VOC emissions (R^2 = 0.9246). These results suggest that exogenous enzymes, especially arazyme, derived from invertebrate gut-associated microbes, enhanced the digestibility of nutrients and affected gut microbiota, which could contribute to VOC-reducing effects.

Conclusion

The supplementation of arazyme in isolation or in combination with xylanase and mannanase had beneficial effects on gut microbiota and led to a reduction of VOCs in an *in vitro* pig intestine continuous fermentation model. The results of this study suggest that the supplementation of exogenous enzymes derived from invertebrate gut-associated bacteria could concurrently improve nutrient digestibility, microbial composition, and reduce odor emission control in livestock industries. Further research would include field practice studies of exogenous enzymes from invertebrate gut-associated bacteria to reduce VOC emissions. The findings of this study provide a theoretical basis for such future studies.

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