Original Article

Declined *Lawsonia intracellularis* in feces by phytogenic feed additive supplementation in fattening pigs in different herds system

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Abstract

The objective of the present study was to determine the efficacy of phytogenic feed additives on the number of *L. intracellularis* in feces and incidence of diarrhea in fattening pigs in different herds system. The present study was carried out in two commercial swine farms located in the northern (Herd A) and western (Herd B) parts of Thailand. In total, 80 fattening pigs (12-week-olds) were randomly allocated into two groups, including the Control group (n=40) and the Treatment group (n=40). The pigs in each group were fed with a conventional diet (Control) and the same diet supplemented with 2 kg/ton of phytogenic feed additive for 14 days (Treatment). A total of 240 individual feces samples from 80 pigs. The feces samples were collected and were scored on days 0, 7, and 14 after supplementation and were determined by quantitative real-time PCR. No effect of phytogenic feed additive on the number of DNA was found in Herd A. In Herd B, the number of DNA in the Treatment group at day 14 (0.8 copies/µl) after supplementation in fattening pigs was lower than at day 0 (5.5 copies/µl; $p = 0.023$) and had a tendency lower than at day 7 (1.4 copies/µl; $p = 0.131$). In Herd A, the average pig fecal consistency in the Treatment group was significantly lower as compared to the Control group on day 7 (0.50 versus 1.20; $p < 0.001$) and was a tendency lower than the Control group on day 14 (0.40 versus 0.07; $p = 0.057$) after supplementation. The incidence of diarrhea in the Treatment group was lower than in the Control group on day 7 (45.0% Versus 90.0%; $p = 0.018$) and day 14 (40.0% versus 70.0%; $p = 0.057$) in Herd A. The pigs in the Treatment group at day 0 (100.0%) had a higher incidence of diarrhea than at day 7 (45.0%, $p < 0.001$) and 14 (40.0%, $p < 0.001$) after supplementation of phytogenic feed additive in Herd A and at day 7 (95.0%, $p = 0.007$) and 14 (80.0%, $p = 0.089$) after supplementation in Herd B. Therefore, phytogenic feed additives may be applied to control *L. intracellularis* in commercial swine farms.

Keywords: Feces, *Lawsonia intracellularis*, phytogenic feed additive, pig

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Introduction

Lawsonia intracellularis is a gram-negative bacterium that causes a common pathogenic bacterial infection in growing to fattening pigs worldwide. It is known as the porcine proliferative enteropathy (PPE). *L. intracellularis* is the most important intestinal disease in pigs. It first occurred in 1944 and was found worldwide in 1992 (Harris, 1992). The pigs show severe diarrhea, dehydration, anemia, low growth performance, and death within 2 days to 3 months after infection (McOrist, 2005). These bacteria resist gastric secretion and rapidly proliferate in crypt of the intestine (McOrist *et al.,* 1995), leading to malabsorption in the ileum and cecum. The infected *L. intracellularis* herd increases the cost by more than 1-5 USD/pig for treatment (McOrist *et al.,* 1997; Veenhuizen *et al.,* 2002). The high prevalence of *L. intracellularis* has been reported in many countries approximately 20-100% (Holyoake *et al.,* 1994; Stege *et al.,* 2000; Lee *et al.,* 2001; Jacobson *et al.,* 2005; Hands *et al.,* 2010; Nuntapaitoon *et al.,* 2021). Therefore, eradicating and controlling the *L. intracellularis* program should be a concern and a priority in swine farms/industries.

Natural growth promoters have been used as antimicrobial, antioxidant, and specific immunomodulatory effects in swine production. The effect of phytogenic feed additives on pig immunity and performance has been reported in previous studies (Delić *et al.,* 2018; Visscher *et al.,* 2018). Many plants are used in combination for enhancing antimicrobial action and gut health, such as *Thymus vulgaris*, *Origanum vulgare*, *Coriandrum sp.*, and extract of *Castanea sativa*. In line with this, natural growth promoters may reduce antimicrobial resistance in humans since declined antibiotic drugs in the pig industry. The herbal extract also provides specific immunomodulatory action, which reduces intestinal bacterial infection in swine (Delić *et al.,* 2018; Draskovic *et al.,* 2018; Bošnjak-Neumüller *et al.,* 2019). The phytogenic feed additive was provided to control diarrhea from bacteria (both *L. intracellularis* and *Brachyspira hyodysenteriae*) in fattening pigs (Delić *et al.,* 2018; Draskovic *et al.,* 2018; 2020; Bošnjak-Neumüller *et al.,* 2019). Previous studies illustrated that phytogenic feed additive supplementation in weaned piglets reduces the number of pathogenic bacteria and improves gut morphology (Draskovic *et al.,* 2018; 2020). However, factors influencing *L. intracellularis* infection were related to many factors, including the pig's age, herd size, management, and health status (Suh and Song; 2005; Bae *et al.,* 2013; Dors *et al.,* 2015). The objective of the present study was to determine the efficacy of phytogenic feed additives in reducing the number of *L. intracellularis* in feces and incidence of diarrhea in fattening pigs in different herds system.

Materials and Methods

Study design and animals: The experimental study was carried out using ethical principles and guidelines for the use of animals for scientific purposes published by the National Research Council of Thailand. All procedures were approved by the Chulalongkorn University Animal Care and Use Committee (animal use protocol number 2031010). The present study was approved by the Chulalongkorn University Biosafety Committee (institutional biosafety number 2031016).

The present study was carried out in two commercial swine farms located in the northern (Herd A) and western (Herd B) parts of Thailand between August and September 2020. The herds used in the present study were detected positive with *L. intracellularis* by multiplex PCR.

Housing and general management: The number of productive sows in Herd A and B was 1,000 and 2,000, respectively. All pigs were born from Landrace x Yorkshire F1 crossbred sows. Herd A pigs were kept in a conventional open-housing system equipped with fans to reduce the impact of high ambient temperature. Outdoor temperature and humidity data were obtained from an official meteorological station within 100 km of the herds. Daily 24-h average temperatures during this period were 29.8 °C. The average minimum-maximum daily temperatures were 26.7 to 34.0 °C. The 24-h average humidity was 75.8%. Herd B pigs were kept in an evaporative cooling-housing system. The automatic temperature and relative humidity regulation in the housing during the experimental period were set at 27.0 °C and 75.0%, respectively. The pigs in Herds A and B were fed a conventional fattening diet to meet or exceed the nutritional requirements (NRC, 2012). Feed was provided twice a day following a standardized feeding pattern. The animals received water ad libitum in a water pipe.

Experimental design: In total, 80 pigs at 12-week-old fattening pigs (40 pigs/herd) were randomly allocated into two groups, including a control group (n=40) and a treatment group (n=40). The fattening pigs in each group were fed a conventional diet (Control) and the same diet supplemented with 2 kg/ton of phytogenic feed additive for 14 days (Treatment). The phytogenic feed additive (the formulation is proprietary of PATENT CO. DOO, Mišicevo, Serbia) is composed of a mix of essential oils (dominantly *Thymus vulgaris*, *Origanum vulgare*, *Coriandrum sp.*), extract of *Castanea sativa*, lysozyme and nicotine amide. The composition included dry matter (min 85%) ashes (min 8.00%), organic matter (min 70.00 %), tannins (min 7%), thymol (min 0.25%), carvacrol (min 0.2%), niacinamide (min 4.5%).

Sample collection: A total of 240 individual feces samples from 80 pigs (40 pigs/herd) were collected alongside the fecal samples from the floor of each group and herd on days 0, 7, and 14 after supplementation. The samples were collected using latex sterile powder-free gloves and were individually identified with each pig. All samples were stored at -20°C until analysis. All samples were analyzed for the detection of *B. hyodysenteriae*, *L. intracellularis,* and *Salmonella* spp. in feces by multiplex PCR and fecal parasite by fecal flotation. Pigs testing positive for *B. hyodysenteriae*, *Salmonella* spp., and parasitic infections in their feces were excluded from the study.

All fecal samples from 4 pigs were pooled in sterile plastic containers based on group, herd, and day of the experiment. The 2 ml pooled feces sample (500 g/pigs) and 8 ml phosphate buffered saline were added to the sterile plastic containers, and the mixture was centrifuged for 10 min at 3,500 rpm. The supernatants were stored at -20 °C until analysis. In total, 60 pooled samples were used for DNA extraction.

Determined the number of DNA copies of L. intracellularis by quantitative real-time PCR: The DNA was extracted using a commercial kit (QIAamp® Fast DNA Stool Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, 1 g of pooled feces samples were melted by 10 ml of InhibitEx buffer. A 1.2 ml of the sample was incubated at 70 °C for 5 min. After centrifugation for 1 min at 3,500 rpm, the 200 µl supernatant was added to 200 µl lysis buffer and 15 µl proteinase K. After that, the samples were incubated at 70 °C for 10 mins. The 200 µl 96% ethanol was put in the samples, transferred to the QIAamp spin column, and centrifuged for 1 min. Further, 500 µl DNA Pre-wash buffer was added; the mixture was centrifuged for 1 min, and 500 µl DNA wash buffer was added; the mixture was centrifuged for 3 min. Finally, 200 µl of the DNA elution buffer was added and incubated for 1 min; the mixture was centrifuged and stored at -20 °C until analysis. The concentration of extracted DNA was performed by NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, US). Determine the purity of DNA by comparing the absorbance ratio at OD260/OD280 in the range of 1.8 and calculate the DNA concentration using the absorbance value at OD260 by machine.

The primer and probes were designed specifically for the 16s ribosomal RNA of the *L. intracellularis* gene (GenBank, accession no.L15739). The forward primer 5'-GCGCGCGTA GGTGGTTATAT-3', reverse primer 5'-GCCACCCTCTCCGATACTCA-3', and TagMan probe 5'-FAM-CACCGCTTAACGGTGGA ACAGCC TT-TAMRA-3' were used according to the previous study (Lindecrona *et al.*, 2002). The total of 25 µl real-time PCR amplification included 10 µl PrecisionFast qPCR Master Mix (Primerdesign, UK) and 5 µl template DNA of *L. intracellularis*. The concentration of primer and probe was 0.9 and 0.2 µM, respectively. The real-time PCR amplification was performed by QuantStudio™ 5 real-time PCR Instrument (Life Technologies, USA). The thermal protocol consisted of 2 steps, including the initial step: Enzyme activation starts at 95 °C for 2 min for 1 cycle; the second step: Denaturation at 95 °C for 10 s and data collection at 60 °C for 60 s, followed by 40 cycles. The number of DNA copies of *L. intracellularis* was determined by Applied Biosystems™ QuantStudio™ Design and Analysis Software. The cycle thresholds (ΔCt) were used to analyze a number of DNA copies of *L. intracellularis*.

Fecal consistency: The feces of pigs were individually scored at days 0, 7, and 14 after supplementation. The fecal consistency was classified into 3 scores, including normal feces (score=0), soft (score=1), and runny and/or watery feces (score=2) (Figure 1). The incidences of diarrhea were classified from the Fecal consistency into two groups (i.e., not found; normal and found; creamy and watery).

Statistical analysis: The all-statistical analyses were performed using SAS 9.0 (SAS, 2002). The number of DNA copies of *L. intracellularis* was tested with the assumption of normal distribution using skewness, kurtosis, and Shapiro–Wilk normality test, and they were logged and transformed. The effect of the phytogenic feed additive on the herd and on the number of DNA copies of *L. intracellularis* were analyzed by using a general linear model (GLM). The following model was applied to analyze data:

$$Yijk = m+Gi+Tj+Hk+PTijk+Oijk$$

Where Yijk is the response variable, m is the overall mean, Gi is the fixed effect of phytogenic feed additive (i.e., control and treatment groups), Tj is the fixed effect of time (i.e., 0, 7 and 14), Hk is the fixed effect of herd (i.e., A and B), GTHijk is the interaction between phytogenic feed additive, herd, and time, and Oijk is the residual error component. The effect of the phytogenic feed additive on the incidence of diarrhea and fecal consistency in fattening pig feces on each day of supplementation in each herd was analyzed by the X2 test and Wilcoxon Rank Sum test, respectively. The data are presented as Least–squared means. Values with $p < 0.05$ were regarded as statistically significant.

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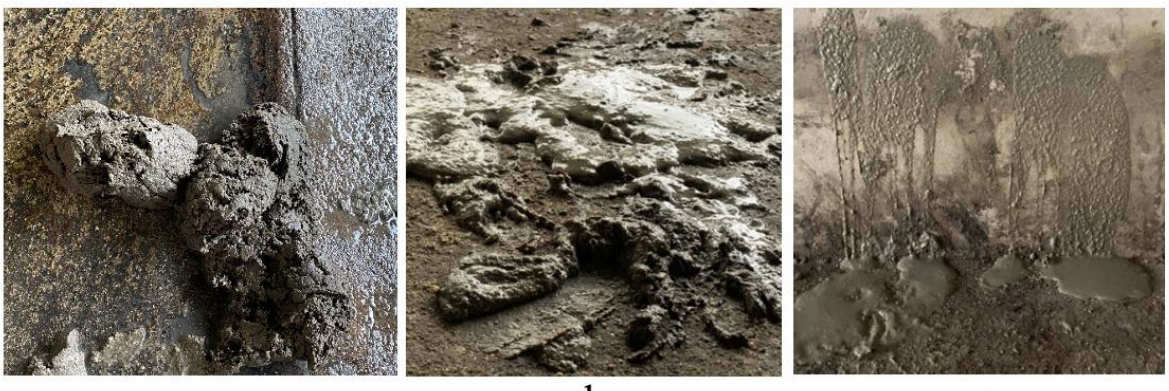

Figure 1 The fecal consistency was classified into 3 scores, including a.) normal feces (score=0), b.) soft (score=1), and c.) runny and/or watery feces (score=2).

Results

The number of DNA of L. intracellularis: The number of DNA copies of *L. intracellularis* is presented in Table 1. In Herd B, the number of DNA copies of *L. intracellularis* in the Treatment group at day 14 (0.8 copies/µL) after supplementation of PFA in fattening pigs was lower than at day 0 (5.5 copies/µL; $p = 0.023$), and it was lower than at day 7 (1.4 copies/µL; $p = 0.131$). No effect of phytogenic feed additive was found in Herd A. In the control group, the number of DNA of *L. intracellularis* did not differ significantly throughout the study in all herds.

Diarrhea: The effect of the phytogenic feed additive on the fecal consistency in fattening pig feces on each day of supplementation in each herd is presented in Figures 2 and 3. In Herd A, the average pig fecal consistency in the treatment group was significantly lower as compared to the control group on day 7 (0.50 versus 1.20; $p < 0.001$) and was a tendency lower than the control group on day 14 (0.40 versus 0.07; $p = 0.057$) after supplementation (Figure 3). The effect of the phytogenic feed additive on the incidence of diarrhea in fattening pig feces on each day of supplementation in each herd is presented in Table 2, respectively. Table 2 demonstrated that the incidence of diarrhea in the treatment group was lower than in the control group at day 7 (45.0% versus 90.0%; $p = 0.018$) and 14 (40.0% versus 70.0%; $p = 0.057$) in Herd A. The pigs in the treatment group at day 0 (100.0%) have a higher incidence of diarrhea than at day 7 (45.0%, $p < 0.001$) and 14 (40.0%, $p < 0.001$) after supplementation in Herd A and at day 7 (95.0%, $p = 0.007$) and 14 (80.0%, $p = 0.089$) after supplementation in Herd B.

Table 1 DNA copy number of *Lawsonia intracelluralis* in a pooled fattening pig feces in the Control (n=40) and Treatment groups (n=40) in each day of supplementation in each herd.

Parameters	Group	Day of Supplementation		
		0	7	14
Herd A	Control	2.0 ± 2.2	3.7 ± 1.7	2.9 ± 2.2
	Treatment	4.2 ± 1.9	0.8 ± 2.2	1.0 ± 2.7
Herd B	Control	2.6 ± 1.9	5.0 ± 2.2	2.3 ± 1.9
	Treatment	5.5 ± 1.7[a]	1.4 ± 2.7[b]	0.8 ± 1.9[b]

[a, b] Different superscript letters within the group indicate significant differences $P < 0.05$.

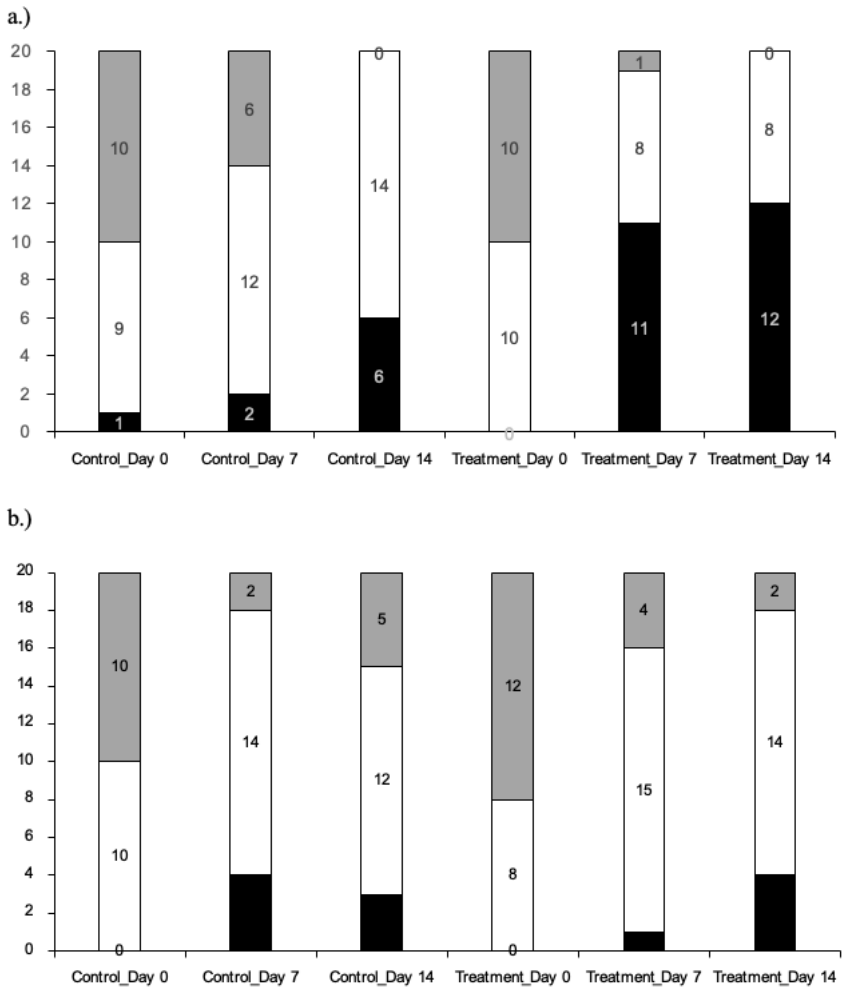

Figure 2 The fecal consistency (normal feces; score=0 (Black), soft; score=1 (White) and runny and/or watery feces; score=2 (Gray)) in control (n=40) and treatment (n=40) groups from 2 farms in Thailand a.) Herd A and b.) Herd B.

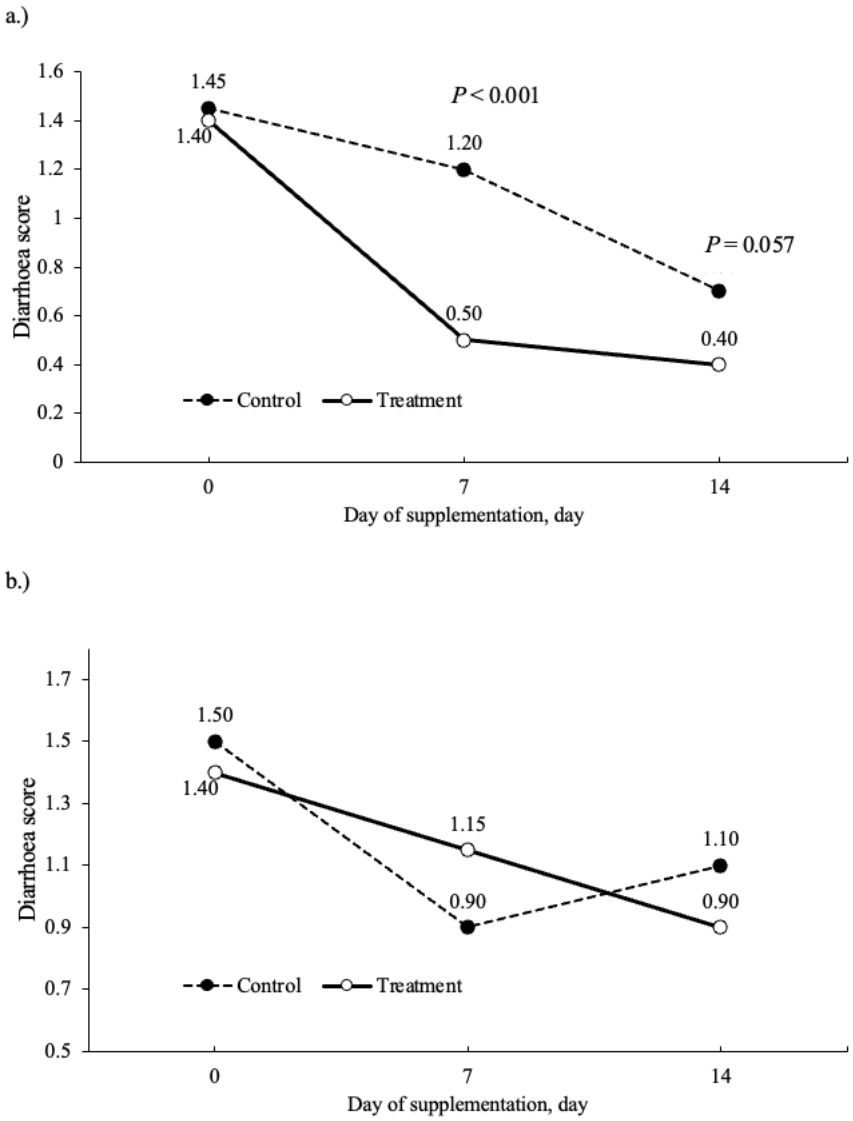

Figure 3 The average fecal consistency throughout the preweaning period in control (n=40) and treatment (n=40) groups from 2 farms in Thailand a.) Herd A and b.) Herd B.

Table 2 Percentage of the incidence of diarrhea (n) (including fecal consistency score 1 and 2) in fattening pig feces in the control (n=40) and treatment groups (n=40) on each day of supplementation in each herd.

Parameters	Group	Day of Supplementation		
		0	7	14
Herd A	Control	95.0% (19)^{Aa}	90.0% (18)^{Aa}	70.0% (14)^{Ab}
	Treatment	100.0% (20)^{Aa}	45.0% (9)^{Bb}	40.0% (8)^{Ab}
Herd B	Control	100.0% (20)^{a}	80.0% (16)^{b}	85.0% (17)^{ab}
	Treatment	100.0% (20) ^{a}	95.0% (19)^{a}	80.0% (16)^{b}

$^{a, b}$ Different superscript letters within the group indicate significant differences $p < 0.05$.

$^{A, B}$ Different superscript letters within period indicate significant differences $p < 0.05$.

Discussion

This is the first report on the efficacy of phytogenic feed supplementation in a short-duration trial on *L. intracellularis* prevention and control under field conditions. The prevalence of porcine proliferative enteropathy was high in a swine commercial herd in Thailand. From our knowledge, 33-85% of farms in Thailand have shown the presence of *L. intracellularis* by multiplex PCR (Raphanaphraiwan *et al.,* 2009; Nuntapaitoon *et al.,* 2021), which is in accordance with previous studies reported in many countries such as Canada, Brazil, South Korea, Taiwan, and European countries. The prevalence of *L. intracellularis* was reported during 1990-2015 about 20-100% (Wilson *et al.,* 1990; Stege *et al.,* 2000; Lee *et al.,* 2001; Merialdi *et al.,* 2003; Jacobson *et al.,* 2005; Suh and Song, 2005; Cizek *et al.,* 2006; Biksi *et al.,* 2007; Reiner *et al.,* 2011; Hands *et al.,* 2010; Dors *et al.,* 2015). However, *L. intracellularis* occurs routinely on farms, so techniques for reducing *L. intracellularis* should be considered.

The effect of the phytogenic feed additive on the number of DNA of L. intracellularis: The number of DNA of *L. intracellularis* represents the shedding chances of *L. intracellularis* within the population. The

decline in the number of DNA copies of *L. intracellularis* in supplemented pigs (12-week-old pigs) on day 14 was found in the present study. Similarly, Draskovic *et al.* (2018) reported that phytogenic feed additive supplemented in 7-week-old pigs over 28 days reduces the *L. intracellularis* excretion in feces. Moreover, phytogenic feed supplementation for 35 days in fattening pigs decreased fecal *L. intracellularis* DNA copies (Bošnjak-Neumüller *et al.*, 2019). Interestingly, the amount of DNA of *L. intracellularis* increased at the end of the study, indicating the spread of this bacteria in the control group. The present study was performed on 12-week-old pigs because the prevalence of *L. intracellularis* was observed in a previous study from 20 herds in Thailand (Nuntapaitoon *et al.*, 2021). We found that the 12-week-old pigs had the highest *L. intracellularis* infection, in accordance with the previous study in Poland (Dors *et al.*, 2015). Differentiation between age and duration of supplementation should be considered for the best prevention program and cost-effective performance. From our results, the use of the phytogenic feed additive fed for 14 days prevented and controlled the porcine proliferative enteropathy in fattening pigs.

The effect of the phytogenic feed additive on the fecal consistency: Herb combination was used to enhance livestock animal production. The *Thymus vulgaris*, *Origanum vulgare*, *Coriandrum sp.*, and extract of *Castanea sativa* were used in the present study. The all-active ingredients of phytogenic feed additives improve digestion and animal immunity. Previous studies found that the phytogenic feed enhances feed palatability, enzyme production, and digestibility (Patel and Srinivasan, 2004; Windisch *et al.*, 2008; Amad *et al.*, 2011). Moreover, herbs also are antioxidant, antimicrobial, and anti-inflammatory in action (Burt, 2004; Jugl-Chizzola *et al.*, 2005; Mueller *et al.*, 2012; Kara *et al.*, 2015). The supplemented pigs showed normal fecal consistency and low incidence of diarrhea in the present study, and these results are in accordance with previous studies (Papatsiros *et al.*, 2009; Draskovic *et al.*, 2018; 2020). The phytogenic feed additive supplementation in weaned pigs improved gut health, which increased crypt depth and the villus-height-to-crypt-depth ratio and reduced fecal expression of *L. intracellularis* in the pig's ilium (Draskovic *et al.*, 2018; 2020). Unfortunately, the present study has no growth performance data. The efficacy of the phytogenic feed on growth performance has been demonstrated in many studies (Frankič *et al.*, 2009; Kroismayr *et al.*, 2008; Delić *et al.*, 2018; Draskovic *et al.*, 2018) because health, digestibility, and absorption in gut's pigs was improved. Therefore, the efficacy of the phytogenic feed may have improved growth and production performance in pigs.

The incidence of diarrhea in Herd A rapidly decreased throughout the experimental period. On the other hand, Herd B showed a gradual decrease in diarrhea. However, the number of DNA copies of *L. intracellularis* in all herds dramatically decreased in the first 7 days after supplementation of PFA. In general, diarrhea in fattening pigs occurs from bacterial and viral infections, internal parasites, or all combinations (Thomson and Friendship, 2019). In line with this, herb supplementation has high efficacy on bacterial infections, including both *L. intracellularis* and *B. hyodysenteriae* (Delić *et al.*, 2018; Draskovic *et al.*, 2018; 2020; Bošnjak-Neumüller *et al.*, 2019). The high incidence of diarrhea in Herd B may have occurred from other infections, coinfection, recurred infection, and general management. Pigs in Herds B were reared in an evaporative cooling system. Moreover, pigs in both herds in the present study were reared within other fattening pigs. Therefore, the efficacy of the phytogenic feed additive should be performed in the whole area to prevent transmission from pen to pen, especially in an evaporative cooling system.

In conclusion, the phytogenic feed additive reduced the number of *L. intracellularis* in fattening pigs in both an evaporative cooling system and an open-housing system. Therefore, phytogenic feed additives may be applied to control *L. intracellularis* in commercial swine farms when the usage of antibiotics is limited.

Conflicts of interest: This work was supported by the Amcovet. Co., Ltd. and Patent Co. DOO and the co-authors (Suphadtra Therarachatamongkoland, Jasna Bosnjak-Neumüller and Marko Vasiljevic) are employees of Amcovet. Co., Ltd. and Patent Co. DOO., respectively. These authors from the company did not influence the study design and results.

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