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The effect of a phytogenic-based feed additive on concurrent *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* infections in pigs

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Abstract

This study investigated the efficacy of a commercial phytogenic-based premixed feed additive (PFA) in treating combined *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* infections in finishing pigs, with tiamulin/lincomycin treatment as the control. Pigs aged 20 weeks were allocated to PFA treatment (11 pens, 45 pigs per pen) and control (7 pens, 43 pigs per pen) groups for a seven-week experimental period. Floor faecal samples and rectal swabs were collected weekly, and the percentage of pigs per pen with diarrhoea was recorded weekly. The bacterial contents of the samples were determined using real-time polymerase chain reaction, and at the end of the experiment, histological changes in ileal samples were examined. There was an intermittent decrease in *L. intracellularis* in the control group (from 4.85 to 0.82 DNA $\log_{10}$ copies/µl) and a continuous reduction in *L. intracellularis* in the PFA group (from 5.69 to 0.64 DNA $\log_{10}$ copies/µl) over a six-week period. *B. hyodysenteriae* was not detected in rectal swabs from the control group at week six, and an intermittent decrease in *B. hyodysenteriae,* from 3.04 to 0.26 DNA $\log_{10}$ copies/µl, was observed in the PFA group. Bacterial DNA in the floor faecal samples declined during the seven-week experimental period, as found for the rectal swabs. There were no cases of diarrhoea from week two onwards in the control group and week three onwards in the PFA group. The results of this study indicate that a PFA rich in essential oils has a therapeutic effect comparable to that of tiamulin/lincomycin in pigs with proliferative enteropathy and swine dysentery.

Keywords: finishing pigs, phytogenic-based premixed feed additive, proliferative enteropathy, real-time polymerase chain reaction, swine dysentery, tiamulin/lincomycin

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Introduction

The occurrence of enteric diseases during the growing and finishing stages results in significant economic losses in the pig industry worldwide (Dors *et al.*, 2015). Of the various intestinal pathogens, two of the most important are *Lawsonia intracellularis* and *Brachyspira hyodysenteriae*. These bacteria primarily affect finisher pigs, gilts, and boars, with the obligate intracellular bacterium *L. intracellularis* causing proliferative enteropathy (PE) (McOrist & Gebhart, 2006), and the gram-negative anaerobic spirochete *B. hyodysenteriae* causing swine dysentery (SD) (Hampson *et al*., 2006). These pathogens, either individually or in combination, result in the development of clinical symptoms such as anorexia, diarrhoea, and growth retardation, leading to decreased welfare and increased production costs. The importance of monitoring and reducing the prevalence of *L. intracellularis* and *B. hyodysenteriae* is thus clear (Stege *et al*., 2001). Significant efforts have therefore been made over the last two decades to understand and identify the transmission routes of *L. intracellularis* and *B. hyodysenteriae* in commercial and feral pig herds worldwide (Phillips *et al.*, 2009; Arnold *et al.*, 2019; Dors *et al.*, 2019; Neirynck *et al.*, 2020; Carranza *et al.*, 2021).

Co-infection with multiple agents is commonly observed in pig herds with enteric health problems (Komine *et al*., 2016; Dors *et al*., 2019; Nuntapaitoon *et al*., 2021). Dors *et al*. (2019) reported the simultaneous presence of *Brachyspira pilosicoli* and *B. hyodysenteriae*, as well as *B. pilosicoli* and *L. intracellularis*, in the faeces of pigs older than seven weeks. Specifically, it was found that *L. intracellularis* infection, which causes primary lesions in the ileum (with potential spread to the colon and jejunum), increases the likelihood of infection with other enteric pathogens (Komine *et al*., 2016). Despite studies detecting mixed infections of *L. intracellularis* and *B. hyodysenteriae* in pigs, and an increasing trend in the use of routine diagnostics tests (Stege *et al*., 2001; Suh & Song, 2005; Phillips *et al*., 2009; Reiner *et al*., 2011), the conditions and pathogenesis of this co-infection remain complex and poorly understood (Daniel *et al*., 2023). In addition to de Groot *et al*. (2022) investigating the pathohistological lesions induced by combined *L. intracellularis* and *B. hyodysenteriae* infections in an *ex vivo* swine colon model, Daniel *et al*. (2023) found a synergistic effect of a mixed infection with these bacteria on clinical symptoms, macroscopic and microscopic lesions, and the faecal microbiome profile in experimentally infected piglets.

The identification of various risk factors has led to the development of different strategies in the management of *L. intracellularis* and *B. hyodysenteriae* infections, as follows: (i) improving building construction and internal biosecurity measures (Jacobson *et al.*, 2010); (ii) developing methods for the quick and easy detection of these bacteria, individually (Nathues *et al.*, 2009) or simultaneously (Nathues *et al.*, 2007); (iii) modifications of the diet in terms of form, ingredient composition, and the supplementation of feed additives (Whitney *et al.*, 2006; Mølbak *et al.*, 2008); and (iv) the use of vaccines and antibiotics (Card *et al.*, 2018). Today, commercially available live attenuated and inactivated bacterin-based *L. intracellularis* vaccines are used in the prophylaxis of PE. While they effectively reduce *L. intracellularis* lesions and shedding, shortcomings in the application and immune response quality persist, often necessitating additional interventions for PE control (Karuppannan & Opriessnig, 2018).

Unlike for *L. intracellularis*, developing a vaccine for controlling SD has encountered several limiting factors (Álvarez-Ordóñez *et al*., 2013a). Therefore, antimicrobial use and biosecurity improvement are still considered primary methods in treatment, control, and eradication programmes for *Brachyspira* spp. infections in pig herds (Massacci *et al*., 2018). The most widely used drugs in practice, which have proven good clinical effects and relatively short withdrawal periods, are the pleuromutilins (tiamulin and valnemulin), tylosin, and lincomycin (Mirajkar *et al.*, 2016). However, the reliance on continuous treatment with antibiotics over extended periods in the past two decades has led to the emergence of multidrug-resistant *Brachyspira* spp. isolates with reduced susceptibility to pleuromutilins, particularly tiamulin, in various countries (Karlsson *et al.*, 2003; Hidalgo *et al.*, 2011; Šperling *et al.*, 2011; Pringle *et al.*, 2012; Joerling *et al.*, 2018). In contrast, because of the difficulty in isolating and establishing *L. intracellularis* in cell culture, there is limited data on its antimicrobial susceptibility (Yeh *et al*., 2011; Luo *et al*., 2020). However, a study by Wattanaphansak *et al*. (2019) reported the reduced susceptibility of *L. intracellularis* strains to lincomycin, gentamicin, trimethoprim, colistin, and bacitracin *in vitro*.

The injudicious use of antimicrobials in swine production has raised concerns for animal and human health in the context of antimicrobial resistance (Lekagul *et al*., 2019). The latest data, reported for 81 countries, is concerning, as it shows that the use of antibiotics in animals increased by 2% globally between 2019 and 2021, following several consecutive years of significant decrease (OIE, 2024).

Therefore, the development of non-antimicrobial alternatives to control *L. intracellularis* and *B. hyodysenteriae* infections has become a practical requirement (Karuppannan & Opriessnig, 2018; Meneguzzi, 2020).

Recent reports have documented that phytogenic-based premixed feed additives (PFA), particularly ones containing essential oils, can play a significant role in reducing or replacing the use of antibiotics (Stevanović *et al*., 2018). In cases of infections caused by *L. intracellularis* or *B. hyodysenteriae*, the effectiveness of dietary supplements like prebiotics, probiotics, and organic acids has been confirmed (Hansen *et al*., 2011; Meneguzzi, 2020; de Groot *et al*., 2022; Xu *et al*., 2023). In addition, supplementation with natural ingredients that possess direct antimicrobial properties has shown promising results (Karuppannan & Opriessnig, 2018). In an *in vitro* study, de Groot *et al*. (2022) demonstrated that a commercial phytogenic product containing a blend of thymol and carvacrol effectively prevented lesions caused by *L. intracellularis* or *B. hyodysenteriae*. The beneficial effects of extracts of *Origanum vulgare* and *Allium sativum* in controlling PE in weaned piglets have also been observed *in vivo* (Papatsiros *et al*., 2009). Furthermore, a few specific field studies have shown that supplementation with the PFA used in this study can lead to a reduced intestinal load, the alleviation of clinical symptoms, and improved performance in herds naturally exposed to either *L. intracellularis* (Drašković *et al*., 2018; Katedangsakulwut *et al*., 2021; Nuntapaitoon *et al*., 2023) or *B. hyodysenteriae* (Delić *et al*., 2018) infection.

To the authors' knowledge, no literature is available on the impact of PFAs on combined *L. intracellularis* and *B. hyodysenteriae* infections under field conditions. Given the rising trend in the detection of this bacterial co-infection in pig herds, along with insufficient data on the susceptibility of *L. intracellularis* and *B. hyodysenteriae* to natural compounds with antimicrobial effects, and the lack of clinical trials to verify the efficacy of phytogenic compounds under practical conditions, the objective of this study was to assess the impact of a PFA in pigs naturally infected with *L. intracellularis* and *B. hyodysenteriae*, and to compare its effectiveness with a control group receiving tiamulin/lincomycin treatment.

Material and methods

A commercially available PFA (the recipe of which is the proprietary information of PATENT CO. DOO, Mišićevo, Serbia) was used in this study. This PFA primarily consists of an essential oil blend (mostly *Thymus vulgaris*, *O. vulgare*, and *Coriandrum* sp.), a *Castanea sativa* extract, and clinoptilolite. The PFA was added to the feed at a dose of 2 kg/t of feed.

The study was conducted on a commercial wean-to-finish pig farm in Tainan City, Taiwan, with a total capacity of 8000 pigs. The farm had a previous record of PE and SD outbreaks, confirmed by polymerase chain reaction (PCR) assay. The experiment was conducted in accordance with local legislation and the Animal Care and Use Committee of the University of Chiayi, Taiwan. The pigs were slaughtered in a slaughterhouse following standard industrial techniques.

The pigs at the farm were divided into two experimental groups: the treatment group received the PFA-supplemented feed (2 kg/t feed) for seven weeks, and the control group was treated with 110 ppm tiamulin in their feed for the first four weeks, followed by lincomycin treatment for a further two weeks. The treatment group consisted of 495 twenty-week-old pigs (11 pens, with 45 pigs per pen), and the control group consisted of 301 twenty-week-old pigs (7 pens, with 43 pigs per pen). The pigs were housed in pens with concrete floors and had free access to feed and water. Both groups of pigs were kept under the same housing conditions throughout the experiment. The presence of *L. intracellularis* and *B. hyodysenteriae* in faecal samples was determined by PCR assay before the animals were introduced into the study.

Floor faecal samples (two samples per pen, 22 samples from the treatment group and 14 samples from the control group) were collected from two randomly selected 25 × 25 cm^2 areas in each pen using sterile gauze rinsed with phosphate-buffered saline, for the evaluation of levels of faecal shedding. Rectal swabs (10 samples from the treatment group and 12 samples from the control group) were collected from randomly selected and marked pigs with observed clinical signs of diarrhoea using sterile cotton swabs. Both floor faecal samples and rectal swabs were collected weekly, on days 0, 7, 14, 21, 28, 35, 42, and 49, while the pigs were 20 to 27 weeks of age. After the first week of the trial,

one pig from the treatment group was excluded because of sudden death. A total of 36 floor faecal samples and 21 rectal swabs per week were thus collected.

The numbers of *L. intracellularis* and *B. hyodysenteriae* bacteria in floor faecal samples and on rectal swabs were determined using real-time PCR. Total DNA was extracted using a commercial kit (DNeasy® PowerSoil® Pro Kit, Qiagen, Hilden, Germany) according to the manufacturers' protocol, and extracted DNA samples were stored at −20 °C until examination. Amplifications were performed using the StepOne™ real-time PCR system (Applied Biosystems®, Foster, USA), and the primers used for the amplification of *B. hyodysenteriae* DNA were, as described by Akase *et al*. (2009):

Forward primer: 5'-TATGAAGAAGGCAGCAGACGTTTAT-3'

Reverse primer: 5'-GTAGGAAGAAGAAATCTGACAATGCA-3'

TaqMan probe: 5'-FAM-ACACAATCATGCTGAAGC-TAMRA-3'.

The primers used to amplify *L. intracellularis* DNA were, as previously described by Lindecrona *et al.*, (2002):

Forward primer: 5′-GCGCGCGTAGGTGGTTATAT-3'

Reverse primer: 5′- GCCACCCTCTCCGATACTCA-3'

TaqMan probe: 5′-FAM-CACCGCTTAACGGTGGAACAGCCTT-TAMRA-3′.

The amplification mixture for real-time PCR consisted of 25 µL of PCR Master Mix (GeneReach Biotechnology, Taiwan), and the cycling programme included one step at 93 °C for 5 min, followed by 40 cycles at 93 °C for 15 sec and 72 °C for 1 min.

The numbers of pigs with clinical signs of diarrhoea in the treatment and control groups were recorded weekly throughout the experiment, from 20 to 27 weeks of age. This was expressed as the percentage of pigs with diarrhoea per pen.

Histological analysis was performed on ileal samples collected from one randomly selected marked pig in each group at the slaughterhouse at the end of the experiment. Small intestine samples were obtained from the electrically stunned and slaughtered pigs at 27 weeks of age. Tissue sections of the distal ileum, measuring 10 cm, were collected and flushed with physiological saline and fixed in neutral formalin solution (37%–40% 10% formalin, 4.0 g $NaH_2PO_4 \cdot H_2O$, and 6.5 g $NaHPO_4 \cdot H_2O$) for 24 hours. After fixation and shaping, intestinal samples were dehydrated in increasing concentrations of ethyl alcohol, cleared with xylene, infiltrated with paraffin, and embedded in paraffin blocks. Standard glass microscope slides were used for mounting. Sections 3 µm thick were placed on glass slides and stained using the routine Mayer's haematoxylin and eosin procedure. Microscopic changes were examined using a light microscope (Olympus BX53) with 40× objective magnification.

The data were analysed using SPSS 20.0 (IBM, Chicago, IL, USA) software. All results were expressed as the mean ± the standard deviation. Continuous variables were examined for normality and homogeneity of variance by examining the residuals using coefficients of skewness and kurtosis, as well as using the Kolmogorov–Smirnov test and the Shapiro–Wilk normality test. The Mann–Whitney U-test and Friedman test were used to assess the significance of differences between the means of the DNA $\log_{10}$ real-time PCR copy numbers for the control and treatment groups. The diarrhoea scores were tested using the Kruskal–Wallis test. A probability value of $P < 0.05$ was considered statistically significant, while Bonferroni correction was applied for the Friedman test, with a post-hoc Wilcoxon test.

Results and discussion

This study focused on combined *L. intracellularis* and *B. hyodysenteriae* infections in growing-finishing pigs, and the mutual susceptibility of these bacteria to antibiotics and a commercial feed additive containing essential oils and *C. sativa* plant extract.

The *L. intracellularis* and *B. hyodysenteriae* contents of the rectal swabs from the control and treatment groups are presented in Table 1. Real-time PCR analyses showed that at the beginning of the experiment (week zero), the number of DNA $\log_{10}$ copies per microlitre in the swabs was higher for *L. intracellularis* than for *B. hyodysenteriae*, and there was no significant difference between the control and treatment groups for either bacterium. This indicates that the control and treatment groups were uniform at the beginning of the experiment, with the level of co-infection being equally pronounced in both groups of pigs. Furthermore, the finding of a higher level of *L. intracellularis* than *B. hyodysenteriae* in both groups of pigs can be partially explained by the hypothesis proposed by Daniel *et al*. (2023). These authors suggested that *L. intracellularis* induces lesions in the large intestine in the early stages

of infection, and impairs the host intestinal immune response, thereby facilitating the colonisation of *B. hyodysenteriae*.

There was a prominent reduction in *L. intracellularis* shedding from week zero to week one (a decrease of 2.65 DNA $\log_{10}$ copies/µL) and from week zero to week two (a decrease of 3.77 DNA $\log_{10}$ copies/µL) in the group treated with tiamulin in their feed. This differed significantly from the treatment group, in which *L. intracellularis* shedding decreased by only 1.57 DNA $\log_{10}$ copies/µL from week zero to week one, and 1.99 DNA $\log_{10}$ copies/µL from week zero to week two. Corroborating our results, Walter *et al.* (2001) demonstrated that tiamulin effectively treated pigs with *L. intracellularis* infection under field-like conditions, eliminating positive faecal PCR results after 14 days of feed-based treatment, initiated at the onset of clinical symptoms. However, by week seven, both groups had had a continuous decrease in the number of *L. intracellularis* in their rectal swabs, with the exception of the control group in week three, which had a 1.47 DNA $\log_{10}$ copies/µL increase in *L. intracellularis* at this time.

This isolated increase cannot be attributed to a decreased sensitivity of *L. intracellularis* to tiamulin, despite the results of Wattanaphansak *et al*. (2019) and Yeh *et al*. (2011). Wattanaphansak *et al*. (2019) indicated that *L. intracellularis* showed potential resistance to certain antibiotics under *in vitro* conditions, and Yeh *et al*. (2011) reported that the effective intracellular and extracellular activities of various antibiotics, including tiamulin, against *L. intracellularis* strains decreased by two to eight times over an eight-year period. Walter *et al*. (2001) previously demonstrated that treating PE with tiamulin could affect faecal PCR diagnostic tests by interfering with faecal shedding, making the interpretation of results ambiguous. Therefore, the confounding results of the present study are most likely due to tiamulin's interference with the PCR procedure or an intermittent trend of excretion of *L. intracellularis* (Schwartz *et al*., 1999; Jacobson *et al*., 2010). In accordance with our results, intermittent faecal shedding lasting up to 12 weeks after the first positive PCR result was observed in growing-finishing pigs experimentally challenged with *L. intracellularis* (Guedes & Gebhart, 2003), and following an acute PE outbreak on a commercial farm (Guedes *et al*., 2002).

After 14 days of lincomycin supplementation and seven days of antibiotic withdrawal (week seven), significantly higher levels of *L. intracellularis* were found in the control (2.38 ± 0.03 DNA $\log_{10}$ copies) than in the PFA treatment group (1.04 ± 0.99 DNA $\log_{10}$ copies). The capacity of non-antibiotic additive premixes to be used until the end of fattening, without a withdrawal period, can thus be considered a competitive advantage. In line with these findings, previous studies similarly determined that the addition of a PFA with a similar composition to the one used in the present study significantly decreased *L. intracellularis* faecal shedding in fattening pigs after two weeks and in weaned piglets after two and four weeks of treatment (Drašković *et al*., 2018; Nuntapaitoon *et al*., 2023). Furthermore, a preparation based on extracts of *O. vulgare* and *A. sativum* proved effective in reducing the prevalence of *L. intracellularis* over a six-week treatment period, with no difference between this group and the control group of piglets, which received tiamulin in their feed throughout the entire experiment (Papatsiros *et al*., 2009).

A significant decrease in the *B. hyodysenteriae* DNA load of the rectal swabs between week zero and week seven of the experiment was found in both groups of pigs, with a difference ($P = 0.018$) between the treatment and the control group only being found in week two (0.68 ± 1.35 and 2.53 ± 1.20 DNA $\log_{10}$ copies/µL, respectively). The *B. hyodysenteriae* faecal load was below the minimum detection level during week six in the control group and week seven in the treatment group. In contrast with the findings for *L. intracellularis*, the decline of *B. hyodysenteriae* DNA $\log_{10}$ copies in the rectal samples was intermittent in the treatment group. However, the sensitivity of *B. hyodysenteriae* and *L. intracellularis* detection can be influenced by various factors, such as intermittent excretion patterns, the bacterial load in the faecal samples or rectal swabs, sample pooling, the sensitivity of the PCR protocol, and the DNA extraction procedures (including inhibitory factors) used, all of which must be considered when interpreting the results (Heinonen *et al*., 2000; Jacobson *et al*., 2003; Grahofer *et al*., 2016).

There is little available *in vivo* data on the antibacterial efficiency of phytogenics for the treatment of pig infections caused by obligatory intracellular agents (Papatsiros *et al.*, 2009; Drašković *et al.*, 2018; Delić *et al.*, 2021; Nuntapaitoon *et al*., 2023), mainly because of the fastidious growth conditions of these agents, and the limited detection methods available (Maele *et al.*, 2015). Additionally, despite the widespread use of phytogenics in animal production, because of their antioxidant and antimicrobial properties and their beneficial impact on growth performance, knowledge about their mode of action is still limited (Silva Júnior *et al*., 2020). Nonetheless, efforts have been made to demonstrate the *in vitro*

susceptibility of *B. hyodysenteriae* and *L. intracellularis* to active compounds composed of different organic acids and essential oils commonly used as feed additives, as well as to partially explain the mechanisms involved in mitigating the intestinal lesions caused by these bacteria (Álvarez-Ordóñez *et al*., 2013b; Maele *et al*., 2015; Gómez-García *et al*., 2020; Meneguzzi, 2020; de Groot *et al*., 2022). Álvarez-Ordóñez *et al*. (2013b) reported the vigorous antibacterial activity of a feed supplement composed of citrus fruit extract against *B. hyodysenteriae* strains, with minimum inhibitory concentration (MIC) values ranging from 10 ppm to 40 ppm. Similarly, Maele *et al.* (2015) found that eugenol, carvacrol, thymol, and cinnamaldehyde had low MIC values against three strains of *B. hyodysenteriae*, with binary essential oil and organic acids combinations having additive effects, but synergism only being observed for a thymol and carvacrol combination. Strong dose-dependent antibacterial activity was reported for thymol and carvacrol, with an approximately 27% higher reduction rate of viable bacterial populations than was found for organic acids (Gómez-García *et al.,* 2020).

It is assumed that multi-component phytogenics based on essential oils, which consist of a wide range of chemical compounds with antimicrobial properties, increase the likelihood of achieving additive or synergistic activities because of their action on different cellular targets (Álvarez-Ordóñez *et al*., 2013b). Thus, in addition to the bacterial cell membrane, which is the primary cellular target of essential oils because of their lipophilic/hydrophobic nature (Trombetta *et al*., 2005), it has been established that specific components in essential oils also affect cell proteins embedded in the cytoplasmic membrane by distorting lipid-protein interactions or directly affecting the hydrophobic regions of these proteins (Sikkema *et al*., 1995). These suggested mechanisms, which exert both physico-chemical effects on bacterial membranes and target specific cellular components, may lower the chances of bacteria developing resistance (Álvarez-Ordóñez *et al*., 2013b). Considering the results obtained in this study, which suggest the effectiveness of a PFA for treating mixed infections, this represents a significant advantage of natural antimicrobials, particularly in controlling SD. This is especially relevant considering the findings of Yeh *et al*. (2018) that *B. hyodysenteriae* isolates from Taiwan have decreased susceptibility to tiamulin and lincomycin, two of the most common antibiotics used in the treatment and control of SD.

It has recently been demonstrated that various non-antimicrobial compounds used as feed additives exhibit beneficial effects in an explant infection model (de Groot *et al*., 2022). In particular, it was found that phytogenics made from a blend of thymol and carvacrol increased epithelial coverage and downregulated interleukin-1α, interferon-γ, and tumour necrosis factor-α in spiral colon explants infected with *B. hyodysenteriae ex vivo*. De Groot *et al*. (2022) suggested that this effect could have been due to the anti-inflammatory properties of carvacrol, thymol, and other phenolic compounds, which are associated with inhibiting the cyclooxygenase-2 cascade. The PFA used in this study, which showed an effect on a combined infection comparable to that of antibiotic treatment, contained a mixture of essential oils from *T. vulgaris* and *O. vulgare*, with thymol and carvacrol as the primary active substances. This suggests that similar effects to those described above might be expected *in vivo*, to a certain extent.

The results for the *L. intracellularis* and *B. hyodysenteriae* DNA loads in the floor faecal samples corroborated the levels of *L. intracellularis* and *B. hyodysenteriae* shedding found in the rectal swabs (Table 2). At week zero and week one of the study, the PFA-supplemented group had significantly higher numbers of *L. intracellularis* DNA $\log_{10}$ copies/µL than the control group. However, in week two, a notable decrease in the *L. intracellularis* contents of the treatment group samples was found, and significant differences between the control group and the PFA-supplemented group were not observed again until week six of the experiment. After the withdrawal of the antibiotic, a significantly lower number of *L. intracellularis* DNA $\log_{10}$ copies/µL were found in the floor faecal samples from the treatment group (2.58 ± 0.94 DNA $\log_{10}$ copies/µL) than from the control group (3.32 ± 0.58 DNA $\log_{10}$ copies/µL). A significant decline in the *L. intracellularis* DNA $\log_{10}$ copies/µL in the floor faecal samples from the beginning of the study was found after four weeks of antibiotic supplementation in the control group and two weeks of PFA supplementation in the treatment group. The long-term presence of pathogenic agents in the environment, shed by animals without visible clinical signs, could be the source of infection for susceptible pigs (Guedes & Gebhart, 2003). This was confirmed by the floor faecal sample results from week two to week seven in this study. Furthermore, *L. intracellularis* could remain viable outside the host for 14 days in faeces at 5–15 °C (Collins *et al.*, 2000).

Table 1 *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* levels (mean ± standard deviation) in rectal swab samples from pigs either treated with tiamulin/lincomycin (control) or a commercial phytogenic-based premixed feed additive (treatment)

Groups	Trial period								*P*-value
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	
***L. intracellularis* (DNA log_{10} copies/µL)**									
Control	$4.85^{A} \pm 1.06$	$2.20^{aBC} \pm 1.66$	$1.08^{aABC} \pm 1.36$	$2.55^{B} \pm 0.86$	$2.05^{BC} \pm 0.83$	$1.64^{BC} \pm 1.03$	$0.82^{C} \pm 1.21$	$2.38^{BC} \pm 0.33$	<0.0001
Treatment	$5.69^{A} \pm 1.13$	$4.12^{bAC} \pm 0.83$	$3.70^{bABCE} \pm 1.24$	$2.87^{BC} \pm 0.40$	$2.40^{CDE} \pm 0.98$	$1.02^{DE} \pm 1.23$	$0.64^{DE} \pm 0.96$	$1.04^{bE} \pm 0.99$	<0.0001
***P*-value**	0.227	0.003	0.001	0.455	0.118	0.380	0.611	<0.0001	
***B. hyodysenteriae* (DNA log_{10} copies/µL)**									
Control	$3.37^{A} \pm 0.44$	$3.11^{AB} \pm 0.32$	$2.53^{aABC} \pm 1.20$	$1.37^{BCD} \pm 1.73$	$0.84^{CD} \pm 1.24$	$0.76^{D} \pm 1.13$	nd	$0.25^{D} \pm 0.86$	<0.0001
Treatment	$3.04^{AC} \pm 1.15$	$2.68^{A} \pm 1.04$	$0.68^{bB} \pm 1.35$	$0.74^{AB} \pm 1.11$	$1.20^{BC} \pm 1.14$	$1.03^{B} \pm 1.23$	$0.26^{B} \pm 0.78$	nd	<0.0001
***P*-value**	0.722	0.569	0.018	0.329	0.875	0.569	-	-	

[a,b] Different lower-case superscript letters within the same column indicate significant differences between groups (P <0.05); [A,B,C,D,E] Different upper-case superscript letters within the same row indicate significant differences within the group during the course of the experiment (P <0.05); nd: not detected.

Table 2 *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* levels (mean ± standard deviation) in floor faecal samples from pigs either treated with tiamulin/lincomycin (control) or a commercial phytogenic-based premixed feed additive (treatment)

Groups	Trial period								*P*-value
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	
***L. intracellularis* (DNA log_{10} copies/µL)**									
Control	$4.22^{aA} \pm 0.56$	$3.98^{aABD} \pm 0.84$	$3.93^{ABD} \pm 0.72$	$3.89^{AB} \pm 0.76$	$3.48^{BC} \pm 0.63$	$3.03^{D} \pm 1.40$	$2.19^{CD} \pm 1.59$	$3.32^{aBD} \pm 0.58$	<0.0001
Treatment	$5.00^{bA} \pm 0.98$	$5.31^{bA} \pm 0.60$	$3.96^{BC} \pm 1.14$	$4.11^{B} \pm 0.37$	$3.77^{BC} \pm 0.61$	$3.25^{CD} \pm 0.95$	$2.84^{D} \pm 1.16$	$2.58^{bE} \pm 0.94$	<0.0001
***P*-value**	0.003	<0.0001	0.721	0.230	0.189	0.808	0.188	0.017	
***B. hyodysenteriae* (DNA log_{10} copies/µL)**									
Control	$4.22^{aA} \pm 0.24$	$3.71^{aB} \pm 0.38$	$3.40^{aBC} \pm 1.04$	$2.23^{C} \pm 1.24$	$0.90^{CD} \pm 1.51$	$0.30^{aD} \pm 0.81$	$0.12^{D} \pm 0.44$	$0.28^{D} \pm 0.73$	<0.0001
Treatment	$3.65^{bA} \pm 0.37$	$3.08^{bB} \pm 1.04$	$3.02^{bBC} \pm 0.87$	$2.92^{BC} \pm 0.81$	$1.85^{CD} \pm 1.51$	$1.48^{bDE} \pm 1.55$	$0.41^{EF} \pm 1.06$	$0.07^{F} \pm 0.35$	<0.0001
***P*-value**	<0.0001	0.005	0.030	0.062	0.080	0.014	0.494	0.294	

[a,b] Different lower-case superscript letters within the same column indicate significant differences between groups (P <0.05); [A,B,C,D,E,F] Different upper-case superscript letters within the same row indicate significant differences within the group during the course of the experiment (P <0.05)

Contrary to the findings for *L. intracellularis*, the *B. hyodysenteriae* DNA log_{10} copies/µL in weeks zero (P <0.0001), one (P = 0.005), and two (P = 0.030) were higher in the floor faecal samples of the control group than in those of the treatment group. A continuous decline in *B. hyodysenteriae* bacteria was noted until week six in the control group and week seven in the PFA-supplemented group. Similarly, Delić *et al.* (2021) showed a positive effect of two essential oil-based phytogenic additives on the degree of infection with *B. hyodysenteriae* that did not differ from tiamulin treatment.

The significantly lower levels of *L. intracellularis* and *B. hyodysenteriae* DNA log_{10} copies/µL found in the floor faecal samples collected during the last week of the study for the PFA-supplemented group can be attributed to the withdrawal of the antibiotics from the feed of the control group for the last seven days of the experiment, as per the required withdrawal periods for animals intended for slaughter. This reflects one of the main advantages of using antibiotic-alternatives in animal feed for the treatment of bacteria for which the development of resistance has not yet been determined (Yang *et al.*, 2015), while the emergence of antibiotic-resistant strains of *L. intracellularis* and *B. hyodysenteriae* suggests an additional advantage (Card *et al.*, 2018; Joerling *et al.*, 2018; Seo *et al.*, 2019).

A recent study reported that experimentally infected six-week-old piglets had more pronounced clinical signs, macroscopic changes, and decreased intestinal microbiome diversity in cases of co-infection with *L. intracellularis* and *B. hyodysenteriae* than in cases of infection with a single pathogen (Daniel *et al*., 2023), with co-infection mainly increasing the severity of SD symptoms. Evidence of the beneficial effects of plant-based feed additives on diarrhoeal disorders caused by *L. intracellularis* or *B. hyodysenteriae* under field conditions is documented in the literature (Papatsiros *et al*., 2009; Delić *et al*., 2018; Drašković *et al*., 2018; Nuntapaitoon *et al*., 2023). However, data on the impact of phytogenics under co-infection conditions remain unavailable. The diarrhoea score results from the present study showed that at the beginning of the experiment, both the control and the treatment groups had pigs with diarrhoea, with a significantly higher incidence in the treatment group (Table 3). After one week of PFA supplementation, the number of pigs with clinical signs had decreased, and there was no significant difference between the control and treatment groups. From week two in the control group and week three in the treatment group, no pigs with diarrhoea were recorded until the end of the experiment. This likely reflects the decrease in the numbers of *L. intracellularis* and *B. hyodysenteriae* detected in the rectal swabs through the course of the study, even though these bacteria were present throughout the study period.

Table 3 Diarrhoea scores (percentage of pigs with diarrhoea per pen) for pigs either treated with tiamulin/lincomycin (control) or a commercial phytogenic-based premixed feed additive (treatment) during a seven-week experiment

Groups	Trial period			
	Week 0	**Week 1**	**Week 2**	**Weeks 3–7**
Control (n = 7)	6.31[a] ± 4.26	2.66 ± 2.62	0.00 ± 0.00	0.00 ± 0.00
Treatment (n = 12)	24.85[b] ± 14.57	6.06 ± 7.10	0.20 ± 0.67	0.00 ± 0.00
***P*-value**	0.002	0.518	0.425	-
Chi square	9.301	0.418	0.636	-
df	1	1	1	-

[a,b] Different lower-case superscript letters within the same column indicate a significant difference between groups (P <0.05)

In experimentally challenged piglets, differences in microscopic changes in the large intestine were observed between piglets infected with a combination of *L. intracellularis* and *B. hyodysenteriae* and piglets infected with only one of the two bacteria, indicating a synergistic effect of these two pathogens (Daniel *et al*., 2023). In addition to moderate diffuse mucohaemorrhagic colitis and severe diffuse fibrin necrohaemorrhagic catarrhal colitis in the large intestines of co-infected piglets, further histological analysis of the caecum and colon revealed more extensive and intense lesions, including superficial necrosis, haemorrhage, goblet cell hyperplasia, crypt abscesses, and lamina propria neutrophil infiltration, in the co-infected piglets, compared to uninfected piglets and those with PE.

However, co-infected piglets only exhibited more pronounced goblet cell hyperplasia than piglets with SD.

In the control group of co-infected pigs treated with antibiotics in the present study, the architecture of the ileum mucosa was improperly organised into intestinal villi. Increased mucosal thickness, with vanishing villi, was observed, the mucosa was composed of adenomatous and hyperplastic crypts, and goblet cells were reduced in number. Crypts were elongated, dilated, and branched, resulting in the aforementioned thickening of the mucosal layer. Furthermore, dilated crypts were filled with inflammatory cells and cell debris, and the lamina propria was hyperaemic (Figure 1A).

In the group of co-infected pigs treated with the PFA, the architecture of the mucosa was properly organised into intestinal crypts and villi, and the mucosal thickening was reduced compared to the distal ileum of the positive control group of piglets. Goblet cells were slightly proliferated, and the lamina propria mucosa was moderately hyperaemic (Figure 1B). These results suggest that the PFA may have exhibited antibacterial effects on both *L. intracellularis* and *B. hyodysenteriae* simultaneously, with a potentially protective effect on the intestinal mucosa. This aligns with the findings of de Groot *et al.* (2022), where a phytogenic supplement containing thymol and carvacrol exhibited anti-inflammatory effects and increased epithelial coverage in explants infected with an obligatory intracellular agent.

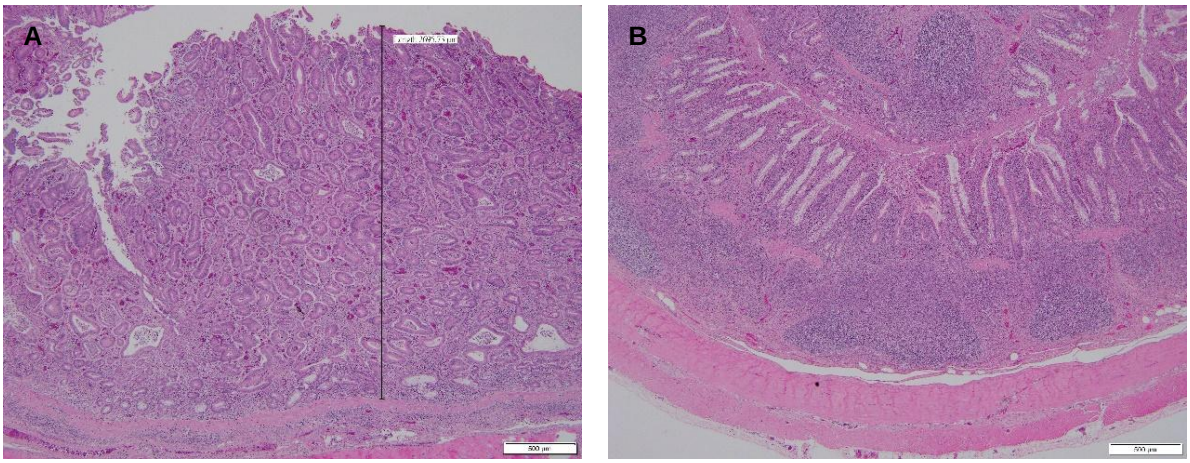

Figure 1 Microscopic changes in the ileums of pigs co-infected with *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* and treated with **(A)** tiamulin/lincomycin, or **(B)** a commercial phytogenic-based premixed feed additive (haematoxylin and eosin staining, 40× magnification with objective lens, scale bar: 500 µm).

Previous studies have shown that PFAs rich in essential oils improve growth performance in pigs with PE or SD with an efficiency comparable to that of tiamulin (Papatsiros *et al.*, 2009; Delić *et al.*, 2018). The results of this study are in agreement with these findings, as, at the end of the experiment, the average body weight of the pigs that received tiamulin/lincomycin was 116 ± 12.83 kg, and that of the pigs supplemented with the PFA in their feed was 117 ± 15.34 kg, with no significant difference observed between these two groups. It can thus be inferred that the PFA had an effect similar to that of the antibiotics on the performance of the pigs infected with both *L. intracellularis* and *B. hyodysenteriae*.

Since this is the first study examining the impact of a PFA on combined *L. intracellularis* and *B. hyodysenteriae* infections under field conditions, its significance lies in providing preliminary insights into the efficacy of natural antimicrobials in a practical setting. However, because of the challenging experimental conditions, several limiting factors must be considered, both in interpreting the results and in conducting additional trials. These include using a larger number of animals to monitor prevalence more accurately, as well as optimising factors that influence the detection of pathogens in faecal samples and rectal swabs through the use of specific PCR procedures, in order to minimise variability in results. Additionally, the use of antibiotics in pigs complicates the detection of infection in medicated populations, necessitating larger sample sizes, as indicated by Schwartz *et al*. (1999).

On farms with concurrent PE and SD infections, it is difficult to separate groups of mono-infected animals. This is another limiting factor of this study that should be considered when elucidating the

efficacy of the PFA, as previous studies suggested a synergic effect between these two pathogens. Previous studies have also highlighted the significant role of the intestinal microbiome in disease development (Daniel *et al*., 2023), underscoring the need to consider the impact of the PFA on dysbiosis. When interpreting production results, the absence of negative controls and the difficulty distinguishing between growth promoting and therapeutic effects complicates the evaluation of the PFA and its comparison to antibiotics. Therefore, monitoring other indicators, such as weight gain, feed intake, and the feed conversion ratio in subclinically infected herds is crucial to determine whether this PFA prevents impaired production.

Conclusions

The present study's findings indicate that a PFA composed of mixtures of different essential oils and plant extracts at a dose of 2 g/kg could effectively control and treat simultaneous *L. intracellularis* and *B. hyodysenteriae* infections in finishing pigs. This represents the first report of the efficacy of this PFA as a treatment for *L. intracellularis* and *B. hyodysenteriae* co-infection in a clinical trial under field conditions. However, further research is needed to address the limitations of this study, including trials with larger sample sizes and incorporating the monitoring of production performance, to elucidate the extent to which this PFA could replace antibiotics, especially pleuromutilins, in the management of PE and SD in a cost-effective manner.

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Authors' contributions

HCK, MV, and JBN conceived and designed the experiment. CFW and HCK conducted the experiment and performed the analyses. MG analysed the data. CFW, HCK, MG, and VD wrote the manuscript draft. All authors reviewed the manuscript and gave their final approval of the manuscript.

Conflict of interest declaration

PATENT CO. DOO provided financial support for this trial; however, this trial was conducted prospectively and the authors had no preconceptions or biases to influence the results of the study. The authors therefore declare that they have no conflicts of interest.

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