Ultra-diluted complex additive in the diet reveals benefits in the intestinal tract of nursery-phase piglets

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Abstract

This study aimed to assess an ultra-diluted (UD) complex as a replacement for an antimicrobial performance enhancer in diets on the growth performance, intestinal health, and inflammatory response of nursery piglets. The experiment lasted 37 d and involved 126 animals weaned at 21±1.3 d, with an initial body weight of 5.62±1.16 kg. Piglets were assigned to 6 dietary treatments in a randomized block design with 7 replicates and 3 piglets per pen as experimental unit. The treatments were: positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC); negative control – basal diet without additives (NC); and NC containing 4.5; 6.0; 7.5 or 9.0 kg of UD additive/ton diet. Performance data were calculated, and daily diarrhea occurrence was observed. Blood was collected for hematological analyses. In the end of experiment, one animal per pen was slaughtered for organ weighing, pH analysis and collection of intestinal samples for histopathology. Feces were collected during experiment, and cecal contents at slaughter for microbiological and antibiogram analysis. There was no difference between treatments on performance. Throughout the study, UD levels were equal to PC regarding diarrhea occurrence. Higher levels of UD complex led to higher values of total leukocytes. The 4.5 treatment showed a reduction in total and thermodurant enterobacteria populations in piglet feces, and an increase in lactic acid bacteria, compared to PC. All treatments showed less duodenal pathological alterations compared to NC. The use of the UD additive, especially at 4.5 kg/ton, may be a good alternative for replacing chlorohydroxyquinoline in piglet diets.

Introduction

The pig production system is highly intensive, which leads to the early weaning of these animals. In the wild, piglets are weaned at 10 to 12 weeks of age, however, depending on facilities, management, and health status of commercial farms, they are weaned within 30 d-old or less (Moeser et al. 2017). The stress caused by this management results in several problems, such as post-weaning diarrhea, loss of performance and even death (Ma et al. 2021). In addition, bacteria such as Escherichia coli further aggravate piglet gastrointestinal tract disorders. Certain subgroups of E. coli have virulence factors that make them capable of causing intestinal diseases (Caldorin et al. 2013). To avoid these problems involving stress and microorganisms, the use of antibiotics as performance enhancers has been recurrent in pig production, being used as a component that maintains health and ensures high levels of production on pig farms (Albernaz-Gonçalves et al. 2022).

However, the frequent use of antibiotics in animals' diets has led to a problem that can affect society as a whole: bacterial resistance. According to the World Health Organization, bacterial resistance occurs when the microorganism changes in response to the use of these drugs. The concern is such that the European Union banned its use in animal production as a performance enhancer (Dewulf et al., 2022). With these restrictions, studies have intensified in search of viable alternatives to these additives, aiming to reduce the productive losses caused by this ban.
The idea of ultra-diluted (UD) remedies was created by Samuel Hahnemann at the end of the 18th century (Hahnemann 1796). There are advantages to its use, such as the ability not to contaminate animal products or the environment (Braccini et al. 2019). Furthermore, some older studies have highlighted the importance of this therapy in weaned piglets (Soto et al. 2008; Camerlink et al. 2010). A current study showed that UD complexes improved nutrient digestibility and feed efficiency in adult pigs (Wendt et al. 2023). A similar study also demonstrated that an UD additive enhanced the performance in finisher pigs without negative effects on meat traits and plasma metabolites (Lima et al. 2022).

Considering that the intestinal health is fundamental for piglets to maintain diet digestion, nutrients absorption and, consequently, increase weight gain (Da Silva et al. 2021a), the use of an UD complex with substances indicated for gastrointestinal disorders was evaluated and compared to an antimicrobial performance enhancer with the aim of verifying its effects on growth performance, intestinal health and inflammatory response of nursery piglets. The hypothesis of this study was that animals that received the UD additive would show enhanced performance, improved intestinal health and inflammatory response.

**Materials and methods**

**Animals, experimental design, and housing**

The experiment was performed at the Swine Research Unit (SRU) located at the Gralha Azul Experimental Farm of the Pontifical Catholic University of Paraná (PUCPR), Fazenda Rio Grande, Paraná, Brazil. The study was double-blind, lasted 37 d and involved 126 animals (castrated males and females) weaned at 21 ± 1.3 d, with an initial weight of 5.62 ± 1.16 kg, which were assigned to 6 treatments in a completely randomized block design with 7 replicates and 3 piglets per experimental unit (sex and initial weight were used as blocking criteria).

During the first 4 d before the start of the trial, all the animals received the same basal feed (a corn, wheat, and soybean meal-based piglet diet), due to the adaptation period to the new facilities. The piglets were housed in suspended pens (1.5 m x 1.5 m) with semi-automatic feeders, water dispensers and plastic slotted floors. The treatments were composed of six experimental diets: positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC); negative control – basal diet without additives (NC); basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of UD complex additive in the feed.

**Composition of the product**

The inclusion of the additive in the diet of animals followed recommendations provided by the manufacturer based on studies of product development. The UD complex (Real H Company - Animal Nutrition and Health; Campo Grande, MS, Brazil) was composed of: *Ignatia amara* (30 CH), *Chamomilla* (15 CH), *Silicea terra* (15 CH), *Staphysagria* (15 CH), *Colibacillinum* (9 CH), *Enterococcinum* (9 CH), *Lac defloratum* (9 CH), *Aethusa cynapium* (6 CH), *Artemisia abrotanum* (6 CH), *Calcarea carbonica* (6 CH),
Thyreoidinum (6 CH), Thymulin (6 CH), vehicle (Calcium carbonate – 1,000 g). The experiment lasted 37 d, and the animals were fed ad libitum until the end of the experiment.

The diets were formulated on the basis of corn and soy, supplemented with industrial amino acids, and fed as bran. They were prepared to meet the piglets' requirements for the pre-starter I (1 to 7 d of experimentation), pre-starter II (8 to 20 d of experimentation) and starter (21 to 37 d of experimentation) phases, following Rostagno et al. (2017). The composition of the experimental diets can be found in the supplementary material (Supplementary Table 1).

Growth performance and diarrhea occurrence

The animals were weighed using a scale (Universal line, Digi-tron, Curitiba, PR, Brazil) on d 1, 7, 21, and 35. The quantification of the diet supplied and wasted was performed at the end of each phase. The body weight (BW) was recorded. The average daily gain (ADG, kg/d), average daily feed intake (ADFI, kg/d) and feed conversion ratio (FCR) of the animals were calculated. Diarrhea occurrence was evaluated daily during the morning by observing the total amount of feces in the pen, and determining how many were diarrhea (watery, without solid pieces) and how many were normal (well-formed consistent feces). Data were collected this way to find the percentage of pens with diarrhea within each treatment. Following observation, the pens were cleaned, and after 24 h it was evaluated again. The results of the data collected were divided into three periods: 1 to 7, 1 to 21 and 1 to 35 d of the experiment. Also, the diarrhea data was analyzed considering three animal sizes: small (mean: 4.57 ± 0.55 kg), midsize (mean: 5.75 ± 0.27 kg) and large (mean: 7.08 ± 0.64 kg). These mean values were based on the average of the blocks in the first day of experiment, considering both males and females. Further analysis was conducted on the overall size.

Blood parameters, inflammatory response, and gut health

On d 36, after 8 h fasting, blood samples of one animal per experimental unit, being 7 animals/treatment, totaling 42 piglets (21 males and 21 females with the average weight of the block considering D21) were collected by jugular vein puncture to evaluate blood metabolites, hemogram, leucogram, and absolute count of peripheral blood cells (phagocytosis process).

The blood samples for analysis of biochemical parameters were centrifuged (FANEM Excelsa Baby I Mod. 206) at 3,000 rpm for 10 min. Then, approximately 3 mL of serum of each sample were transferred to previously identified and frozen eppendorf polyethylene microtubes for analysis of urea (colorimetric-enzyme method), total protein (Colorimetric-Biuret method), glucose (enzymatic-colorimetric method, Trinder), alkaline phosphatase (kinetic-colorimetric method), blood ALT (Kinetic-UV method) and AST (kinetic-UV method). These analyses were determined by spectrophotometry (Bel SPECTRO S05) using specific Gold Analisa Diagnóstica kits (Belo Horizonte, MG, Brazil).

For determination of absolute proportion and count of peripheral blood cells, 1 mL of blood collected with anticoagulant (EDTA) was used for purification of peripheral blood mononucleated cells (PBMCs) by the RBC lysis method by use of ammonium chloride solution. After purification, PBMCs were incubated for 30
min (37°C) with 50 µL of a florescent particle conjugate for phagocytosis (pHrodo™ Green Zymosan Bioparticles™ Conjugate for Phagocytosis, from ThermoFisher Scientific, 1 mg/mL). Samples were incubated with pHrodo™ for 30 min at 37°C and, then, fixed with 1% paraformaldehyde for 30 min at 4°C. Subsequently, the already marked and fixed cells were resuspended with 2 mL of saline phosphate buffer (PBS) + 1% Bovine Serum Albumin (BSA) + 0.01% Azide sodium, for conservation until reading and analysis by flow cytometry (Citometer - BD FACSCalibur™). For absolute quantification of circulating leukocytes, 50 µL of absolute counting microspheres were added (C36950, CountBright from ThermoFisher Scientific).

**Slaughter and collection of samples**

On d 37, 42 animals (21 males and 21 females with the average weight of the block considering D21) were slaughtered, after fasting for 12 h, for data and biological samples collection. The pH measurement of stomach, cecum and colon content was performed using a peagameter (HI 99163 Hanna Instruments, Barueri, SP, Brazil). The final portion of each organ was sealed using twine so that the contents wouldn't mix and compromise the measurements. Then, the digestive organs (liver with gallbladder, stomach, small and large intestine that were empty), as well as the spleen, were weighed to calculate the relative weight of the organs, according to the live weight of the animal measured at d 35. The organs were weighed on digital scales (Prix Toledo 2095, São Bernardo do Campo, SP, Brazil).

**Microbiological analysis and antibiogram**

On the second day of the experiment, feces samples were collected directly from the rectum of 10 piglets (5 males and 5 females), as baseline. On the 21st d of the experiment, fecal samples were also collected from 42 piglets (21 males and 21 females with the average weight of the block considering D21). Also, caecal samples were collected from these 42 piglets after slaughter (D37). The samples were stored in sterile, identified and refrigerated universal collectors for microbiological analysis. The microbiological profile was determined by counting colonies of total and thermotolerant enterobacteria and lactic acid bacteria populations. For this purpose, aliquots of collected feces were homogenized in 0.1% peptone water followed by plating on MacConkey Agar (MAC; for enterobacteria) (Macconkey 1905) and Man, Rogosa and Sharpe Agar (MRS; for lactic acid bacteria). The inoculated plates were incubated at 37°C (total enterobacteria and lactic acid bacteria) and 45°C (thermotolerant enterobacteria) for 24–48 h and the bacterial populations were determined in CFU/g of feces.

To identify the isolated bacteria, three colonies with morphology compatible with *E. coli* were isolated from each sample. For the selection of each one, the study carried out by Barcella (2016) was followed, selecting pink colonies with a bile precipitation halo. These were subjected to differentiation by inoculation in Rugai with lysine. The tube contained nine biochemical tests present in one test tube: indole reaction, deamination of L-tryptophan, sucrose fermentation, glucose fermentation, urea hydrolysis, gas production, hydrogen sulfide (H₂S) production, lysine decarboxylation and motility. After observing the results found in each of the tests, the IDENTAX Bacterial Identification System (v. 12) software was used for identification.
The technique used to determine the susceptibility of *E. coli* was the Mueller-Hinton agar disk-diffusion method. In Petri plates containing Mueller-Hinton agar, 100 µL of a solution containing 10^8 CFU/mL of each bacterium identified as *E. coli* were inoculated and evaluated separately. After complete drying, 4 discs were placed with the previously chosen antibiotics (2 discs for each antimicrobial). The following antibiotics were used: amikacin (30 µg), amoxicillin/clavulamic acid (30 µg), ampicillin (10 µg), azithromycin (15 µg) ciprofloxacin (5 µg), chloramphenicol (30 µg), streptomycin (10 µg), gentamicin (10 µg), erythromycin (15 µg), sulfazotrim (25 µg), tetracycline (30 µg) and tobramycin (10 µg). After all the processes, the plate was incubated at 37°C for 24h, for subsequent reading of the results.

**Histopathological alterations**

Immediately after removal of the organs, fragments of approximately 3 cm in length from the duodenum (removed 15 cm from the stomach sphincter) and jejunum (removed from the medial portion of the jejunum) were collected, washed with saline solution, and stored in 50 mL tubes containing buffered formalin solution. For the analysis of intestinal morphology, the scoring system methodology (ISI) adapted from Kraieski et al. (2017) was followed: each histopathological alteration contains an impact factor ranging from 1 to 3; the higher the factor, the more harmful the alteration (Table 1). Also, the extent of each lesion or frequency observed, if compared with unaffected tissues, was also evaluated with scores ranging from 0 to 3, being: score 0 - no lesion found, score 1 - alteration of up to 25% of the tissue, score 2 - alteration between 26–50% of the tissue, score 3 - alteration of more than 50% of the tissue. For the final value of the ISI methodology, the impact factor of each alteration was multiplied by the respective score, and the results of all alterations were summed.

<table>
<thead>
<tr>
<th>Alteration</th>
<th>Impact Factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory infiltrate</td>
<td>3</td>
</tr>
<tr>
<td>Congestion</td>
<td>1</td>
</tr>
<tr>
<td>Desquamation</td>
<td>2</td>
</tr>
<tr>
<td>Coccidiosis</td>
<td>3</td>
</tr>
<tr>
<td>Bacterial clumps</td>
<td>3</td>
</tr>
<tr>
<td>Bacillus</td>
<td>3</td>
</tr>
<tr>
<td>Cystic crypts</td>
<td>2</td>
</tr>
<tr>
<td>Mucus</td>
<td>1</td>
</tr>
<tr>
<td>Necrosis</td>
<td>3</td>
</tr>
<tr>
<td>Edema</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1  
Impact factor of each alteration assessed according to the ISI methodology.
* Adapted from Kraieski et al. (2017).

**Statistical analysis**

The initial body weight of the piglets was compared using general linear model and it was included as a covariate for growth performance considering NC and UD levels (quantitative variable), also, the PC was compared with each treatment using Dunnett's test. Relative organ weights, pH of the digestive contents, hemogram, leucogram, biochemical parameters, phagocytic profile, and microbiology variables were also analyzed using general linear models (without the covariate) and Dunnett's test. Diarrhea occurrence was analyzed using Chi-square test: the analysis was performed between weight groups (small, midsize, and large) within each treatment, and the diarrhea occurrence between treatments within each weight group. Regarding non-parametric variables of the phagocyte profile by flow cytometry, Kruskal-Wallis test was used, followed by Dunn's multiple comparisons post-test. Intestinal histopathology data were analyzed by the Mann-Whitney test performing a pairwise comparison between all treatments. All analysis was performed with statistical software SPSS 25 (IBM Corp 2017). P values less than 0.05 were considered significant.

**Results**

**Growth performance and diarrhea occurrence**

For all periods, there was no difference between treatments for BW, ADG, ADFI, and FCR (Table 2). For the evaluation of diarrhea occurrence (DO), the treatments that had lower values in the overall animal population on d 1 to 7 were PC, NC, 4.5, and 7.5. On d 1 to 21, the treatments with lower values of diarrhea occurrence were NC, 4.5, and 7.5. Throughout the entire experimental period, only NC showed lower values, while all UD levels were equal to PC (P < 0.05) (Table 3). When the different animal sizes were compared, treatments 4.5 and 9.0 showed no difference in the three periods evaluated, while the diarrhea percentage of PC and NC treatments, in the periods from d 1 to 7 and 1 to 21, were lower for the large animals.
Table 2
Growth performance of nursery piglets fed ultra-diluted complex feed additive in the diets.

<table>
<thead>
<tr>
<th>Treatments¹</th>
<th>PC</th>
<th>NC</th>
<th>4.5</th>
<th>6.0</th>
<th>7.5</th>
<th>9.0</th>
<th>SEM²</th>
<th>P-Value³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight† (kg)</td>
<td>5.94</td>
<td>6.16</td>
<td>5.67</td>
<td>5.46</td>
<td>5.35</td>
<td>5.17</td>
<td>0.104</td>
<td>-</td>
</tr>
<tr>
<td>d 1 to 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW⁴ 7</td>
<td>7.29</td>
<td>7.24</td>
<td>6.86</td>
<td>6.46</td>
<td>6.54</td>
<td>6.14</td>
<td>0.127</td>
<td>0.832</td>
</tr>
<tr>
<td>ADG⁵ (kg/d)</td>
<td>0.19</td>
<td>0.15</td>
<td>0.17</td>
<td>0.14</td>
<td>0.17</td>
<td>0.13</td>
<td>0.009</td>
<td>0.832</td>
</tr>
<tr>
<td>ADFI⁶ (kg/d)</td>
<td>0.19</td>
<td>0.18</td>
<td>0.20</td>
<td>0.17</td>
<td>0.18</td>
<td>0.16</td>
<td>0.003</td>
<td>0.828</td>
</tr>
<tr>
<td>FCR⁷</td>
<td>1.17</td>
<td>1.15</td>
<td>1.32</td>
<td>1.20</td>
<td>1.19</td>
<td>1.30</td>
<td>0.053</td>
<td>0.351</td>
</tr>
<tr>
<td>d 1 to 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW21 (kg)</td>
<td>12.25</td>
<td>12.56</td>
<td>12.03</td>
<td>11.53</td>
<td>11.50</td>
<td>11.16</td>
<td>0.221</td>
<td>0.545</td>
</tr>
<tr>
<td>ADG (kg/d)</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.28</td>
<td>0.29</td>
<td>0.28</td>
<td>0.007</td>
<td>0.545</td>
</tr>
<tr>
<td>ADFI (kg/d)</td>
<td>0.39</td>
<td>0.38</td>
<td>0.38</td>
<td>0.37</td>
<td>0.37</td>
<td>0.35</td>
<td>0.006</td>
<td>0.125</td>
</tr>
<tr>
<td>FCR</td>
<td>1.39</td>
<td>1.26</td>
<td>1.41</td>
<td>1.38</td>
<td>1.33</td>
<td>1.30</td>
<td>0.030</td>
<td>0.613</td>
</tr>
<tr>
<td>d 1 to 35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW35 (kg)</td>
<td>21.85</td>
<td>21.58</td>
<td>21.11</td>
<td>20.28</td>
<td>20.27</td>
<td>20.31</td>
<td>0.321</td>
<td>0.291</td>
</tr>
<tr>
<td>ADG (kg/d)</td>
<td>0.45</td>
<td>0.44</td>
<td>0.44</td>
<td>0.42</td>
<td>0.42</td>
<td>0.43</td>
<td>0.007</td>
<td>0.291</td>
</tr>
<tr>
<td>ADFI (kg/d)</td>
<td>0.65</td>
<td>0.63</td>
<td>0.62</td>
<td>0.61</td>
<td>0.61</td>
<td>0.60</td>
<td>0.006</td>
<td>0.066</td>
</tr>
<tr>
<td>FCR</td>
<td>1.47</td>
<td>1.48</td>
<td>1.47</td>
<td>1.48</td>
<td>1.50</td>
<td>1.42</td>
<td>0.022</td>
<td>0.575</td>
</tr>
</tbody>
</table>

¹ Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of ultra-diluted additive in the feed. ² Standard error of the mean. ³P-value for linear regression. ⁴Body Weight. ⁵Average daily gain. ⁶Average daily feed intake. ⁷Feed conversion ratio. † Linear effect (P < 0.05) of the ultra-diluted additive levels on the Initial body weight (y = −0.109452x + 6.15606 R²= 0.0859).
Table 3
Diarrhea occurrence (%) by size of nursery piglets fed ultra-diluted complex feed additive in the diets.

<table>
<thead>
<tr>
<th>Piglet size</th>
<th>Treatments¹</th>
<th>PC</th>
<th>NC</th>
<th>4.5</th>
<th>6.0</th>
<th>7.5</th>
<th>9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 1 to 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (%)</td>
<td>9.21bA</td>
<td>5.41bA</td>
<td>8.93bA</td>
<td>19.61aA</td>
<td>9.26bA</td>
<td>7.69bA</td>
<td></td>
</tr>
<tr>
<td>Midsize (%)</td>
<td>12.96aA</td>
<td>9.30abA</td>
<td>5.45bA</td>
<td>6.52abB</td>
<td>0.00cB</td>
<td>15.56aA</td>
<td></td>
</tr>
<tr>
<td>Large (%)</td>
<td>0.00bB</td>
<td>0.00bB</td>
<td>6.49aA</td>
<td>0.00bc</td>
<td>10.20aA</td>
<td>10.20aA</td>
<td></td>
</tr>
<tr>
<td>Overall (%)</td>
<td>8.09abc</td>
<td>4.30c</td>
<td>6.91bc</td>
<td>11.86a</td>
<td>7.35bc</td>
<td>10.69ab</td>
<td></td>
</tr>
<tr>
<td>d 1 to 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (%)</td>
<td>4.59bA</td>
<td>1.96dA</td>
<td>4.79bA</td>
<td>9.62aA</td>
<td>2.51cdA</td>
<td>4.49bCA</td>
<td></td>
</tr>
<tr>
<td>Midsize (%)</td>
<td>8.89aA</td>
<td>6.67abA</td>
<td>3.82bCA</td>
<td>2.99cdB</td>
<td>1.74dA</td>
<td>8.16aA</td>
<td></td>
</tr>
<tr>
<td>Large (%)</td>
<td>1.59bcB</td>
<td>0.00dB</td>
<td>1.90bCA</td>
<td>0.88cDB</td>
<td>3.82abA</td>
<td>5.43aA</td>
<td></td>
</tr>
<tr>
<td>Overall (%)</td>
<td>5.01a</td>
<td>2.00b</td>
<td>3.24b</td>
<td>6.13a</td>
<td>2.70b</td>
<td>5.89a</td>
<td></td>
</tr>
<tr>
<td>d 1 to 35</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (%)</td>
<td>2.82bcAB</td>
<td>1.14dA</td>
<td>3.64bA</td>
<td>5.82aA</td>
<td>1.71cdA</td>
<td>2.49bcdA</td>
<td></td>
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<tr>
<td>Midsize (%)</td>
<td>4.84aA</td>
<td>4.15abA</td>
<td>2.36bCA</td>
<td>1.67cB</td>
<td>2.19bCA</td>
<td>4.67abA</td>
<td></td>
</tr>
<tr>
<td>Large (%)</td>
<td>0.84cDB</td>
<td>0.00dB</td>
<td>1.38cA</td>
<td>1.40cB</td>
<td>6.51aA</td>
<td>3.50bA</td>
<td></td>
</tr>
<tr>
<td>Overall (%)</td>
<td>2.86ab</td>
<td>1.33c</td>
<td>2.32b</td>
<td>3.75a</td>
<td>3.15ab</td>
<td>3.48a</td>
<td></td>
</tr>
</tbody>
</table>

¹ Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of ultra-diluted additive in the feed.

abcd Different lowercase letters on the same row indicate statistical difference by Chi-square test (P < 0.05).

AB Different capital letters on the same column indicate statistical difference by Chi-square test (P < 0.05).

**Blood parameters, inflammatory response, and gut health**

Regarding hemogram, the higher the levels of the UD additive, the higher the values of hemoglobin, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) (P < 0.05) (Table 4). Serum biochemical parameters and leucogram showed no difference between treatments.
Concerning flow cytometry analysis, piglets that received the negative control diet treatment had a higher percentage of monocytes in relation to the total number of circulating leukocytes compared to 7.5 and 9.0, and showed no difference to PC, 4.5 and 6.0 treatments (P < 0.05). For the degree of activity for phagocytic monocytes, levels 6.0, 7.5 and 9.0 of UD additive showed lower values when compared to PC (P < 0.05). Furthermore, it was possible to identify a difference between the levels: the greater was the amount of UD complex in the feed, the lower was the phagocytic activity of monocytes (P < 0.05). For the quantification of total leukocytes, animals from the negative control treatment (NC) showed a lower quantity of defense cells when compared to the positive control. Also, the higher was the level of the UD additive, the greater was the amount of leukocytes found (P < 0.05) (Fig. 1).

Table 4
Hemogram and leucogram values of nursery piglets fed ultra-diluted complex feed additive in the diets.

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatments¹</th>
<th>SEM²</th>
<th>P-Value³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
<td>NC</td>
<td>4.5</td>
</tr>
<tr>
<td>Hemogram</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cells (Log million/µL)</td>
<td>6.80</td>
<td>6.81</td>
<td>6.82</td>
</tr>
<tr>
<td>Hemoglobin (g/dL) †</td>
<td>12.21</td>
<td>10.88</td>
<td>11.86</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>36.42</td>
<td>35.00</td>
<td>37.16</td>
</tr>
<tr>
<td>MCV (fL) ⁴</td>
<td>53.73</td>
<td>54.62</td>
<td>55.38</td>
</tr>
<tr>
<td>MCH (pg) ⁵ † †</td>
<td>17.70</td>
<td>16.96</td>
<td>17.93</td>
</tr>
<tr>
<td>MCHC (%) ⁶ † † †</td>
<td>33.55</td>
<td>31.10</td>
<td>32.43</td>
</tr>
<tr>
<td>Leucogram</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Segmented (%)</td>
<td>45.28</td>
<td>45.14</td>
<td>44.71</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.00</td>
<td>0.85</td>
<td>1.42</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>2.57</td>
<td>1.14</td>
<td>1.85</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>45.57</td>
<td>45.14</td>
<td>45.00</td>
</tr>
<tr>
<td>Plasma proteins (g/dL)</td>
<td>6.12</td>
<td>5.80</td>
<td>5.97</td>
</tr>
<tr>
<td>Platelets (Log mm³)</td>
<td>5.70</td>
<td>5.66</td>
<td>5.64</td>
</tr>
</tbody>
</table>

¹ Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of ultra-diluted additive in the feed. ² Standard error of the mean. ³ P-value for linear regression; ⁴ Mean corpuscular volume; ⁵ Mean corpuscular hemoglobin; ⁶ Mean corpuscular hemoglobin concentration; † Linear effect (P < 0.05) of the ultra-diluted
additive levels on the Hemoglobin variable ($y = 0.178095x + 10.9838 \ R^2=0.2486$). $\dagger \dagger$ Linear effect ($P < 0.05$) of the ultra-diluted additive levels on the MCH variable ($y = 0.183907x + 17.0677 \ R^2= 0.1772$). $\dagger \dagger \dagger$ Linear effect ($P < 0.05$) of the ultra-diluted additive levels on the variable MCHC ($y = 0.195377x + 31.2997 \ R^2=0.1168$).

**Microbiological analysis and antiibiogram**

It was observed an increase in the population of lactic acid bacteria on d 21 compared with d 2 (baseline) ($P < 0.05$), regardless of the treatment received by the animals (Table 5). In addition, animals of groups NC, 4.5, 7.5 and 9.0 also showed an increase of these populations when compared to PC on d 21. A further result is that the higher the level of UD complex, the lower the populations of lactic acid bacteria found. Regarding thermotolerant enterobacteria, there was no difference between d 2 and d 21 for any groups of animals. On d 21, the animals receiving 4.5 and 6.0 kg/ton of UD additive showed lower population than the PC ($P < 0.05$). For total enterobacteria, piglets from 4.5 treatment showed lower population counts on d 21 compared to baseline. On d 21, animals that received 4.5 treatment had a lower population than the PC ($P < 0.05$). There was no statistical difference ($P > 0.05$) in bacterial culture count for caecal content samples (data not shown).
Table 5
Population (mean ± standard deviation Log CFU/g) of lactic acid bacteria and thermotolerant and total enterobacteria from feces collected from nursery piglets fed ultra-diluted complex feed additive in the diets.

<table>
<thead>
<tr>
<th>Treatments (^1)</th>
<th>PC</th>
<th>NC</th>
<th>4.5</th>
<th>6.0</th>
<th>7.5</th>
<th>9.0</th>
<th>SEM(^2)</th>
<th>P-Value(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid bacteria (Log CFU/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 21(^†)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermotolerant enterobacteria (Log CFU/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total enterobacteria (Log CFU/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>d 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of ultra-diluted additive in the feed.\(^2\) Standard error of the mean. \(^3\)P-value for linear regression. \(^AB\)Statistical difference by the Dunnett test (P < 0.05) for baseline (d 2). \(^*\) Statistical difference by the Dunnett test (P < 0.05) for PC. \(^†\) Linear effect (P < 0.05) of the ultra-diluted additive levels on the lactic acid bacteria (y = −0.0396821x + 9.80066 R²= 0.0795).

Regarding the susceptibility of *E. coli* found in feces from D2 sampling, out of 10 feces collected, only one of these detected the growth of *E. coli*. Bacterial resistance for *E. coli* was observed to eight antimicrobials, which were ampicillin, azithromycin, ciprofloxacin, chloramphenicol, erythromycin, streptomycin, sulfazotrim and tetracycline. In the samples from D21, the highest percentage of resistance was found for the antimicrobial tetracycline (PC; NC; 4.5; 6.0 and 7.5 = 100% resistance), only the last UD
level showed a rate of 85.7% resistance. In the collection of caecal content, a total of 12 *E. coli* were identified, and none was identified in the animals that received the first level of the UD additive. When analyzing the results, a great resistance to antimicrobials is observed. Among the 12 antibiotics used, *E. coli* showed 100% resistance to 4 of them (amoxicillin, erythromycin, streptomycin, and tetracycline).

**Intestinal parameters**

As for the histopathology data, it was possible to observe that all treatments showed less pathological alterations in the duodenum compared to the NC (Table 6). The organ weights and pH of digestive contents showed no statistical difference between treatments.

Table 6

<table>
<thead>
<tr>
<th>Items</th>
<th>PC</th>
<th>NC</th>
<th>4.5</th>
<th>6.0</th>
<th>7.5</th>
<th>9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological alterations</td>
<td>8.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jejunum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological alterations</td>
<td>4.85</td>
<td>12.57</td>
<td>11.00</td>
<td>8.71</td>
<td>5.42</td>
<td>4.71</td>
</tr>
</tbody>
</table>

¹ Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of ultra-diluted additive in the feed. <sup>ab</sup> Different letters on the same row indicate statistical difference by Mann-whitney test (P < 0.05).

**Discussion**

Due to the growth-promoting properties of the antimicrobials, it was expected that the piglets in the positive control group would show some improvement over the negative control regarding performance, which did not occur in the current study. Similar results can be found in the literature, with the negative control animals showing the same performance as those given chlorohydroxyquinoline (Andrade et al. 2016; Gois et al. 2016). The efficacy of these medications varies according to the pig raising conditions, with better results being obtained in commercial farms than in experimental facilities (Sbardella et al. 2016). Furthermore, other studies have shown that chlorohydroxyquinoline did not present positive effects on the performance of weaned piglets (Perina et al. 2014; Cairo et al. 2018). The authors justified that this result was mainly due to the strict sanitary control of the facilities, which resulted in a lower level of challenge for the animals. Regarding the UD additive, our findings do not align with those of Lima et al. (2022), who reported positive results on performance in finisher pigs after 97 d of experimental period. Our study focused on piglets, which possess a less mature intestinal tract compared to adult animals.
(Santos et al. 2022), and this distinction is a possible reason for the discrepancy in results, since some of the UD compounds were similar.

Regarding diarrhea, the results may have different interpretations. The ability of treatments 4.5 and 9.0 to maintain values for all animal sizes suggests that these treatments may be useful in environments where variability in piglet size is common, ensuring a consistent approach in diarrhea prevention. However, treatments PC and NC also exhibited qualities, particularly among large piglets, demonstrating targeted effectiveness. When the results of the overall animals on d 1 to 35 are observed, it is noticeable that the NC treatment presents advantages over all treatments. This outcome once again prompts us to contemplate the conditions under which the animals were being kept. The facilities are unable to fully represent the environment of a commercial farm, since given the prolonged vacancy, the environment lacked the challenges necessary to fully unlock the potential of the additives. It is important to emphasize that the effects of UD remedies manifest themselves when the pathways related to the pathology are triggered (Khuda-Bukhsh 2003). The levels 4.5 and 7.5 of the UD additive also showed satisfactory values, moreover, both levels were equal to or even better than the positive control in certain periods of the experiment. The ability of the UD complex to act on the digestive system is directly linked to the substances present in its composition. Among them are *Silicea terra* and *Enterococcinum*, compounds that have already been used in studies, reducing diarrhea, and improving nutrient digestibility (Fortuoso et al. 2017; Wendt et al. 2023).

It must be noted that the main problem concerning blood analysis is the stress that handling can cause to the animals, which can easily change the values, being the largest source of hematologic variation (Dubreuil et al. 1990). When the animal is handled under high stress, there is an increase of neutrophils, hemoglobin, and other parameters in the blood (Brooks et al. 2022). In our study, neutrophils showed no increase. Regarding hemoglobin, as the UD levels increased, there was a rise in hemoglobin, MCH, and MCHC levels. The UD additive used in the study aims to promote the development of piglets. Although many components of the additive act on the intestinal tract, other components can act on different parts of the body. *Thyreoidinum*, a substance used in our UD complex, has effects on the thyroid and is a possible method of hastening the healing process in primary hypothyroidism (Kiruthiga 2018). Thyroid dysfunctions cause alterations in hemoglobin and MCH (Kawa et al. 2010), leading to a reduction in these parameters in hypothyroidism. The use of *Thyreoidinum* may explain the proportional increase in these parameters. Even so, the values of these variables are within the species standard (Thorn 2000).

Interesting results were found in flow cytometry, with all levels of the UD additive being equal to the positive control for total leukocytes. Different potencies of UD remedies with antineoplastic properties (*Thuja occidentalis, Carcinosinum* and *Ruta graveolens*) have already been tested on mice and the amount of total leukocytes in the groups treated with UD remedies increased compared to the negative control (Remya and Kuttan 2015). For the authors, the gradual rise in leukocytes, reaching its highest point during or shortly after treatment, followed by a gradual decline, signifies the drug-induced boost in white blood cell production. This augmentation enhances the effectiveness of the immune system. Our
study demonstrated that large quantities of the UD additive are also more effective in increasing leukocytes.

Regarding total and thermotolerant enterobacteria, the 4.5 treatment allowed a reduction in the number of colonies compared to the positive control. Ultra-diluted remedies such as *Ignatia* and *Calcarea carbonica* have modulating effects on the microbiota (Vacaras et al. 2023). A substance that most probably could have assisted the piglets was *Lac defloratum* and *Chamomilla*, their potential makes them an effective UD treatment for gastrointestinal disorders (Raak et al. 2019). As for the bacterial population in the feces, comparing d 2 vs. d 21, there was a significant increase in the population of lactic acid bacteria in all treatments. The greater bioavailability of nutrients, due to the adaptation in the consumption of solid diet, could be one of the possible explanations for the observed increase, since lactic acid bacteria are present mainly in the small intestine, where the greatest absorption of nutrients occurs (Collier et al. 2003). Furthermore, the lower population of lactic acid bacteria in piglets on PC compared to the other treatments is directly related to the broad-spectrum property of the antibiotic, which influences both populations of beneficial and harmful bacteria (Gois et al. 2016).

Regarding bacterial resistance, tetracycline showed the highest rate among the 12 antimicrobials tested, reaching 100% resistance in many cases and presenting as the lowest level of resistance 85.7%. In the same proposal, Moredo et al. (2017) described the high resistance (62%) of *E. coli* to antibiotics commonly used in pig farming, highlighting resistance to ampicillin, streptomycin, chloramphenicol, and tetracycline. The same occurs with the study of Brito and Tagliari (2000), when finding less than 30% of sensibility to the antimicrobials streptomycin, tetracyclines, and chloramphenicol. The present study corroborates with both studies described, being the resistance values of *E. coli* even higher for the antimicrobials tested. It is important to highlight that UD additives do not promote bacterial resistance, which is one of their major advantages (Braccini et al. 2019).

The finding that the UD levels and the positive control showed lower pathological alterations in the duodenum compared to the negative control reveals that both additives can act positively in the context of histopathologies. The UD complex provided to the treated animals contained *Colibacillinum*, a substance already used in a study demonstrating the improvement it causes in the intestinal tract, leading to a lower incidence risk of digestive problems (Da Silva et al. 2021b). The statement for UD remedies presenting these benefits is related to the electrostatic model of homeopathy, which explains how UD complexes can have effects even when highly diluted (Shahabi and Borneman 2022). For chlorohydroxyquinoline, by reducing pathogenic bacteria, the antibiotic promotes microbiome control, resulting in less production of toxins that would affect the intestinal mucosa (Budiño et al. 2005). Besides its strengths, the analysis had also limitations. We can cite the initial weight of the piglets, which, even after randomization, presented disparity between treatments. This inaccuracy was fixed by adding the initial weight as a covariate in the statistical model. In addition, a point that should also be considered is that the animals did not go through challenging situations, which did not allow the additives to show their full potential.
Conclusions

With the results of the present study, we accept the hypothesis that the UD complex improves the intestinal health of piglets in the nursery phase. Our study showed that the animals given 4.5 kg/ton of UD complex provided equal or better results than the positive control (basal diet + 120 mg/kg of chlorohydroxyquinoline) regarding diarrhea, also resulting in lower populations of total and thermotolerant enterobacteria and higher populations of lactic acid bacteria in the feces compared to PC. In relation to the NC (basal diet without additives), the 4.5 treatment exhibited fewer histopathological alterations in the duodenum and a higher quantity of total leukocytes in the flow cytometry analysis. None of the treatments influenced performance. We conclude that the first level of the UD complex additive (4.5 kg/ton) may be a good alternative for replacing chlorohydroxyquinoline in piglet diets.

Declarations

Acknowledgments

None.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contribution

G. Z. de Paula: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. I.C.C. Bez: Investigation, Methodology, Writing – review & editing. L. F. C. Garrido: Investigation, Methodology. C. C. Rodrigues: Data curation, Formal analysis, Investigation, Methodology. A. C. F. de Oliveira: Conceptualization, Data curation, Methodology, Writing – original draft. P. E. Rupolo: Conceptualization, Methodology. L. B. de Azevedo: Conceptualization, Methodology. E. M. E. Hernandez: Conceptualization, Methodology, Writing – review & editing. J. L. Genova: Conceptualization, Formal analysis, Methodology, Writing – review & editing. S. H. Weber: Formal analysis, Writing – review & editing. P. L. O. Carvalho: Conceptualization, Methodology, Resources, Writing – review & editing. L. B. Costa: Conceptualization, Investigation, Methodology, Supervision, Resources, Writing – review & editing.

Data Availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval**

The study was approved by the Ethics Committee on Animal Use (CEUA) of PUCPR under protocol number: 01720 (2nd version). The animals were only handled when needed and by trained and qualified personnel. The piglets were not subjected to any unnecessary discomfort throughout the experimental period. There was no loss of animals throughout the entire experiment.

**Consent to participate**

Not applicable.

**Consent to publish**

Not applicable.

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Figures
Quantitative results in percentage of cells, in relation to the total number of circulating leukocytes, for phagocytic monocytes (A), quantitative results regarding the degree of activity for phagocytic monocytes (B), and quantification of total undifferentiated leukocytes (C) for analysis of the phagocytic profile by flow cytometry for nursery piglets fed ultra-diluted complex feed additive in the diets.

¹ Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of ultra-diluted additive in the feed. ab Different letters represent statistical differences according to the Kruskal-Wallis Test followed by Dunn's multiple comparisons post-test (P<0.001). * Statistical difference by the Dunnett test (P<0.05) for PC. † Linear effect (P<0.001) of the ultra-diluted additive levels on phagocytic activity of monocytes (y= -
0.0843791x + 12.1144 R² = 0.7534). †† Linear effect (P<0.05) of the ultra-diluted additive levels on total leukocytes (y = 0.044186x + 5.45453 R² = 0.3990)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx