

## Research Article

# Effects of Chlorophyll-Derived Efflux Pump Inhibitor Pheophorbide *a* and Pyropheophorbide *a* on Growth and Macrolide Antibiotic Resistance of Indicator and Anaerobic Swine Manure Bacteria

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Natural plant compounds, such as the chlorophyll a catabolites pheophorbide a (php) and pyropheophorbide a (pyp), are potentially active in the gastrointestinal tracts and manure of livestock as antimicrobial resistance-modifying agents through inhibition of bacterial efflux pumps. To investigate whether php, a known efflux pump inhibitor, and pyp influence bacterial resistance, we determined their long-term effects on the MICs of erythromycin for reference strains of clinically relevant indicator bacteria with macrolide or multidrug resistance efflux pumps. Pyp reduced the final MIC endpoint for *Staphylococcus* (*S.*) *aureus* and *Escherichia* (*E.*) *coli* by up to 1536 and 1024  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$  or 1.4- and 1.2-fold, respectively. Estimation of growth parameters of *S. aureus* revealed that pyp exerted an intrinsic inhibitory effect under anaerobic conditions and was synergistically active, thereby potentiating the effect of erythromycin and partially reversing high-level erythromycin resistance. Anaerobe colony counts of total and erythromycin-resistant bacteria from stored swine manure samples tended to be lower in the presence of pyp. Tylosin, php, and pyp were not detectable by HPLC in the manure or medium. This is the first study showing that pyp affects growth and the level of sensitivity to erythromycin of *S. aureus*, *E. coli*, and anaerobic manure bacteria.

## 1. Introduction

Agricultural antimicrobial drug use is regarded a major driver of one of today's foremost global public health challenges: more frequent clinical antimicrobial treatment failures due to resistant microorganisms [1–4]. In the U.S. swine and other livestock production, much of the use of antimicrobials is nontherapeutic and/or occurs in the form of free-choice medicated feeds and water [1, 2, 5]. This results in exposures of the animals' gastrointestinal tract and waste microbiota to inconsistent, often sublethal or subinhibitory concentrations [1, 6]. As even ultralow ( $\ll$  MIC) antimicrobial concentrations can confer a selective pressure towards the persistence of resistance in microbial communities [7–12], induction of gut and waste microbial resistance is an inevitable collateral effect of oral antimicrobials in animal agriculture [13–16].

More than 335 million tons (dry weight) of manure, a valuable fertilizer, are produced by U.S. agriculture per year [17]. Soil amendment with manure presents a significant route of transmission of antimicrobial resistance from livestock bacteria to human clinical pathogens [4, 18–24]. Of greatest concern in this context is the increasing prevalence of multidrug resistance (MDR), especially in Gram-positive pathogens, such as *Staphylococcus* (*S.*) *aureus*, *Streptococcus pneumoniae*, and enterococci [25, 26]. MDR is frequently caused by bacterial efflux pumps that primarily confer broader, compound nonspecific functions unrelated to antimicrobials and are ubiquitous among bacteria [22, 25, 27–29]. MDR is a baseline resistance for the emergence of further resistance mechanisms, and, due to its physiological determination, it naturally persists [29–32].

Plants have recently been recognized as an important source for the discovery and development of compounds with efflux pump inhibitor (EPI) activity [26, 33]. The pharmacological inhibition of active efflux by adjuvant application of phytogetic EPIs presents a promising strategy for the mitigation of bacterial MDR [29, 31–34]; however, due to intrinsic toxicity among other factors, so far no EPI/antimicrobial drug combination is used clinically [29, 35]. In this study, we aimed at investigating whether pheophorbide *a* (php) and pyropheophorbide *a* (pyp), catabolites of the major green plant pigment chlorophyll *a* [36], would influence resistance of clinically relevant indicator bacteria and anaerobic bacteria from stored swine manure to the macrolide antibiotics erythromycin and tylosin.

Php and pyp can be ingested preformed in various green foods and feedstuffs [37–41], or in humans swine and other nonruminant livestock species can be produced from chlorophyll *a* or chlorophyllide *a* by acidity in the stomach (php) and microbial enzymes in the large intestine (php; pyp) (Figure 1) [36, 42–44]. Prior research further indicates that despite apparently undergoing an enterohepatic circulation [45–48], php and pyp are mostly excreted with no change and hence appear in a dietary concentration-dependent manner as the predominant chlorophyll catabolites in feces [43, 49, 50]. The EPI activity of php was first deduced from its berberine- and norfloxacin-potentiating, antimicrobial effect against *S. aureus* bearing the NorA MDR pump [51]. Later on, this effect was extended to ciprofloxacin and other strains of *S. aureus*, *S. epidermidis*, *Escherichia (E.) coli*, and *Pseudomonas aeruginosa* [52]. An EPI activity of pyp has not been reported at the time of writing.

Discovered in 1952 as the first macrolide antibiotic, erythromycin is now the representative of its class. Although its application in human medicine has diminished over time due to increased bacterial resistance [53], it is still an important alternative against human respiratory and food-borne infections [31, 54–56]. In U.S. agriculture, erythromycin is approved for in-feed application for swine, cattle, and poultry. It is also routinely used in the corn ethanol industry, entailing the risk of inadvertent exposure of livestock to residues in distillers' by-products, such as dried distillers' grains with solubles (DDGS) [57–59]. Erythromycin is a 14-membered ring macrolide and a narrow-spectrum antibiotic, as it is effectively extruded by MDR efflux pumps in many Gram-negative bacteria [60]. In Gram-positive bacteria, efflux is the main mechanism of erythromycin resistance besides rRNA target modification [61]. The related veterinary 16-membered ring macrolide tylosin can spur bacterial resistance to erythromycin [62–64]. It is estimated to be quantitatively the second most common in-feed antimicrobial in the U.S. swine production [5] and is also approved for cattle and poultry [59].

## 2. Materials and Methods

**2.1. Bacterial Reference Strains.** *S. aureus* ATCC 29213, *Enterococcus (Ent.) faecalis* ATCC 29212, *Salmonella (Sal.) enterica* serovar Typhimurium ATCC 14028, and porcine *E. coli*

strains P286.10.99.C3 and P475.10.99.C3 were used for the determination of the effects of php and pyp on the MIC of erythromycin. The previously reported MIC of erythromycin ( $\mu\text{g mL}^{-1}$ ) was 0.25–0.5 (*S. aureus*), 2–256 (*Ent. faecalis*), 128–>256 (*Sal. Typhimurium*), and >256 (*E. coli*) [65–67]. The strains are best characterized as possessing the following MDR and macrolide efflux pumps: NorA (*S. aureus*), EmeA (*Ent. faecalis*), AcrAB-TolC (*S. Typhimurium*), and Mef(B) (*E. coli*) [27, 67].

**2.2. Serial Passage Selection for Induced Erythromycin Resistance.** Cultures with induced resistance to erythromycin were generated by serial passage of the parental (naïve) reference strains through progressively escalating doses of erythromycin. Starting at half the lower reported MIC, the strains were repeatedly subcultured depending on growth in 10 mL volumes of Iso-Sensitest broth (ISB, Oxoid, Ontario, Canada) with erythromycin (E6376, Sigma-Aldrich, St. Louis, MO, USA) for two (*Ent. faecalis*) to eight (*Sal. Typhimurium*) weeks under static aerobic conditions at 37°C. Next, the strains were subcultured aerobically as well as anaerobically in an anaerobic chamber (Coy Laboratories, Ann Arbor, MI, USA) in a 96% carbon dioxide, 4% hydrogen atmosphere for another two (*Ent. faecalis*) to four (*Sal. Typhimurium*) weeks—until no further increase of the tolerated erythromycin concentration was obtained by repeated attempts.

**2.3. MICs of Erythromycin over Time.** The MICs of erythromycin were determined for the naïve and induced reference strains under aerobic and anaerobic conditions by using the Clinical and Laboratory Standards Institute-recommended broth macrodilution method [65]. The antibiotic range of the duplicate assays routinely comprised four and maximally ten concentrations of erythromycin in ISB in doubling ( $0.125\text{--}1024 \mu\text{g mL}^{-1}$ ) or incremental (by  $512 \mu\text{g mL}^{-1}$ ;  $1024\text{--}5632 \mu\text{g mL}^{-1}$ ) steps. Assays were supplemented with either 0 (ethanol control), 0.5, or 50  $\mu\text{g}$  php or pyp (both from Frontier Scientific, Logan, UT, USA)  $\text{mL}^{-1}$ . 1:100 dilutions in ISB of 0.5 McFarland-equivalent aqueous suspensions of cells grown overnight on antibiotic-free agar medium (ISB + 8 g antibiotic-free agar-agar (Merck, Darmstadt, Germany)  $\text{L}^{-1}$ ) were used as inocula. Incubation was performed for seven or 14 (induced *S. aureus* under anaerobic conditions) days protected from light and as described above. The MIC range defined as between the maximum tolerated concentration (MTC, highest concentration of erythromycin with visible growth in both duplicate assays) and the MIC endpoint (lowest concentration of erythromycin with no visible growth in both duplicate assays) was recorded at 20 h, and then every 24 h. Experiments were repeated twice.

**2.4. Growth of *S. aureus*.** The effect of pyp on the growth kinetics of naïve and erythromycin-induced cultures of *S. aureus* was analyzed using a spectrophotometric method. Aliquots of overnight cultures in antibiotic-free ISB diluted to approximately  $10^7 \text{CFU mL}^{-1}$  [68] were inoculated into five volumes of prewarmed ISB with 0, 0.0625 (naïve) or

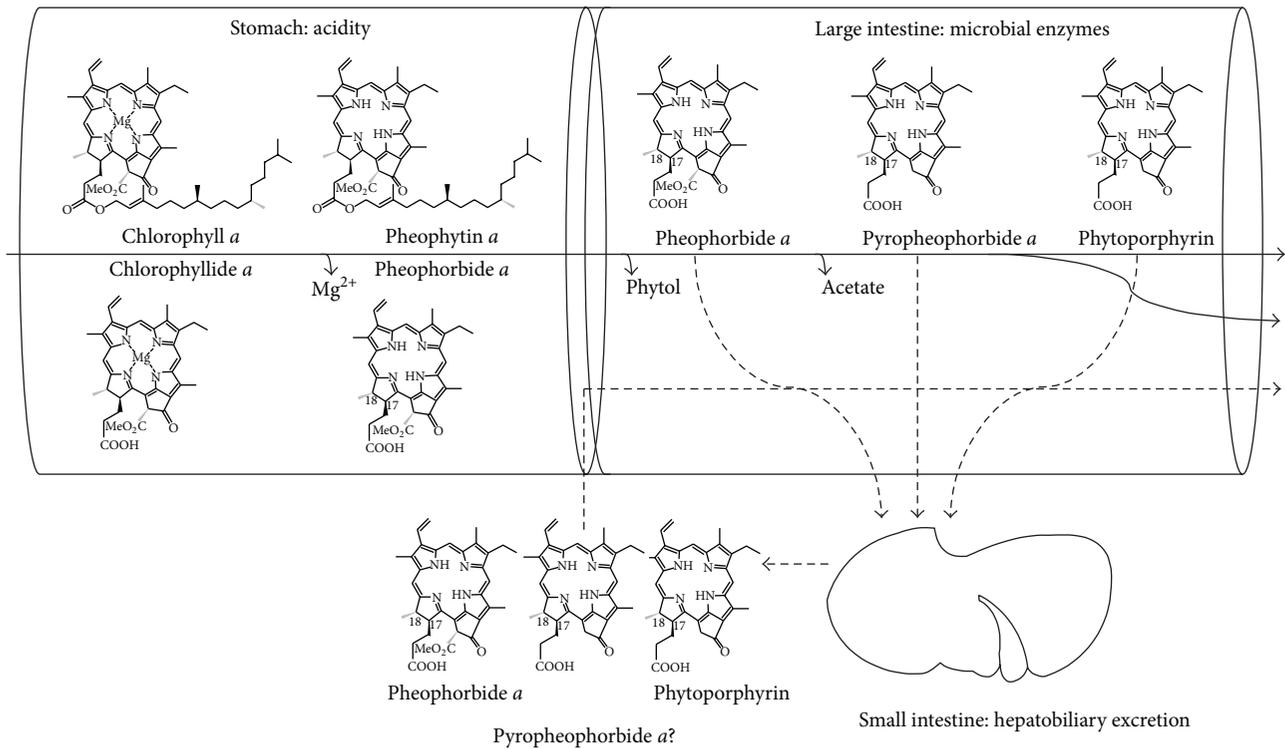


FIGURE 1: Enterohepatic metabolism of dietary chlorophyll *a* and its catabolic derivatives in humans and nonruminant livestock.

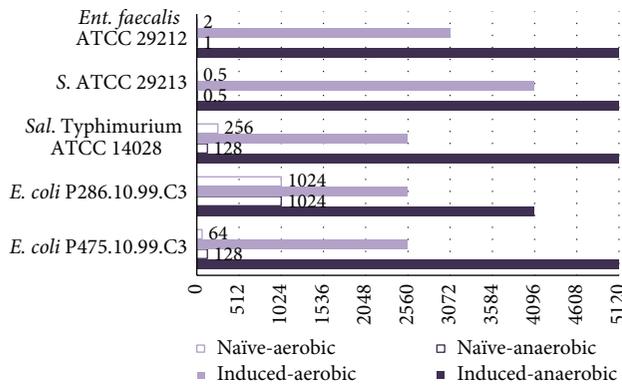


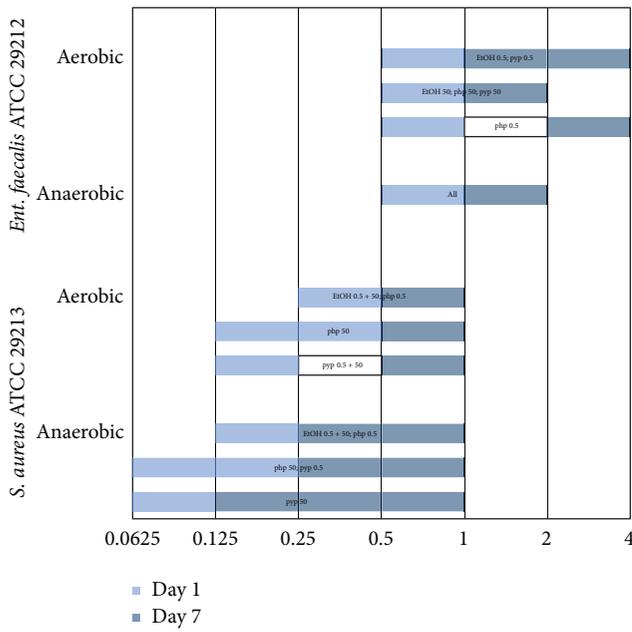
FIGURE 2: Overall maximum tolerated concentration of erythromycin ( $\mu\text{g mL}^{-1}$ ) and induction factor for reference strains of indicator bacteria.

2048 (induced)  $\mu\text{g erythromycin mL}^{-1}$  and supplemented with 0 (ethanol control), 0.5 or 50  $\mu\text{g pyp mL}^{-1}$ . The assays were incubated as described above, and the culture OD was initially measured at 660 nm with a Beckman DU-600 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and then measured every 60 min over the entire incubation period (6.5 h and 9.5 h for naïve cultures under aerobic and anaerobic conditions, resp.) or every 120 min between 24.5 and 36.5 h of incubation (induced cultures under anaerobic conditions). The total yield (maximum OD–initial OD) was calculated, as well as the average and maximum/minimum generation time, specific growth rate, and division rate [69].

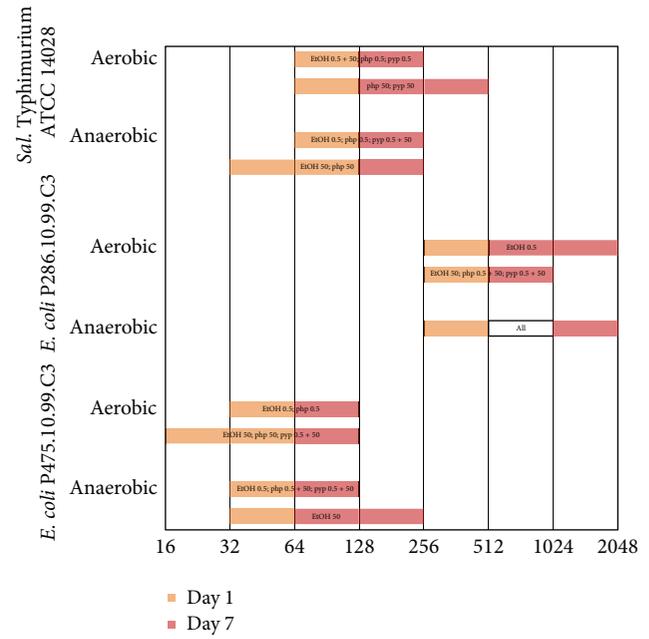
All experiments were performed in duplicate and repeated three times. Results displayed in Figures 3, 4, and 5 are means plus or minus standard deviations to show the variability and reproducibility of the data.

**2.5. Collection and Preparation of Swine Manure Samples.** A swine farm near Peoria, IL, was used as the source of manure pit (slurry) samples. Samples from a concrete underfloor manure storage pit were collected at up to 2.4 meters (bottom of the pit) depth using sterile screw-capped 50 mL plastic tubes attached to a Tank Sampler (NASCO, Fort Atkinson, WI, USA). During the two-week sampling period, 875–668 finisher pigs of 21–23 weeks of age that were fed a corn-soybean-DDGS-based diet were kept in two barns above the manure pit. The pit, with a full capacity volume of approx.  $1.8 \times 10^6$  L, had been emptied on day 31 of an “all out phase” of the farm’s all in-all out by building production system. This 31st day was two days before the new pigs were introduced and 94, 101, and 108 days prior to the three days of sampling. Samples were protected from light while returning to the laboratory (approx. 30 min) and there immediately taken into a Coy anaerobic chamber. Approximately 25 mL manure was added to 2.5 g sterilized glass beads (Novagen ColiRollers Plating Beads, Merck) in a sterile 50 mL tube, vortexed gently for 10–20 s, and allowed to stand for 15 min. Sample collection, preparation, and subsequent analytical steps were repeated twice.

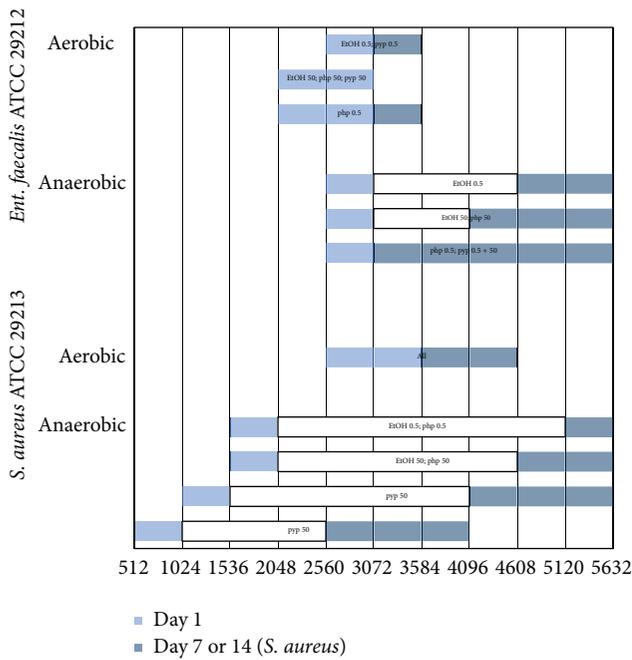
**2.6. Data on Zootechnical Additives and Application of Antimicrobials.** Data concerning the use of feed additive premixes,



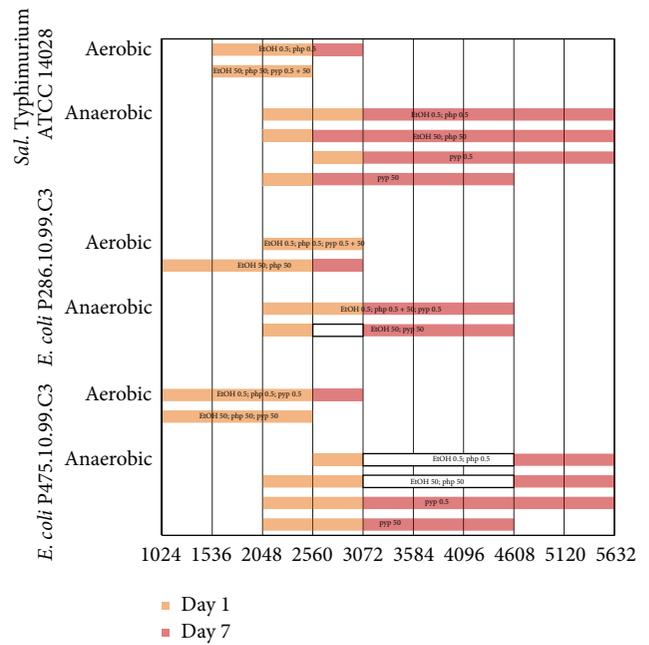
(a)



(b)



(c)



(d)

FIGURE 3: Initial (day 1) and final (day 7 or 14) MIC ranges of erythromycin ( $\mu\text{g mL}^{-1}$ ) for naïve (a), (b) and induced (c), (d) Gram-positive and -negative bacterial reference strains. Assays were supplemented with either 0 (EtOH 0.5, EtOH 50), 0.5, or 50  $\mu\text{g}$  pheophorbide *a* (pyp 0.5, pyp 50) or pyropheophorbide *a* (pyp 0.5, pyp 50)  $\text{mL}^{-1}$ .

including those with antimicrobials, and of antimicrobials by other routes of administration (in water, by injection) were obtained by request from the farm staff. The total amounts of administered antimicrobial compounds were quantified between the day of pit emptying (day 0) and the last day of sampling (day 108).

2.7. Total Cell Counts of Microorganisms from Stored Swine Manure. Microorganisms present in the manure samples were enumerated by direct microscopic examination using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA, USA). A 10 mL-volume of a 1 : 10 dilution of the prepared manure supernatant in a detergent saline solution

(9 g sodium chloride (NaCl) L<sup>-1</sup>, SDS and dibasic potassium phosphate until foaming and pH 7.2–7.4, resp.) [70, 71] was added to 1 g of glass beads, suspended by gentle vortexing for 10–20 s, and allowed to stand for 15 min. Aliquots of the supernatant were mixed 1:1 with 0.1 M hydrogen chloride (HCl), and 2  $\mu$ L of the resulting mixture was used to fill the counting chamber. At least 400 cells counted under an Olympus BX51 phase-contrast microscope (Olympus, Center Valley, PA, USA) at  $\times 2000$  magnification with oil immersion were taken into account for the calculation of the number of cells per mL of original sample [70].

**2.8. Viable Cell Counts of Anaerobic Bacteria from Stored Swine Manure.** To determine the numbers of anaerobic bacteria by viable plate counts, a 40 mL volume of a 1:100 dilution of the prepared manure supernatant in a sterile, anaerobic phosphate-buffered saline solution (PBS, 0.15 M NaCl, 0.07 M sodium phosphate, pH 7.0) was added to 2.5 g of sterilized glass beads in a sterile 50 mL tube and vortexed gently for 10–20 s. Further serial dilutions in PBS were performed on this suspension, and aliquots were plated onto anaerobically prepared [72] modified swine manure slurry medium (Slurry medium [73] containing 50% (v/v) clarified swine manure slurry and supplemented with 1 g of porcine gastric mucin (M2378, Sigma-Aldrich) and 50 mL swine feed hydrolysate (from approx. 1 g swine feed, prepared as described below) L<sup>-1</sup>). The nonselective, habitat-simulating slurry medium has been proven to yield the highest viable counts of anaerobic swine manure storage organisms, predominantly *Firmicutes* bacteria [73]. Pyp (10  $\mu$ g mL<sup>-1</sup>) was included individually and in combination with erythromycin (10  $\mu$ g mL<sup>-1</sup>) or tylosin (10  $\mu$ g mL<sup>-1</sup>, T-6134, Sigma-Aldrich) in some of the media. Plates were incubated anaerobically in a Coy anaerobic chamber at 37°C, and growth on plates was examined regularly for 4 weeks and numbers of colonies enumerated.

**2.9. Preparation of Hydrolyzed Swine Feed for Swine Manure Slurry Medium.** A pepsin-pancreatin enzymatic hydrolysis was performed on a sample of nonmedicated swine feed obtained from the swine farm used for manure sampling following a previously described protocol [74, 75]. This pepsin-pancreatin enzymatic hydrolysis mimics the endogenous (host-derived) digestion in the porcine upper gastrointestinal tract. 1 g, aliquots of the fine grained feed were mixed with 25 mL of phosphate buffer solution (0.1 M, pH 6.0) and 10 mL 0.2 M HCl. The pH was adjusted to 2.0 with 1 M HCl and 1 M sodium hydroxide (NaOH), and 1 mL of a freshly prepared porcine pepsin solution containing 25 mg pepsin (516360, Calbiochem, San Diego, CA, USA) mL<sup>-1</sup> was added. The mixtures were transferred into 50 mL glass serum bottles with rubber stoppers, and the crimp-sealed bottles were placed for 2 h in a gently rotating stainless steel beaker reactor system equipped with infrared heating (Labomat BFA-12 v200, Werner Mathis, Concord, NC, USA) set to 39°C. After the pepsin hydrolysis, 10 mL of phosphate buffer solution (0.2 M, pH 6.8) and 5 mL 0.6 M NaOH were added to the hydrolysis mixtures. The pH was adjusted to 6.8,

and 1 mL of a freshly prepared porcine pancreatin solution containing 100 mg pancreatin (P-1750, Sigma) mL<sup>-1</sup> was added. The hydrolysis step using the Labomat reactor system was repeated at 39°C for 4 h. Finally, the hydrolysates were filtered through three layers of cheesecloth, and the filtrate was stored at –18°C for later use.

**2.10. Concentrations of Php, Pyp, and Tylosin in Stored Swine Manure, Hydrolyzed Feed, and Agar Medium.** Php, pyp, and tylosin concentrations in the stored swine manure, hydrolyzed swine feed, and unsupplemented agar medium were determined on a wet weight basis using HPLC analysis of methanol extracts from dried samples. The concentrations of erythromycin were not analyzed, as there had been no history of erythromycin application on the farm used for manure sampling. To obtain dry materials and wet weight/dry weight ratios, the remaining manure sample and unautoclaved feed hydrolysate were spread out evenly in a plastic tray, weighed, and put in a convection oven (Blue M Electric Company, Blue Island, IL, USA) until they dry (approx. 48 h) at 40 and 60°C, respectively. 2 agar plates of the unsupplemented medium were weighed and then freeze-dried in 50 mL tubes with perforated lids using a Labconco Bulk Tray Dryer (Labconco, Kansas City, MO, USA) for approximately 48 h. After reweighing, the dried samples were ground with mortar and pestle, and 3  $\times$  0.3  $\pm$  0.01 g aliquots of the resulting powders were mixed with 2 mL HPLC grade methanol in 20 mL disposable scintillation vials. The vials were capped, wrapped with sealing tape, sonicated for 30 min in a Branson 2510 ultrasonic cleaner (Branson Ultrasonics, Danbury, CT, USA), and allowed to stand overnight at room temperature and to be protected from light. Aliquots of the extraction supernatants were passed through a 0.45  $\mu$ m nylon chromatography syringe filter (Fisher Thermo Scientific, Pittsburgh, PA, USA) into 1.5 mL screw-thread vials (SUN-Sri, Rockwood, TN, USA) for HPLC analysis for both pheophorbides, php and pyp, and tylosin.

HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector, running under Shimadzu LCSolutions version 1.22 chromatography software, Shimadzu, Columbia, MD, USA). The column used was an Inertsil ODS-3 reverse phase C-18 column (5  $\mu$ M, 250  $\times$  4.6 mm, Varian Lake Forrest, CA, USA). For php and pyp analysis, the initial conditions were from 50% acetonitrile and 50% water with 0.025% trifluoroacetic acid (TFA) at a flow rate of 1 mL per minute. The effluent was monitored at 410 nm on the photodiode array (PDA) detector. After injection (typically 25  $\mu$ L), the column was developed to 100% acetonitrile and 0.025% TFA in a linear gradient over 30 minutes. Standard curves based on micrograms injected were prepared from a preparatory standard of php from chlorophyll *a* (C5753, Sigma-Aldrich) [76] and pure standard of pyp purchased commercially from Frontier Scientific. For tylosin analysis, the initial conditions were 10% acetonitrile, 90% water, with 0.025% TFA at a flow rate of 1 mL per minute. The effluent was monitored at 285

and 210 nm on the PDA detector. After injection (typically 25  $\mu\text{L}$ ), the column was held at the initial conditions for 5 minutes and then developed to 100% acetonitrile and 0.025% TFA in a linear gradient over 30 additional minutes. Standard curves based on  $\mu\text{g}$  tylosin injected were prepared from commercially obtained standard (Sigma-Aldrich).

### 3. Results

**3.1. Serial Passage Selection for Induced Erythromycin Resistance.** Figure 2 shows the MTCs of erythromycin for the naïve strains and after serial passage selection in progressively increasing erythromycin concentrations for 4 (*Ent. faecalis*) to 12 weeks (*Sal. Typhimurium*). The level of parental but not of induced resistance was higher in the Gram-negative strains (parental: 64–1024, induced: 2560–5120  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$ ) than in the Gram-positives (parental: 0.5–2, induced: 3072–5120  $\mu\text{g}$   $\text{mL}^{-1}$ ). For all strains, induction was higher under anaerobic than under aerobic conditions, with the maximum factor being 10240 (*S. aureus*).

**3.2. MICs of Erythromycin over Time.** The most notable effects on the MICs of erythromycin were observed in pyp-supplemented assays of *S. aureus*. The initial MIC range of naïve cultures of *S. aureus* was 2-fold lower both under aerobic (0.25–0.5 in controls versus 0.125–0.25 in assays with pyp at 0.5 and 50  $\mu\text{g}$   $\text{mL}^{-1}$ ) and anaerobic (0.125–0.25 in control versus 0.0625–0.125 in assays with pyp at 50  $\mu\text{g}$   $\text{mL}^{-1}$ ) conditions (Figure 3(a)). For induced cells under anaerobic conditions, the initial MIC range was reduced by 512  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$  or 1.3–1.5-fold (pyp at 0.5  $\mu\text{g}$   $\text{mL}^{-1}$ ) to 1024  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$  or 2–3-fold (pyp at 50  $\mu\text{g}$   $\text{mL}^{-1}$ ) (Figure 3(c)). Using the higher concentration of pyp, this initial effect was stable over time (up to 14 days) with a reduction of the final MIC end point by 1536  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$  or 1.4-fold (Figure 3(c)). Pyp was more effective against *S. aureus* under anaerobic conditions, and insofar as, in naïve cultures, the increase of MIC endpoints to the final value of 1 occurred later (day 7) than under aerobic conditions (days 3–4) (Figure 3(a), details not depicted), and insofar as no differences occurred on aerobically grown-induced cultures (Figure 3(c)). The effect of pyp (at 50  $\mu\text{g}$   $\text{mL}^{-1}$ ) was less on *E. coli* P475.10.99.C3 (reduction of the final MIC endpoint of induced cultures by 1024  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$  or 1.2-fold under anaerobic conditions, Figure 3(d)) and not significant on the other strains. MICs in pyp-supplemented assays were unaffected or insignificantly different (Figures 3(a)–3(d)).

**3.3. Growth of *S. aureus*.** In general, results from the spectrophotometric analysis of the growth kinetics of *S. aureus* confirmed a concentration-dependent inhibitory effect of pyp. In naïve cultures under aerobic conditions, the growth-retarding effect of 0.0625  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$  was synergistically potentiated by 50  $\mu\text{g}$  pyp  $\text{mL}^{-1}$ , resulting in a significantly longer generation time  $g$  (minimum  $g$  ( $g_{\min}$ ) = 54.6  $\pm$  3.7 min, average  $g$  ( $g_{\text{ave}}$ ) = 71.1  $\pm$  2.0 min) and reduced growth rate  $k$  (maximum  $k$  ( $k_{\max}$ ) = 0.33  $\pm$  0.02  $\text{h}^{-1}$ ,

average  $k$  ( $k_{\text{ave}}$ ) = 0.25  $\pm$  0.01  $\text{h}^{-1}$ ) compared with cultures that contained only erythromycin ( $g_{\min}$ ,  $g_{\text{ave}}$ ,  $k_{\max}$  and  $k_{\text{ave}}$  = 49.3  $\pm$  0.9 min, 61.9  $\pm$  1.6 min, 0.37  $\pm$  0.01  $\text{h}^{-1}$  and 0.29  $\pm$  0.01  $\text{h}^{-1}$ , resp.) (Figures 4(a), 4(b), and 5(a)). Under anaerobic conditions, 0.5 and 50  $\mu\text{g}$  pyp  $\text{mL}^{-1}$  exerted an additive to synergistic effect in combination with 0.0625  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$ . Using the higher pyp concentration significantly increased  $g_{\min}$  and  $g_{\text{ave}}$  (from 63.7  $\pm$  5.0 to 73.1  $\pm$  3.5 min and 79.7  $\pm$  2.1 to 85.9  $\pm$  1.5 min, respectively, Figure 4(a)) and reduced  $k_{\max}$  and  $k_{\text{ave}}$  (from 0.29  $\pm$  0.02 to 0.25  $\pm$  0.01  $\text{h}^{-1}$  and 0.23  $\pm$  0.01 to 0.21  $\pm$  0.00  $\text{h}^{-1}$ , resp., Figure 4(b)), as reflected by a lower total yield (0.84  $\pm$  0.03 versus 0.68  $\pm$  0.08, Figures 4(c) and 5(b)). Furthermore, pyp at 50  $\mu\text{g}$   $\text{mL}^{-1}$  exhibited an intrinsic growth-inhibitory effect, thereby leading to an increased  $g_{\text{ave}}$  (from 74.1  $\pm$  1.3 to 79.4  $\pm$  1.9 min, Figure 4(a)) and reduced  $k_{\text{ave}}$  (from 0.24  $\pm$  0.00 to 0.23  $\pm$  0.01  $\text{h}^{-1}$ , Figure 4(b)) and total yield (from 1.07  $\pm$  0.02 to 0.85  $\pm$  0.02, Figures 4(c) and 5(b)). In erythromycin-induced cultures, the combinatory effect of 0.5 and 50  $\mu\text{g}$  pyp  $\text{mL}^{-1}$  was consistently synergistic and thus partially reversed high-level erythromycin resistance (Figure 5(c)).  $g_{\min}$  (319.8  $\pm$  12.3 min) and  $g_{\text{ave}}$  (342.3  $\pm$  14.2 min) were significantly longer and  $k_{\max}$  (0.06  $\pm$  0.00  $\text{h}^{-1}$ ),  $k_{\text{ave}}$  (0.05  $\pm$  0.00  $\text{h}^{-1}$ ), and the total yield (0.40  $\pm$  0.06) significantly reduced in the presence of both 50  $\mu\text{g}$  pyp  $\text{mL}^{-1}$  and 2048  $\mu\text{g}$  erythromycin  $\text{mL}^{-1}$  relative to cultures exposed to only erythromycin ( $g_{\min}$ ,  $g_{\text{ave}}$ ,  $k_{\max}$ ,  $k_{\text{ave}}$ , and total yield = 273.7  $\pm$  6.0 min, 299.0  $\pm$  6.0 min, 0.07  $\pm$  0.00  $\text{h}^{-1}$ , 0.06  $\pm$  0.00  $\text{h}^{-1}$  and 0.59  $\pm$  0.03, resp.) (Figures 4(a)–4(c), and 5(c)).

**3.4. Data on Zootechnical Additives and Application of Antimicrobials.** The antimicrobial compounds applied between the day when the manure storage pit was emptied and the last day of the sampling period were oxytetracycline hydrochloride, lincomycin hydrochloride, tiamulin hydrogen fumarate, and penicillin G (benzylpenicillin) procaine in the following decreasing quantities: 6.90 kg oxytetracycline hydrochloride by feed, water, and injection, 5.82 kg lincomycin hydrochloride by feed and injection, 0.31 kg tiamulin hydrogen fumarate by feed and 0.15 kg penicillin G procaine by injection. There was no history of the use of erythromycin on the farm, and the last contamination of the manure pit with tylosin occurred 31 days before the pit emptying. Other applied zootechnical feed additives with antimicrobial activity were copper sulfate (4.93 kg) and zinc oxide (67.77 kg). Besides antimicrobials, 78.47 kg of an algae-containing prebiotic feed additive (Intivate, Alltech, Ames, IA, USA) as well as 90.04 kg of MicroSource S, a direct-fed microbial feed supplement containing  $1.47 \times 10^8$  spores of *Bacillus* (*B.*) *subtilis* and *B. licheniformis*  $\text{g}^{-1}$  (DSM, Parsippany, NJ, USA) [77] were applied. Intivate had first been introduced 9 months and 10 days before the pit emptying, and the amount used during this time was 53.07 kg. Only lincomycin hydrochloride (approx. 0.12 kg by injection) and MicroSource S (17.24 kg) were administered during the two-week sampling period.

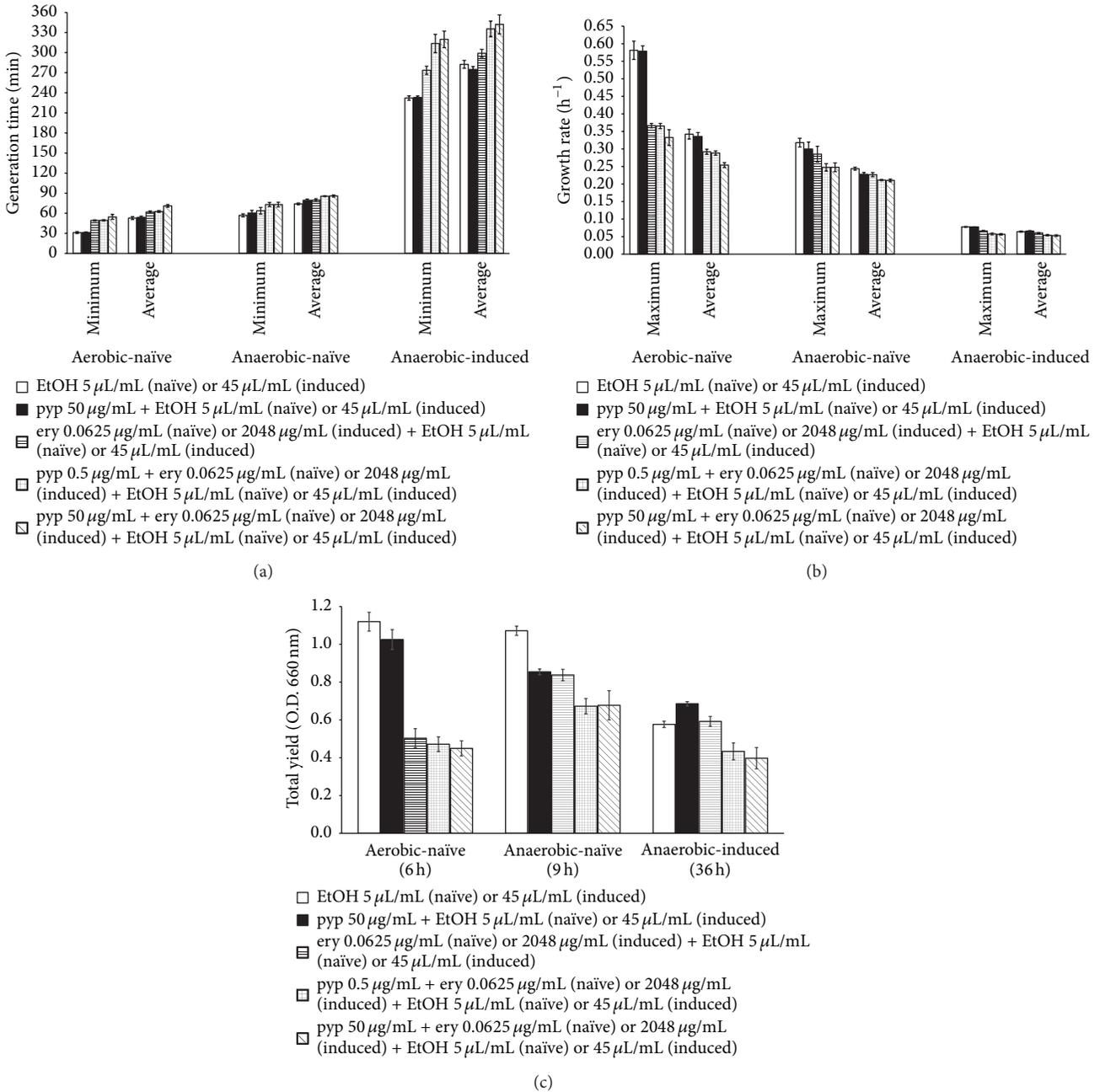


FIGURE 4: Effects of pyrophephorbide *a* (pyp, 0.5 and 50 µg mL<sup>-1</sup>) alone and in combination with erythromycin (ery, 0.0625 and 2048 µg mL<sup>-1</sup>) on growth parameters ((a) generation time; (b) growth rate; (c) total yield) of naïve and erythromycin-induced *S. aureus* ATCC 29213 under aerobic and anaerobic conditions. Values are averages and standard deviations from three repeated experiments.

TABLE 1: Culture counts of anaerobic bacteria from stored swine manure.

|      | Viable counts: swine slurry medium* |                            |                        |                            |                        |                            |
|------|-------------------------------------|----------------------------|------------------------|----------------------------|------------------------|----------------------------|
|      | +H <sub>2</sub> O/EtOH              |                            | +erythromycin          |                            | +tylosin               |                            |
| -pyp | 1.26 × 10 <sup>9</sup>              | (±0.13 × 10 <sup>9</sup> ) | 1.01 × 10 <sup>9</sup> | (±0.30 × 10 <sup>9</sup> ) | 1.07 × 10 <sup>9</sup> | (±0.15 × 10 <sup>9</sup> ) |
| +pyp | 8.99 × 10 <sup>8</sup>              | (±1.69 × 10 <sup>8</sup> ) | 8.90 × 10 <sup>8</sup> | (±2.66 × 10 <sup>8</sup> ) | 1.09 × 10 <sup>9</sup> | (±0.22 × 10 <sup>9</sup> ) |

\* (CFU mL<sup>-1</sup>) on swine slurry medium supplemented with pyrophephorbide *a* (pyp, 10 µg mL<sup>-1</sup>) alone and in combination with erythromycin, tylosin (10 µg mL<sup>-1</sup>, individually) or water and ethanol (H<sub>2</sub>O/EtOH, control). Values are averages and standard deviations from three repeated experiments with duplicates.

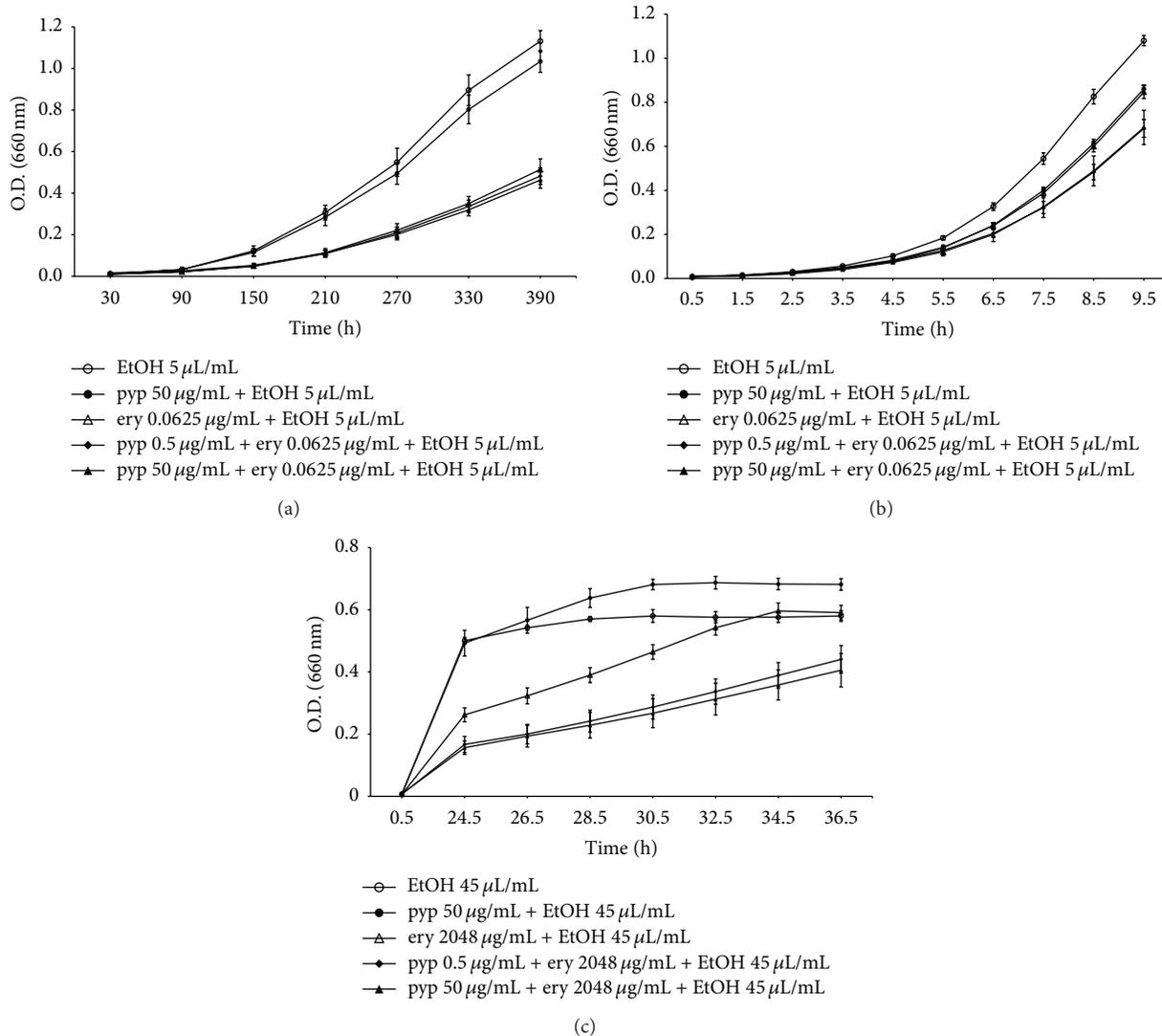


FIGURE 5: Effects of pyropheosphorbide *a* (pyp, 0.5 and 50 µg mL<sup>-1</sup>) alone and in combination with erythromycin (ery, 0.0625 and 2048 µg mL<sup>-1</sup>) on growth curves of naïve (a), (b), and erythromycin-induced (c) *S. aureus* ATCC 29213 under aerobic (a) and anaerobic (b), (c) conditions. Values are averages and standard deviations from three repeated experiments.

3.5. Total Cell Counts of Microorganisms and Viable Cell Counts of Anaerobic Bacteria from Stored Swine Manure. Direct microscopic counts of microorganisms in stored swine manure were  $5.49 \pm 0.41 \times 10^9$  mL<sup>-1</sup>, and the recovery rate on anaerobic swine slurry medium was about 23% ( $1.26 \pm 0.13 \times 10^9$  CFU, Table 1). About 80% ( $1.01 \pm 0.30 \times 10^9$  CFU) and 85% ( $1.07 \pm 0.15 \times 10^9$  CFU) of the organisms were capable of growing in erythromycin and tylosin containing media, respectively. Pyp exerted a significant intrinsic inhibitory effect which was greater ( $8.99 \pm 1.69 \times 10^8$  CFU) than the antibiotic effect of erythromycin or tylosin. CFU on media supplemented with both pyp and erythromycin tended to be lower ( $8.90 \pm 2.66 \times 10^8$ ) than on those containing only erythromycin. This combinatorial effect was indifferent, that is, equal to the effect of pyp alone. Pyp in combination with tylosin produced an antagonistic effect, resulting in increased

and equivalent counts ( $1.09 \pm 0.22 \times 10^9$  CFU) relative to the number of CFU on media containing only pyp and tylosin, respectively.

3.6. Concentrations of Php, Pyp, and Tylosin in Stored Swine Manure, Hydrolyzed Feed, and Agar Medium. Php and pyp concentrations in stored swine manure samples and unsupplemented swine slurry agar media were consistently below the general detection limit of 5 µg injected or  $\leq 10$  µg g<sup>-1</sup> sample (wet weight). The concentration of tylosin or a compound with the same retention time in the unsupplemented agar media was  $56.3 \pm 2.2$  µg g<sup>-1</sup> wet weight, whereas tylosin was not detected in the manure samples. Subsequent HPLC analysis of the hydrolyzed swine feed used for media preparation did not detect php, pyp, or tylosin and hence

ruled out that the feed accounted for the possible background concentration of tylosin in the media.

#### 4. Discussion

In the course of the serial passage experiments, the overall MTCs of erythromycin for naïve cultures of *S. aureus* and *Ent. faecalis* were below the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values of  $\leq 1$  and  $4 \mu\text{g mL}^{-1}$ , respectively (Figure 2 and EUCAST [78, 79]). This was presumably due to the absence of intrinsic resistance mechanisms and, because of their broad substrate specificities [80], downregulated basal expression levels of existing MDR efflux pumps, such as NorA in *S. aureus* and EmeA in *E. faecalis*. NorA and EmeA are homologous to each other [27, 81]; however, while EmeA is known to contribute to erythromycin resistance [61, 81], such a function has merely been indicated for NorA [60, 82, 83]. As with other macrolides, erythromycin is classically considered as bacteriostatic, but it has shown bactericidal activity against *S. aureus* at higher drug/cell ratios [84–86]. Bactericidal antimicrobials at sublethal concentrations induce mutational rather than adaptive resistance [10]. It is likely that the serial exposure of *S. aureus* to increasing concentrations of erythromycin ultimately resulted in the selection of cells bearing additive, high-level resistance-conferring mutations from naturally occurring subpopulations with, due to overexpression of the regular cellular machinery, low-level phenotypic adaptive resistance [10, 29, 87, 88]. High-level resistance to erythromycin can be predominant among *S. aureus* strains in individual studies (approx. 60% of strains with MIC  $> 1024 \mu\text{g mL}^{-1}$  [89]), but, generally it is rare (approx. 3% of strains with MICs  $\geq 512 \mu\text{g mL}^{-1}$  according to EUCAST [78]).

For all facultatively anaerobic bacterial reference strains examined, the serial passage experiments led to higher MTCs under anaerobic (96% carbon dioxide, 4% hydrogen atmosphere) than under aerobic conditions (Figure 2). Susceptibility testing of macrolides in the presence of carbon dioxide can result in elevated resistance, because the carbon dioxide decreases the pH of the medium and consequently erythromycin activity [90, 91]. However, in our experiments, the pH was controlled by sodium carbonate in the media ( $4 \text{ g L}^{-1}$ ), and therefore the “pH effect” of incubation in carbon dioxide can be ruled out. Instead, reactive oxygen species (ROS) generated by aeration might account for the observed differences between aerobic and anaerobic MTCs. ROS have antimicrobial activity and contribute to dysregulation primarily of MDR efflux pump expression by oxidative inactivation of global regulator proteins [29, 32]. For example, in *S. aureus*, reduced aeration causes an increase of *norB* expression and hence increased resistance to antimicrobial NorB substrates via an effect on MgrA, an oxidative stress-sensitive global regulator of 350 genes including *norA* and *norB* [92–94]. Thirdly, efflux-mediated antimicrobial resistance is controlled by the metabolic condition of bacteria and can be altered by a switch from an aerobic to an anaerobic metabolism through the influence of metabolically integrated global regulators, different endogenous cellular metabolites,

such as ROS from aerobic respiration or anaerobic fermentation end products which often times are the natural pump substrates, and an altered transmembrane electrochemical proton gradient as energy source for secondary active transporters [27, 28, 32, 80]. Hence, it can be presumed that the higher level of induced resistance in *S. aureus* under anaerobic conditions was contingent on a switch from glycolysis, the pentose phosphate pathway and ROS generating tricarboxylic acid cycle under aerobiosis to anaerobic glucose fermentation and ATPase-mediated proton efflux, generating a greater motive force for erythromycin-proton antiporters [28, 80, 94], such as LmrS, MdeA, Mef(A), and conceivably NorA [27, 32, 95–97].

In the broth macrodilution assays, pyp exerted significant effects on the MICs of erythromycin for *E. coli* P475.10.99.C3 and especially *S. aureus* (Figure 3). Subsequent spectrophotometric determination of growth parameters of *S. aureus* confirmed that pyp in combination with erythromycin was most effective, that is, synergistic already at the lower concentration of  $0.5 \mu\text{g mL}^{-1}$ , in highly resistant cultures under anaerobic conditions (Figures 4 and 5(c)) and revealed that pyp was intrinsically inhibitory to anaerobic, naïve cultures (Figures 4 and 5(b)). As could be expected [8, 94], the growth rate in the absence and at a low concentration of erythromycin ( $0.0625 \mu\text{g mL}^{-1}$ ) (Figure 4(b)) was greatest in the aerobic, naïve cultures and lowest for the highly resistant strain—the latter effect being due to a “fitness cost” as entailed by most antimicrobial resistance mechanisms [88]. The intrinsic growth inhibition of anaerobic, naïve cultures of *S. aureus* indicates that pyp, provided it equals php in its function as an EPI [51, 52], acts against a metabolically regulated MDR pump with broader, compound nonspecific functions unrelated to antimicrobials. It can be concluded that this hypothetical MDR efflux pump is one other than NorA, as there is no strong indication of erythromycin being a NorA substrate [29, 60, 82, 83] and as we observed no significant effect of pyp against *Ent. faecalis* bearing the NorA homologue EmeA (Figures 3(a) and 3(c)). The *S. aureus* genome comprises at least 30 genes for putative drug transporters, and approximately 17 of these encode MDR efflux pumps, most of which are still unknown [98, 99] and two of which, LmrS and MdeA, contribute to erythromycin resistance [27, 32, 97, 100] and are therefore possible candidates for inhibition by pyp. Pyp in combination with erythromycin only partially reversed high-level resistance of *S. aureus* and did not completely inhibit its growth (Figures 3(a), 3(c), and 5(c)). This indicates that the high-level resistance is depended on the combined effects of more than one efflux pump and/or other resistance mechanisms [80]. There are at least 17 genes encoding for protein efflux systems for macrolides, lincosamides, and streptogramins [20]. Msr(A) and Mef(A) are well known to confer erythromycin resistance in strains of *S. aureus* [27, 95, 96, 101, 102]. The plasmid-borne Msr(A) transporter, even though mainly responsible for erythromycin efflux in *S. aureus* [103], is not present in the strain used in our study, *S. aureus* ATCC 29213 [104]. Mef(A) is closely related to Mef(B), the erythromycin efflux pump in *E. coli* P475.10.99.C3 [67], and as pyp decreased the final

MIC endpoint for induced cultures of this strain (Figures 3(b) and 3(d)), it can be concluded that Mef(A) is an additional candidate for inhibition by pyp in *S. aureus*.

The basal levels of resistance to erythromycin (approx. 80%) and tylosin (approx. 85%) of anaerobic bacteria from stored swine manure samples (Table 1) were considerably higher than those found earlier in manure samples from the same farm (max. 21% and 32%) [73]. However, according to other previous studies [105, 106], background levels of erythromycin and macrolide resistance in nonmedicated swine fecal and manure bacterial communities can already be as high as approximately 40% and are enhanced further by the administration of compounds with antimicrobial activities [62–64]. In our study, tylosin had last been used 31 days and 4–4.5 months prior to the last pit emptying and days of sampling, respectively, and consequently was not detected by HPLC in the manure. Also, it can be concluded that instead of tylosin a different compound with the same retention time was detected in the unsupplemented agar media, as both the manure and hydrolyzed swine feed could be ruled out as a source of contamination and as any tylosin present in the media would, due to its heat lability [107], probably have been destroyed by autoclaving (121°C, 20 min). There had been no history of the use of erythromycin on the farm, but a low concentration in the manure due to erythromycin-contaminated DDGS in the swine feed is possible and might have contributed to the observed high basal resistance level. More likely, however, the high levels of erythromycin and tylosin resistance were partly the outcome of the recent administration of oxytetracycline, lincomycin, and the heavy metals copper and zinc, these compounds can indirectly enhance antimicrobial incl. macrolide resistance through coselection [105, 108–113].

Pyp in combination with erythromycin (10 µg mL<sup>-1</sup>, individually) tended to reduce the number of erythromycin resistant bacteria cultured from stored swine manure (Table 1); however, this effect was indifferent and less significant than the synergistic effects observed on *S. aureus* (Figures 4 and 5). The level of tylosin resistance remained unchanged when pyp and tylosin were combined (Table 1). Experiments with *S. aureus* had shown that pyp acts in a concentration-dependent manner between 0.5 and 50 µg mL<sup>-1</sup> (Figures 3–5), and therefore it can be presumed that more significant effects on macrolide resistance of manure bacteria can be achieved with higher concentrations of pyp. Alternatively, the lower effectiveness might be due to cross- or coresistance between pyp and the macrolide antibiotics. The development of bacterial resistance to EPIs themselves is a recognized concern [29]. As in the experiments with *S. aureus* (Figures 4 and 5(b)), pyp was intrinsically inhibitory to the growth of anaerobic manure bacteria (Table 1). It is not an uncommon feature for an EPI from natural sources to possess a direct antibacterial effect [26, 35]. For example, the aerobic MIC of pyp was found to vary between 4 µg mL<sup>-1</sup> (*S. epidermidis*) and 500 µg mL<sup>-1</sup> (*S. aureus* ATCC 29213) [52], and in another study the death percentage of *S. aureus* ATCC 26923 in 20 µg pyp mL<sup>-1</sup> was 32% [114]. It is conceivable that, as discussed above for *S. aureus*, pyp exerted its intrinsic effect

on the manure microbiota through inhibition of a MDR efflux pump, such as LmrS, MdeA, and possibly NorA, as these and their homologues occur in *B. subtilis*, a major component of MicroSource S, and are widespread among other low mol percentage guanine-cytosine Gram-positive (phylum *Firmicutes*) bacteria [27, 81, 97, 100, 115–117], the predominant culturable anaerobic microorganisms from swine manure [73, 118, 119].

Neither php nor pyp was detectable by HPLC in the swine manure or corn-soybean-DDGS-based feed (without Intivate). These results confirm previous findings that the fecal concentrations of php and pyp are diet-dependent and range between <1 and 180 µg g<sup>-1</sup> in feces (dry matter) from swine and herbivore livestock with a low and high intake of chlorophyll *a*, respectively [50]. Consequently, in order to achieve the effects indicated by our results (bacterial growth inhibition, potentiation of antimicrobials, and partial reversal of bacterial antimicrobial resistance) inside the gastrointestinal tracts of swine and/or in manure storage pits, both “hotspot habitats” for the selection and spread of bacterial resistance genes [4, 16, 120], it is necessary to include green plant feedstuffs, such as alfalfa and grass, or microalgae. Even though, in this study, the use of the algae-containing feed additive Intivate was not sufficient, supplementation with microalgae is a suitable natural means of obtaining significant concentrations of php and pyp in swine manure. Microalgae, such as *Chlorella vulgaris*, which can be autochthonous to swine manure habitats [121, 122] presents a good source of chlorophyll *a*, php, and pyp [38, 40, 123] as well as a valuable substitute for conventional protein sources [123, 124]. Their direct addition to swine waste lagoons, ponds, or other treatment bioreactors can be combined with the photodegradative bioremediation of residual antimicrobial substances [125, 126].

## 5. Conclusions

To conclude, this is the first study showing that pyropheophorbide derived from chlorophyll affects growth and the level of sensitivity to erythromycin of *S. aureus*, *E. coli*, and anaerobic manure bacteria. Addition of chlorophyll-containing plant material to animal diets has the potential to reduce antibiotic resistance in the animal gut and eventual stored manure.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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