Organic Acids and Nature Identical Compounds Can Increase the Activity of Conventional Antibiotics Against *Clostridium Perfringens* and *Enterococcus Cecorum In Vitro*

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Primary Audience: Nutritionists, Researchers, Veterinarians

SUMMARY

In a global context of increased antibiotic resistance, feed additives with enhanced antimicrobial properties are a useful and increasingly needed strategy. Organic acids (OA) and botanical molecules such as nature identical compounds (NIC) have been shown to be effective against bacterial infections based on their antimicrobial activity. The aim of this study was to evaluate whether the combination of OA or NIC with conventional antibiotics in poultry could increase antibiotic efficacy against *Clostridium perfringens* and *Enterococcus cecorum*. These organisms are the major poultry pathogens responsible for necrotic enteritis and bacterial chondronecrosis with osteomyelitis, respectively, and they have developed resistance to several antibiotics worldwide. A set of antimicrobial tests showed that both species had variable antibiotic sensitivity. Alternatively, OA and NIC were always effective in a dose-dependent manner, even when the antibiotics failed. For several strains, selected combinations of OA or NIC with antibiotics increased the bacterial sensitivity to antibiotics. Therefore, OA and NIC have potential to enhance the efficacy of conventional antibiotics against *C. perfringens* and *E. cecorum*.

Key words: antibiotic resistance, minimal inhibitory concentration, *Clostridium perfringens*, necrotic enteritis, *Enterococcus cecorum*, bacterial chondronecrosis with osteomyelitis, organic acids, nature identical compounds

INTRODUCTION

Necrotic enteritis and bacterial chondronecrosis with osteomyelitis (BCO) are recognized as major diseases in poultry production, causing a reduction in growth performance and considerable economic losses [1, 2]. *Clostridium perfringens* is the causative agent of necrotic enteritis which leads to necrosis and inflammation of the gastrointestinal tract [3]. The most common antibiotics used to treat necrotic enteritis are bacitracin, lincomycin, neomycin, penicillin, tylosin, and virginiamycin [4], although resistance to these antibiotics has been reported around the world [5–13]. *Enterococcus*

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cecorum is the main microorganism responsible for BCO. Clinically, BCO is characterized by necrosis of cartilaginous tissue and inflammation of the bone and bone marrow, which result in lameness and growth retardation. Although *E. cecorum* has been recently recognized as a causative agent of BCO, antibiotic resistance including multidrug resistance has already been reported [14–22]. Currently, the use of antibiotics in livestock is regulated by several legislative measures, i.e., Regulation 1831/2003 in Europe [23] or the recent Veterinary Feed Directive by the Food and Drug Administration in the US [24]. Improper use of antibiotics in food animals aggravates the issue of antibiotic resistance, contributing to the loss of efficacy of therapeutic treatments in both animals and humans. Therefore, it is essential to study antimicrobial alternatives, such as adjuvant molecules, alone and in combination with antibiotics, with the aim of supporting the limited existing antibiotics or replacing them entirely. Among these substances, organic acids (OA) and botanical molecules, such as nature identical compounds (NIC), have garnered growing interest in the field of feed additives thanks to their antimicrobial properties [25–27].

The aim of this study was to evaluate the in vitro susceptibility of *C. perfringens* and *E. cecorum* to selected antibiotics and antimicrobial compounds. In particular, we investigated whether specific OA and NIC that are commonly found in feed additives could be used as alternative or adjuvant molecules along with conventional antibiotics against *C. perfringens* and *E. cecorum*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

A total of 10 strains of *C. perfringens* and 10 strains of *E. cecorum* were obtained from clinical cases (necrotic enteritis and BCO, respectively) that occurred in commercial Italian broiler operations. The *C. perfringens* strains were isolated from broiler intestine, while the *E. cecorum* strains were isolated from broiler femur head (4 strains), intestinal tract (4 strains) and vertebral column (2 strains); and the strains were then identified with specific biochemical kits [28].

The *C. perfringens* and *E. cecorum* strains were grown in brain heart infusion broth (BHI) [29] (pH 6.5) at 37°C under anaerobic conditions and counted via plating 10-fold serial dilutions onto BHI agar.

Chemicals and Test Solutions

The antibiotics used in this study were ampicillin, bacitracin, doxycycline, lincomycin, penicillin G, tiamulin, tylosin and 2 broad-spectrum antibiotics, amoxicillin and neomycin [30]. Antibiotic stock solutions were prepared in BHI. Citric acid, sorbic acid, benzoic acid, butyric acid, hexanoic acid, octanoic acid, decanoic acid, dodecanoic acid, thymol, vanillin, carvacrol and eugenol were obtained from the same source [31]. Stock solutions of the bioactive compounds were prepared in BHI (citric acid, sorbic acid, benzoic acid, butyric acid, hexanoic acid) or BHI supplemented with 70% (v/v) ethanol at a final concentration ≤3.5% (v/v) to increase their solubility (octanoic acid, decanoic acid, dodecanoic acid, thymol, vanillin, carvacrol, and eugenol). All solutions were buffered to pH 6.5, filter-sterilized [32], and then diluted in fresh sterile BHI to reach the final concentrations tested.

Antimicrobial Assay 1 – Individual Compounds

The minimal inhibitory concentrations (MICs) of the antibiotics, OA and NIC were determined using a broth microdilution method in 96-well microtiter plates [33]. The *C. perfringens* and *E. cecorum* strains were tested over a range of concentrations (2-fold dilutions) with various concentrations of the compounds: antibiotics (64–0.5 mg/L); citric acid, sorbic acid, benzoic acid, butyric acid, and hexanoic acid (100–1.56 mM); octanoic acid, decanoic acid, dodecanoic acid, thymol, vanillin, carvacrol and eugenol (7.5–0.12 mM). The bacterial strains (10^5 cfu/mL) were incubated with the test substances under anaerobic conditions at 37°C for 24 h. Control strains were grown in medium containing 3.5% (v/v) ethanol to exclude the possibility of inhibitory effects of the ethanol. After incubation, the absorbance at 630 nm was read with a spectrophotometer [34] to measure the bacterial growth. For each substance, the

MIC was defined as the lowest concentration that resulted in null absorbance after 24 h of incubation.

Antimicrobial Assay 2 – Combinations

After the primary screening analysis, *C. perfringens* and *E. cecorum* cells were challenged with combinations of antibiotics and OA or NIC in a 3 × 3 checkerboard design. For *C. perfringens*, bacitracin (1, 0.75, 0.50 mg/L) and lincomycin (64, 32, 16 mg/L) were combined with citric acid (12.5, 6.25, 3.13 mM), sorbic acid (50, 25, 12.5 mM), benzoic acid (50, 25, 12.5 mM), dodecanoic acid (0.06, 0.03, 0.015 mM), thymol (1.40, 0.94, 0.70 mM), vanillin (5.62, 3.75, 2.81 mM), carvacrol (1.40, 0.94, 0.70 mM), and eugenol (2.81, 1.87, 1.40 mM). For *E. cecorum*, lincomycin, tylosin, and neomycin (64, 32, 16 mg/L) were combined with citric acid (6.25, 3.13, 1.56 mM), sorbic acid (50, 25, 12.5 mM), benzoic acid (50, 25, 12.5 mM), dodecanoic acid (0.06, 0.03, 0.015 mM), thymol (0.94, 0.47, 0.23 mM), vanillin (1.87, 0.94, 0.47 mM), carvacrol (0.94, 0.47, 0.23 mM), and eugenol (1.87, 0.94, 0.47 mM). Incubation and absorbance measurements were performed as described for antimicrobial assay 1.

Statistical Analysis

The experiments were performed in triplicate for each strain and the values presented are the means. The data were analyzed with one-way ANOVA followed by the Tukey post hoc test [35], and differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Antimicrobial Assay 1 – Individual Compounds

Antibiotic resistance is a worrying problem that is dangerous for animals and humans. As a consequence of improper antibiotic use, microorganisms develop resistance, and therapeutic treatments become ineffective. Different antibiotic classes are characterized by different mechanisms of action, such as blocking cell wall synthesis, inhibiting protein or nucleic acid synthesis, and interfering with the energetic metabolism of bacteria. Bacteria can acquire resistance to antimicrobial agents via several mechanisms: (1) chemically altering the antibiotic molecular structure or even disrupting it; (2) limiting the accumulation of the drug within the bacterial cell via activation of efflux pumps or decreasing cell permeability to block antimicrobial uptake; (3) altering the target site of the drug; or (4) adapting the entire cell to resist the challenge via multiple mechanisms [36, 37]. The activities of the antibiotics against 10 *C. perfringens* strains and 10 *E. cecorum* strains are presented in Table 1. All strains of the *C. perfringens* strains were susceptible to amoxicillin, ampicillin, and penicillin G and resistant to neomycin up to 64 mg/L. Bacitracin and doxycycline were effective at MIC values between 1–2 mg/L and 0.5–8 mg/L, respectively. Bimodal trends were observed for lincomycin, tiamulin, and tylosin. Similarly, all 10 *E. cecorum* strains were susceptible to amoxicillin, ampicillin, and penicillin G. A total of 8 strains were resistant to neomycin since no MIC was detected among the tested concentrations, while the MIC value was 64 mg/L for the other 2 strains. Bacitracin, doxycycline, and tiamulin were effective at MIC values between 0.5–1, 0.5–8, and 0.5–2 mg/L, respectively. Bimodal trends were observed for lincomycin and tylosin. The penicillin group was very active against both bacterial species, while neomycin was ineffective at all doses tested. Several studies have reported neomycin resistance in *C. perfringens* and *E. cecorum* [8, 19]. The resistance patterns to lincomycin, tiamulin, and tylosin were very variable among the strains, highlighting the heterogeneity of antibiotic resistance. Indeed, bimodal distributions in MIC values, which have been frequently observed with lincomycin against *C. perfringens* [11, 13, 38, 39], likely result from the emergence and dissemination of bacterial resistance [40].

The antimicrobial activities of OA and NIC against *C. perfringens* and *E. cecorum* are reported in Figures 1 and 2, respectively. For *C. perfringens*, the MIC values of citric acid, sorbic acid, benzoic acid, hexanoic acid, octanoic acid, decanoic acid, and dodecanoic acid were 25, 100, 50–100, 100, 7.5, 1.87, and 0.23–0.94 mM, respectively (Figure 1A). Thymol and carvacrol

Table 1. Frequency of Minimal Inhibitory Concentration Values of 9 Antibiotics Against 10 Strains of *Clostridium perfringens* and 10 Strains of *Enterococcus cecorum*.

| | Clostridium perfringens | | | | | | | | | Enterococcus cecorum | | | | | | | | |
| | mg/L | | | | | | | | | mg/L | | | | | | | | |
	>64	64	32	16	8	4	2	1	0.5	>64	64	32	16	8	4	2	1	0.5
AMX									10									10
AMP									10									10
BAC							8	2									3	7
DOX					3	3			4						6			4
LIN	6				1	1		2		6							1	3
NEO	10									8	2							
PEN									10									10
TIA	3						2		5							1	1	8
TYL		1	2			1	2	2	2	6							2	2

AMX = amoxicillin; AMP = ampicillin; BAC = bacitracin; DOX = doxycycline; LIN = lincomycin; NEO = neomycin; PEN = penicillin G; TIA = tiamulin; TYL = tylosin.

(A) Organic acids

(B) Nature Identical Compounds
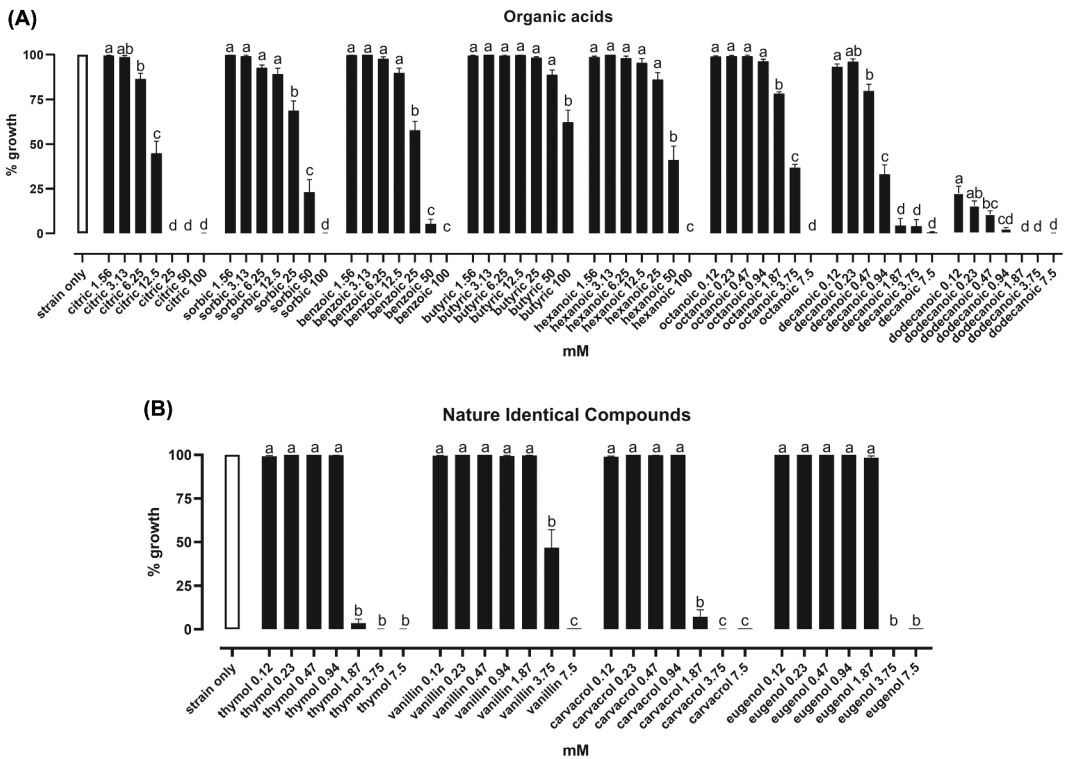
Figure 1. *Clostridium perfringens* growth after 24 h in the presence of OA (A) or nature identical compounds (B). *Clostridium perfringens* growth is expressed as a percentage relative to the control (strain only), and values are presented as the mean ± SEM (n = 10). Data were analyzed with one-way ANOVA and, within each compound, columns with different letters were significantly different ($P \leq 0.05$).

completely inhibited *C. perfringens* growth at concentrations ranging from 1.87 to 3.75 mM, whereas the values for vanillin and eugenol were 3.75–7.5 mM and 3.75 mM, respectively (Figure 1B). The MIC values of citric acid, sorbic acid, benzoic acid, hexanoic acid, octanoic acid, decanoic acid, and dodecanoic acid against *E. cecorum* were 12.5–25, 100, 100, 100, 7.5, 1.87, and 0.23–0.94 mM, respectively (Figure 2A). The MIC values of thymol and carvacrol were
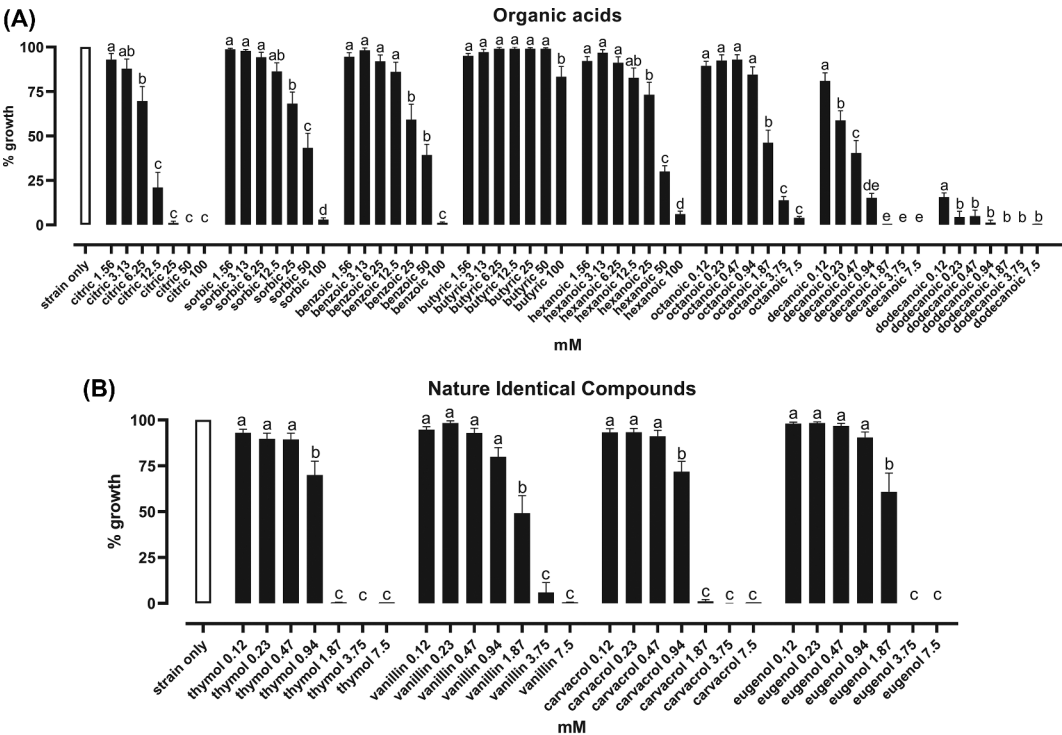
Figure 2. *Enterococcus cecorum* growth after 24 h in the presence of organic acids (A) or nature identical compounds (B). *Enterococcus cecorum* growth is expressed as a percentage relative to the control (strain only), and values are presented as the mean $\pm$ SEM (n = 10). Data were analyzed with one-way ANOVA and, within each compound, columns with different letters were significantly different ($P \leq 0.05$).

both 1.87 mM, and the values for vanillin and eugenol were both 3.75 mM (Figure 2B). Butyric acid was ineffective against both species up to 100 mM. Previous studies have reported positive effects for botanicals (blends of essential oils) and OA in controlling the intestinal damage associated with *C. perfringens* mainly in in vivo necrotic enteritis models [41–44]. Regarding *E. cecorum*, literature is still lacking; however, antimicrobial properties have been demonstrated in vitro for carvacrol and mixtures of medium chain fatty acids, including hexanoic, octanoic, decanoic, and dodecanoic acid [45]. The antibacterial mode of action of NIC is primarily due to their pore-forming activity, which results in the release of cell contents and cytoplasm coagulation [46]; furthermore, their attachment on the bacterial cell surface alters the structural integrity of the membrane, disturbing metabolism and causing cell death [47]. Organic acids exert their antimicrobial activity against pH-sensitive bacteria by passing through

the bacterial membrane in their undissociated form. Once inside the bacteria, OA dissociate their H^+ ions, resulting in a decrease in the intracellular pH. The bacteria then activate proton pumps to restore the normal pH, which consumes energy [48]. Furthermore, the anion $RCOO^-$ can be toxic to DNA replication, disrupt metabolic functions and increase osmotic cell pressure [49, 50].

Antimicrobial Assay 2 – Combinations

The inhibition of *C. perfringens* and *E. cecorum* growth by combinations of antibiotics and OA or NIC is represented in Tables 2 and 3, respectively. For *C. perfringens*, bacitracin and lincomycin were chosen to be tested in combination with OA and NIC due to their extensive use in treating necrotic enteritis [51–53]. In the 6 strains shown to be lincomycin resistant, the antimicrobial activity of lincomycin was enhanced in combination with eugenol. Eugenol at

Table 2. Percentage of Inhibition of *Clostridium perfringens* Growth in the Presence of Combinations of Antibiotics and Antimicrobial Compounds.

	%	LIN 16 mg/L 5	LIN 32 mg/L 5	LIN 64 mg/L 8	BAC 0.5 mg/L 11	BAC 0.75 mg/L 36	BAC 1 mg/L 65
CIT 3.13 mM	14	17	12	13	12	15	14
CIT 6.25 mM	24	26	28	34*	21	23	21
CIT 12.5 mM	57	79***	73***	76***	60***	62	56
SOR 12.5 mM	27	52*	49	44	29	63	92
SOR 25 mM	42	65**	63**	61**	48**	73**	97*
SOR 50 mM	92	96***	99***	100***	93***	97***	99**
BEN 12.5 mM	24	34	37	37	82***	96***	95**
BEN 25 mM	92	96***	96***	97***	100***	100***	100***
BEN 50 mM	95	100***	100***	100***	100***	100***	99***
DOD 0.015 mM	1	2	18	13	54*	81*	85
DOD 0.03 mM	3	26	28	29	80***	91***	89
DOD 0.06 mM	16	33	34	35	96***	98***	98
THY 0.70 mM	3	6	6	7	82***	99***	98***
THY 0.94 mM	4	7	6	12	82***	100***	100***
THY 1.40 mM	5	20	19	34*	100***	100***	100***
VAN 2.80 mM	13	19	20*	20	52***	86***	93*
VAN 3.75 mM	18	23**	24**	25**	56***	87***	99**
VAN 5.62 mM	28	33***	33***	34***	75***	97***	98**
CAR 0.70 mM	8	9	10	10	86***	99***	100***
CAR 0.94 mM	9	10	12	13	87***	100***	100***
CAR 1.40 mM	11	22	22	37*	100***	100***	100***
EUG 1.40 mM	3	3	5	2	88***	95***	93***
EUG 1.87 mM	4	21	15	18	95***	100***	100***
EUG 2.80 mM	5	95***	100***	100***	100***	100***	100***

Values are presented as the means, n = 6 for lincomycin (resistant strains) and n = 10 for bacitracin.

Data were analyzed with one-way ANOVA and, within each combination, asterisks indicate values that are different from those obtained with the antibiotic alone (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

LIN = lincomycin; BAC = bacitracin; CIT = citric acid; SOR = sorbic acid; BEN = benzoic acid; DOD = dodecanoic acid; THY = thymol; VAN = vanillin; CAR = carvacrol; EUG = eugenol.

2.80 mM in combination with all lincomycin concentrations resulted in a 95–100% growth inhibition, whereas 2.80 mM eugenol alone was only 5% effective. Bacitracin at concentrations lower than the MIC showed enhanced antimicrobial activity in combination with eugenol, thymol, carvacrol, and dodecanoic acid. The antimicrobial activity of bacitracin at 0.5 mg/L was increased by 88–100% with low doses of eugenol (1.40–2.80 mM) and by 82–100% and 86–100% with low doses of thymol and carvacrol (0.70–1.40 mM), respectively. The same concentration of bacitracin (0.5 mg/L) in combination with 0.03 and 0.06 mM dodecanoic acid inhibited bacterial growth by 80% and 96%, respectively. OA and NIC were tested in combination with lincomycin, tylosin, and neomycin against *E. cecorum* (Table 3). The first 2 antibiotics were chosen due to their bimodal spectrum of action, whereas neomycin was chosen due to its broad resistance profile. The antimicrobial activity of neomycin was enhanced when combined with dodecanoic acid. The combination of neomycin (64 mg/L) with 0.03 mM dodecanoic acid resulted in an 82% reduction in *E. cecorum* growth, while neomycin (32 mg/L) with 0.06 mM dodecanoic acid resulted in an 86% reduction. Figure 3 presents the results for the only 2 strains sensitive to 64 mg/L neomycin, whose activity was enhanced in combination with carvacrol and thymol. *E. cecorum* growth was fully inhibited by neomycin (16 mg/L) combined with 0.47–0.94 mM carvacrol or 0.94 mM thymol and by neomycin (32 mg/L)

Table 3. Percentage of Inhibition of *Enterococcus cecorum* Growth in the Presence of Combinations of Antibiotics and Antimicrobial Compounds.

	%	NEO 16 mg/L 18	NEO 32 mg/L 15	NEO 64 mg/L 38	TYL 16 mg/L 10	TYL 32 mg/L 16	TYL 64 mg/L 21	LIN 16 mg/L 39	LIN32 mg/L 37	LIN64 mg/L 40
CIT 1.56 mM	13	18	23	43	17	20	11	46	45	52
CIT 3.13 mM	17	24	25	55	32	24	26	55	54	59
CIT 6.25 mM	32	35	55**	86**	33	39	41	52	56	56
SOR 12.5 mM	14	22	48*	67	20	20	16	28	26	28
SOR 25 mM	27	55***	71***	91***	35	40	43	45	41	47
SOR 50 mM	58	84***	94***	100***	61	63	68	66	68	74
BEN 12.5 mM	17	23	48**	69	23	24	19	29	24	34
BEN 25 mM	38	57***	74***	98***	42	41	43	47	49	49
BEN 50 mM	60	90***	99***	100***	70**	75**	79***	72	69	76
DOD 0.015 mM	8	6	14	33	14	17	4	43	34	47
DOD 0.03 mM	8	16	33	82*	9	13	13	38	37	45
DOD 0.06 mM	31	56**	86***	98***	37	41	38	55	55	55
VAN 0.47 mM	8	7	15	31	6	10	10	38	33	36
VAN 0.94 mM	10	14	19	38	11	9	12	36	36	37
VAN 1.87 mM	22	25	34	42	23	26	28	47	47	46
EUG 0.47 mM	6	7	9	34	4	13	6	43	31	41
EUG 0.94 mM	12	17	21	58	12	15	16	42	44	44
EUG 1.87 mM	20	31	54**	66	22	24	26	48	51	50

Values are presented as the means, n = 6 for tylosin and lincomycin (resistant strains) and n = 10 for neomycin.

Data were analyzed with one-way ANOVA and, within each combination, asterisks indicate values that are different from those obtained with the antibiotic alone (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

NEO = neomycin; TYL = tylosin; LIN = lincomycin; CIT = citric acid; SOR = sorbic acid; BEN = benzoic acid; DOD = dodecanoic acid; VAN = vanillin; EUG = eugenol.
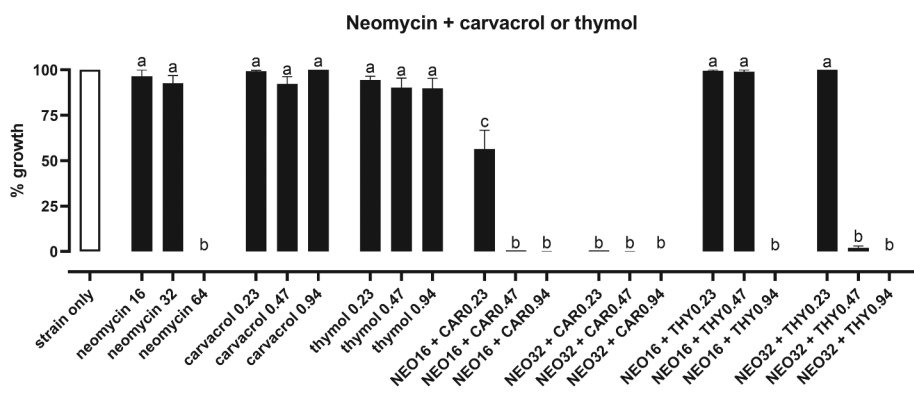
Figure 3. Growth of 2 strains of *Enterococcus cecorum* after 24 h in the presence of either neomycin, carvacrol, thymol, or combinations of neomycin and carvacrol or thymol. *Enterococcus cecorum* growth is expressed as a percentage relative to control (strain only), and values are presented as the mean ± SEM (n = 2). Data were analyzed with one-way ANOVA and, within each neomycin + carvacrol or thymol combinations, columns with different letters were significantly different ($P \leq 0.05$). NEO = neomycin (mg/L); CAR = carvacrol (mM); THY = thymol (mM).

combined with all of the tested concentrations of carvacrol or thymol (0.47–0.94 mM). It is noteworthy that these 2 strains were isolated from femur heads, while the remaining neomycin resistant strains were all isolated from vertebral columns and intestines. Consistent with our findings, other labs have reported that pathogenic *E. cecorum* strains isolated from

spinal lesions can have increased neomycin resistance [15]. A possible correlation between isolation sites and sensitivity to certain antibiotics may exist, but this possibility must be confirmed via further investigation. While vanillin was entirely ineffective at increasing the antimicrobial activity of neomycin against *E. cecorum*, the effects of combinations of neomycin with benzoic, citric, and sorbic acid, and eugenol, were very variable among the strains. The antimicrobial activities of lincomycin and tylosin were generally not affected by combination with OA or NIC, except for citric acid, which improved their activities against only a few strains; however, this combination had no effect on the average of the 10 strains.

Our results indicate that selected combinations of OA or NIC and antibiotics can increase the efficacy of conventional antibiotic against *C. perfringens* and *E. cecorum*. It appears that the bacterial strains tested in this study primarily develop resistance to antibiotics that inhibit protein synthesis. Drugs that interfere with cell wall synthesis, i.e., the penicillin group and bacitracin, were effective at lower concentrations, while neomycin, lincomycin, tylosin, or tiamulin all had high MIC values. The synergistic effects of components of essential oils and antibiotics against resistant bacteria can occur via several mechanisms, including targeting different sites in the bacterial cell (multitarget effect) or targeting specific bacterial resistance mechanisms [54]. It has been demonstrated that NIC facilitate the entry of OA into bacterial cells via their pore-forming action, resulting in synergistic antibacterial effects [55]. The same mechanism could also occur with antibiotics. It is possible that the antibiotic, assisted by NIC, enters the bacterial cell more easily, thus bypassing resistance systems of the microorganism. In addition, NIC can inhibit efflux pumps, limiting the expulsion of antibiotics from bacterial cells [56, 57]. The roles of OA in increasing antibiotic efficacy are less clear. A possible synergy could result from enhanced bacterial adenosine triphosphate (ATP) consumption due to OA exposure [58]. Due to this wasted energy, bacterial resistance responses could be impaired and the antibiotic effects, i.e., inhibition of protein synthesis, could be enhanced.

CONCLUSIONS AND APPLICATIONS

1. Since the ban of antibiotic use for promoting growth in animal production, the global challenge is now to reduce the use of therapeutic antibiotics as much as possible via the use of alternative compounds and adjuvants that increase the efficacy of existing antibiotics.

2. The responses of *C. perfringens* and *E. cecorum* strains to conventional antibiotics were highly variable among the strains, demonstrated by different resistance patterns. By contrast, the effects of OA and NIC were species-specific rather than strain-specific, and they were always effective in a dose-dependent manner, even when the antibiotics failed.

3. Several combinations of antibiotics and antimicrobial compounds showed highly synergic effects that sensitized the *C. perfringens* and *E. cecorum* strains to the conventional antibiotics tested.

4. OA and NIC are promising tools to fight antibiotic resistance. Used alone, OA and NIC can function as an alternative to antibiotics. When used in combination with antibiotics, OA and NIC can act as adjuvants and increase antibiotic efficacy at the same time lowering the amount of antibiotic required.

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