NOTE *Bacteriology*

In vitro susceptibility of *Brachyspira hyodysenteriae* to organic acids and essential oil components

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ABSTRACT. The antibacterial potential of organic acids and essential oil components against *Brachyspira hyodysenteriae*, the causative pathogen of swine dysentery, was evaluated. Minimum inhibitory concentrations (MIC) of 15 compounds were determined at pH 7.2 and pH 6.0, using a broth microdilution assay. In addition, possible synergism was determined. MIC values for the three tested strains were similar. For organic acids, MIC values at pH 6.0 were lower than at pH 7.2. *B. hyodysenteriae* was most sensitive to cinnamaldehyde and lauric acid, with MIC values <1.5 mM. Most antibacterial effects of binary combinations were additive, however, for thymol and carvacrol, synergism could be observed. *In vitro* results demonstrate the antibacterial action of certain essential oil components and organic acids against *B. hyodysenteriae*.
KEY WORDS: *Brachyspira hyodysenteriae*, essential oil, minimum inhibitory concentration (MIC), organic acid

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Swine dysentery is a gastro-intestinal disease mostly affecting fattening pigs, with a significant economic impact on the worldwide pig industry. The disease is caused by the fastidious, anaerobic spirochete, *Brachyspira hyodysenteriae* [1], colonizing the crypts of the large intestine of pigs and causing a muco-hemorrhagic inflammation of the hindgut [5]. Symptoms can vary widely from a severe diarrhea stained with blood and mucus, to a mild gastro-intestinal disease or even a sub-clinical infection [6]. At present, there are only a few effective drugs available for the treatment of swine dysentery, and reduced efficacy of these antimicrobials is an emerging concern for the pig industry [26]. Isolates of *B. hyodysenteriae* showing increasing acquired resistance towards the limited number of registered antimicrobials have been reported in many countries [8, 15, 18, 21]. Consequently, alternative measures besides antimicrobials are wanted to control swine dysentery. A lot of research on organic acids and natural components as alternatives to antibiotics has already been done [4, 19, 25]. In pigs, supplementation of organic acids and essential oils to the feed can improve performance, support intestinal health and counteract infections with enteric pathogens, such as *E. coli* and *Salmonella* [12, 23]. However, until now, little or no information on the potential antimicrobial activity of essential oil components [28] or organic acids against *B. hyodysenteriae* is available, probably due to the fastidious growth conditions required for this bacterium.

The aim of this study was to evaluate the effect of different organic acids and essential oil components on *in vitro* growth of *B. hyodysenteriae*. Screening for products with a direct inhibiting effect is a first step in the selection of compounds that might be used as feed additives in the prevention or treatment of swine dysentery. Moreover, some combinations of products were tested to examine possible synergistic effects.

Three different *B. hyodysenteriae* strains, two field isolates, 6bI and 8dII, and a reference strain, B78 (ATCC 27164^T), were used during this study. For fifteen compounds with potential antibacterial effects (shown in Table 1), the minimum inhibitory concentration (MIC) was determined using a broth microdilution method, as originally described by Karlsson *et al.* for antimicrobial susceptibility testing of *B. hyodysenteriae* [11]. Standard solutions and twofold dilutions of the 15 different compounds were prepared in brain heart infusion broth (BHI) (EMD Millipore, Billerica, MA, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone, Thermo Scientific, Cramlington, U.K.). To exclude any interfering effects of pH on bacterial growth during the inhibition test, the pH of all standard solutions and dilutions was adjusted with 3 M NaOH or 1 M HCl. In a first experiment, the antibacterial effects were tested at regular pH of BHI-broth, pH 7.2. Additionally, for strain 8dII, antibacterial effects at pH 6.0 were investigated, which is a more physiological pH in the large intestine of swine.

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Table 1. Minimum Inhibitory Concentration (MIC) values in mM of selected organic acids and essential oil components against *Brachyspira hyodysenteriae* strains B78, 8dII and 6bI, at pH 7.2 and at pH 6 against *B. hyodysenteriae* strain 8dII

Product	Tested range (mM)	pH	*B. hyodysenteriae* strain		
			B78	6bI	8dII
Formic acid	(2.5–320)	7.2	320	320	320
Citric acid	(0.63–80)	7.2	40	20	40
Benzoic acid	(0.31–40)	7.2	40	40	40
Lactic acid	(2.5−320)	7.2	320	160	320
		6	-	-	80
Acetic acid	(2.5– 320)	7.2	160	320	320
		6	-	-	80
Propionic acid	(2.5−320)	7.2	160	160	160
		6	-	-	40
Butyric acid	(2.5−320)	7.2	40	80	80
		6	-	-	10
Caproic acid	(0.31−40)	7.2	20	20	20
		6	-	-	5
Caprylic acid	(0.31−40)	7.2	10	10	20
		6	-	-	2.5
Capric acid	(0.31−40)	7.2	2.5	1.25	2.5
		6	-	-	0.63
Lauric acid	(0.08−10)	7.2	0.63	0.63	0.63
		6	-	-	0.31
Eugenol	(0.16−20)	7.2	2.5	2.5	2.5
		6	-	-	2.5
Carvacrol	(0.16–20)	7.2	1.25	1.25	1.25
		6	-	-	0.63
Thymol	(0.08–10)	7.2	1.25	1.25	1.25
		6	-	-	0.63
Cinnamaldehyde	(0.08–10)	7.2	0.31	0.31	0.31
		6	-	-	0.16

Wells of 48-well culture plates (Greiner Bio-One, Frickenhausen, Germany) were loaded with 100 μl of each twofold dilution (8 different concentrations, see range in Table 1). Addition of 400 μl inoculum to each well, prepared as previously described [27], was done in an anaerobic workstation (Invivo$_2$ 500, Ruskinn Technology, Bridgend, U.K.). Strains were tested in duplicate within panels, and positive and negative control wells were included. All panels were incubated for 4 days in the anaerobic atmosphere of the workstation (84% N_2, 8% CO_2 and 8% H_2) at 37°C, on a shaking platform (approximately 100 rotations per minute). The MIC was read after 4 days of incubation as the lowest concentration of the compound that prevented visible growth of *B. hyodysenteriae*, determined by comparing the visual turbidity of the wells to that of the positive control wells with an enlarging mirror. All growth control wells were also checked microscopically for purity [11]. Tests were repeated three times, with interexperimental variabilities of maximum one twofold dilution in obtained MIC value. MICs listed in Table 1 are the MIC values that were most common between the replications. The range in MIC values never exceeded the represented value plus or minus one twofold dilution. In addition, possible interactions between two products with high antibacterial effect, selected based on results of MIC-tests, were investigated. The inhibitory effect of seven, randomly chosen, binary combinations on growth of *B. hyodysenteriae* was tested on one occasion, using a checkerboard method [3]. In this method, dilutions of two products are combined in a two dimensional raster. Briefly, in 48-well plates, horizontally 5 wells of each row were filled with 50 μl of a twofold dilution of product A. Vertically, 50 μl of a twofold dilution of product B was added to five columns of the 48-well plate. In this way, 25 unique combinations of the two products A and B were obtained in a checkerboard type arrangement, as shown in Table 2. Control wells with twofold dilutions of only product A or only product B were included to verify MIC-values. Addition of inoculum and incubation of the panels occurred as described above. Tests were performed for one *B. hyodysenteriae* strain, 8dII, both at pH 7.2 and at pH 6.0. After 4 days, the lowest concentrations required to prevent growth were visually determined. To define actual interactions between products, the fractional inhibitory concentrations (FIC) for product A and product B were calculated

($FIC_A = \frac{MIC_{A\ with\ B}}{MIC_{A\ without\ B}}$ and $FIC_B = \frac{MIC_{B\ with\ A}}{MIC_{B\ without\ A}}$) for each of the wells on the growth-no growth interface.

The resulting FIC index ($=FIC_A+FIC_B$) in these wells was interpreted as follows [3, 14]. Synergism was presumed if the FIC index ≤ 0.5 and antagonism if the FIC index ≥ 4. If the FIC index was between these values ($0.5 <$ FIC index <4), the interactive effect was considered to be additive, meaning that the combined effect is equal to the sum of the effects of the individual compounds.

Minimum inhibitory concentration (MIC) values of the organic acids and essential oil components against *B. hyodysenteriae* are shown in Table 1. Between the organic acids tested at pH 7.2, a large difference in MIC values was observed, ranging from 320 mM for the least potent organic acids (formic, acetic and lactic acid) to 0.63 mM for lauric acid. The antibacterial activity was correlated with the length of the carbon chain: High MIC values were seen for all short chain fatty acids, whereas medium chain fatty acids showed lower inhibitory concentrations. The four essential oil components showed very low MIC values, ranging between 2.5 and 0.31 mM.

Between the three *B. hyodysenteriae* strains tested at pH 7.2, no significant differences in susceptibility could be observed. Inhibitory concentrations of all compounds were very similar for all three strains, and for each compound, a maximum difference in MIC value of one twofold dilution was observed (See Table 1). This can be considered as test variability, since a variation in endpoints of one twofold dilution is acceptable [11] and was also seen between repetitions in this study. Although the number of strains in this study was limited, distribution of the observed MICs for *B. hyodysenteriae* strains is suspected to be monomodal. Indeed, a lack of variation in susceptibility to fatty acids and essential oils between strains was also suggested for other bacteria [2, 9], and in contrast with antimicrobial agents,

Table 2. Binary combinations of organic acids and essential oil components at different concentrations tested in a checkerboard array and their lowest Fractional Inhibitory Concentration (FIC) Index (=FIC_A+FIC_B) against *B. hyodysenteriae* strain 8dII

Product A	(Tested range) (mM)	Product B	(Tested range) (mM)	Lowest FIC Index (FIC_A + FIC_B) at pH 7.2	Lowest FIC Index (FIC_A + FIC_B) at pH 6
Cinnamaldehyde	(1.25−0.08)	Butyric acid	(80−5)	0.56 (0.5 + 0.06)	N.D.
Cinnamaldehyde	(1.25−0.08)	Lauric acid	(1.25−0.08)	1.13 (1 + 0.13)	1.25 (1 + 0.25)
Capric acid	(2.5−0.16)	Thymol	(1.25−0.08)	0.63 (0.13 + 0.5)	0.75 (0.5 + 0.25)
Thymol	(1.25−0.08)	Carvacrol	(1.25−0.08)	0.5 (0.25 + 0.25)	0.38 (0.25 + 0.13)
Butyric acid	(80−5)	Lauric acid	(1.25−0.08)	0.63 (0.5 + 0.13)	0.75 (0.5 + 0.25)
Lauric acid	(80−5)	Carvacrol	(1.25−0.08)	0.63 (0.13 + 0.5)	0.75 (0.5 + 0.25)
Cinnamaldehyde	(1.25−0.08)	Carvacrol	(1.25−0.08)	0.56 (0.5 + 0.06)	1.06 (1 + 0.06)

N.D. = not done.

there have been no reports suggesting acquired resistance against organic acids or essential oils. Moreover, for a commercial product derived from garlic, no differences were found in the *in vitro* antimicrobial activity against 47 strains of *B. hyodysenteriae* with either high or low sensitivity to antibiotics [20]. Due to this comparable susceptibility between *B. hyodysenteriae* strains at pH 7.2, only strain 8dII was tested at pH 6.0. The observed MIC values of organic acids against *B. hyodysenteriae* strain 8dII at pH 6.0, were 4- to 8-fold lower compared to values at pH 7.2 (Table 1). This effect of pH has been described for other bacteria as well [2, 7] and is related to the proposed antibacterial working mechanism of acids. Undissociated acids are able to enter the bacterial cells, where they dissociate in the more alkaline interior and compromise bacterial homeostasis, resulting in cell death [19]. One exception was lauric acid: At pH 6.0, the MIC value of lauric acid against *B. hyodysenteriae* strain 8dII was only one twofold dilution lower than the MIC value at pH 7.2. For the essential oil components, there was no pH dependency of their antibacterial action against *B. hyodysenteriae*. Minimum inhibitory concentrations were the same at both pH values or were only one twofold dilution lower at pH 6.0 compared to pH 7.2. Although the inhibitory activity of organic acids against the growth of *B. hyodysenteriae* was enhanced at lower pH, the antibacterial activity of essential oil components and lauric acid appears to be less influenced by pH, which has also been observed by Skřivanová *et al.* for *Clostridium perfringens* [22].

The binary combination tests revealed a synergistic effect between thymol and carvacrol, both at pH 7.2 (lowest FIC Index=0.5, for the combination 0.31 mM carvacrol–0.31 mM thymol) and at pH 6.0 (lowest FIC Index=0.38, for the combinations 0.16 mM carvacrol–0.31 mM thymol and 0.31 mM carvacrol–0.16 mM thymol). For the other combinations of essential oil components or for the combinations between an organic acid and an essential oil component, only an additive effect could be observed with FIC Indices varying between 0.56 and 1.25 (Table 2).

Synergistic effects between thymol and carvacrol, as were observed in this study, have been described before for other bacteria [16]. Due to synergistic effects, it can be assumed that essential oils containing both thymol and carvacrol, like oregano or thyme oil [10], will have enhanced antibacterial activity against *B. hyodysenteriae*. However, a disadvantage of such essential oils is the variability in their composition by harvesting season or geographical location, etc. [29]. The combined use of two products with a merely additive antibacterial effect can also be advantageous. In this way, lower concentrations of each individual product can be combined in one feed additive. Since high concentrations of an aromatic substance in the diet of pigs can affect palatability and lead to decreased feed intake, lowering individual product concentrations can be preferable [24, 29].

Probably, mainly due to its fastidious growth characteristics, up till now, no information was available about susceptibility of *B. hyodysenteriae* to organic acids and essential oil components. During this *in vitro* study, interesting antibacterial properties against this anaerobic spirochete could be observed for some MCFA (capric and lauric acid) and essential oil components (thymol, carvacrol and cinnamaldehyde). These *in vitro* antibacterial properties suggest the potential of some products or product combinations, as alternatives to the commonly used antimicrobials, in control strategies for swine dysentery. Theoretically, for intestinal contents, if comparable to broth with pH 6.0, concentrations of 0.06 mg/m*l* lauric acid or 0.02 mg/m*l* cinnamaldehyde would have an antibacterial effect on present *B. hyodysenteriae* bacteria. Obviously, the *in vivo* situation is far more complex, and various factors can interfere with the luminal concentration and with efficacy of antibacterial compounds. Early absorption can at least in part be overcome by coating of feed additives [17]. In chickens, curative addition of 500 mg/kg coated cinnamaldehyde to the feed, proved to lower *Brachyspira intermedia* in the ceca [28]. Besides direct antibacterial effects, essential oils and organic acids can possibly have an indirect effect on pathogenic bacteria like *B. hyodysenteriae*, by changing gut conditions and intestinal microbiota [13]. Such indirect effects can influence the antibacterial effects of essential oils and organic acids *in vivo*. To evaluate the *in vivo* efficacy of (coated) products as part of a control strategy for swine dysentery, clinical trials should be performed, focusing primarily on lauric acid and cinnamaldehyde.

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