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

Lien VANDE MAELE1,2*, Marc HEYNDRICKX1,2, Dominiek MAES3, Nele DE PAUW1, Maxime MAHU1, Marc VERLINDEN1, Freddy HAESEBROUCK1, An MARTEL1, Frank PASMANS1 and Filip BOYEN1

1) Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820, Merelbeke, Belgium
2) Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit-Food Safety, Brusselsesteenweg 370, 9090, Melle, Belgium
3) Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820, Merelbeke, Belgium

(Received 9 June 2015/Accepted 2 September 2015/Published online in J-STAGE 14 September 2015)

**ABSTRACT.** The antibacterial potential of organic acids and essential oil components against *Brachyspira hyodysenteriae*, the causative pathogen of swine dysentery, was evaluated. Minimum inhibitory concentration (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 lactic 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

doi: 10.1292/jvms.15-0341; J. Vet. Med. Sci. 78(2): 325–328, 2016

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 hind-gut [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 27164T), 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.
Wells of 48-well culture plates (Greiner Bio-One, Frickenhausen, Germany) were loaded with 100 \( \mu l \) of each twofold dilution (8 different concentrations, see range in Table 1). Addition of 400 \( \mu l \) inoculum to each well, prepared as previously described [27], was done in an anaerobic workstation (Invivo2 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.

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

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

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 \( \mu l \) of a twofold dilution of product A. Vertically, 50 \( \mu l \) 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:

\[
FIC_A = \frac{MIC_{A \text{ with } B}}{MIC_{A \text{ without } B}} \quad \text{and} \quad FIC_B = \frac{MIC_{B \text{ with } A}}{MIC_{B \text{ without } A}}
\]

The resulting FIC index (=FIC\(_A\)+FIC\(_B\)) in these wells was interpreted as follows [3, 14]. Synergism was presumed if the FIC index \( \leq 0.5 \) and antagonism if the FIC index \( \geq 4 \). 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,
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 \textit{B. hyodysenteriae} with either high or low sensitivity to antibiotics [20]. Due to this comparable susceptibility between \textit{B. hyodysenteriae} strains at pH 7.2, only strain 8dII was tested at pH 6.0. The observed MIC values of organic acids against \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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/ml lauric acid or 0.02 mg/ml cinnamaldehyde would have an antibacterial effect on present \textit{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 \textit{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 \textit{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.

**ACKNOWLEDGMENT.** This research was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT Vlaanderen), Brussels, Belgium (Grant IWT LO-100850).
REFERENCES

1. Alvarez-Ordóñez, A., Martínez-Lobo, F. J., Argüello, H., Carvajal, A. and Rubio, P. 2013. Swine dysentery: aetiology, pathogenicity, determinants of transmission and the fight against the disease. *Int. J. Environ. Res. Public Health* 10: 1927–1947. [Medline] [CrossRef]

2. Boyen, F., Haesebrouck, F., Vanparys, A., Volf, J., Mahu, M., Van Immerseel, F., Rychlik, I., Dewulf, J., Ducatelle, R. and Pasman, F. 2008. Coated fatty acids alter virulence properties of *Salmonella* Typhimurium and decrease intestinal colonization of pigs. *Vet. Microbiol.* 132: 319–327. [Medline] [CrossRef]

3. Davidson, P. M. and Parish, M. E. 1989. Methods for Testing the Efficacy of Food Antimicrobials. *Food Technol.* 43: 148–155.

4. Dorman, H. J. D. and Deans, S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* 88: 308–316. [Medline] [CrossRef]

5. Glock, R. D., Harris, D. L. and Kluge, J. P. 1974. Localization of spirochetes with the structural characteristics of *Treponema hydysenteriae* in the lesions of swine dysentery. *Infect. Immun.* 9: 167–178. [Medline]

6. Hildalgo, A., Carvajal, A., Vester, B., Pringle, M., Naharro, G. and Rubio, P. 2011. Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of *Brachyspira hydysenteriae* in Spain. *Antimicrob. Agents Chemother.* 55: 3330–3337. [Medline] [CrossRef]

7. Hermans, D., Martel, A., Van Deun, K., Verlinden, M., Van Immerseel, F., Garmyn, A., Messens, W., Heyndrickx, M., Haesebrouck, F. and Pasman, F. 2010. Intestinal mucus protects Campylobacter jejuni in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids. *Poult. Sci.* 89: 1144–1155. [Medline] [CrossRef]

8. Hulánková, R. and Bořilová, G. 2011. In vitro combined effect of oregano essential oil and caprylic acid against *Salmonella* serovars, *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Listeria monocytogenes*. *Acta Vet. Brno* 80: 343–348. [CrossRef]

9. Hyldgaard, M., Mygind, T. and Meyer, R. L. 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front. Microbiol.* 3: 12. [Medline]

10. Karlsson, M., Oxberry, S. L. and Hamson, D. J. 2002. Antimicrobial susceptibility testing of Australian isolates of *Brachyspira hydysenteriae* using a new broth dilution method. *Vet. Microbiol.* 84: 123–133. [Medline] [CrossRef]

11. Li, S. Y., Ra, Y. J., Liu, M., Xu, B., Pérorn, A. and Shi, X. G. 2012. The effect of essential oils on performance, immunity and gut microbial population in weaner pigs. *Livest. Sci.* 145: 119–123. [CrossRef]

12. Michiels, J., Missotten, J. A. M., Fremaut, D., De Smet, S. and Dierick, N. A. 2009. In vitro characterisation of the antimicrobial activity of selected essential oil components and binary combinations against the pig gut flora. *Anim. Feed Sci. Technol.* 151: 111–127. [CrossRef]

13. Odds, F. C. 2003. Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother.* 52: 1. [Medline] [CrossRef]

14. Ohya, T. and Sueyoshi, M. 2010. In vitro antimicrobial susceptibility of *Brachyspira hydysenteriae* strains isolated in Japan from 1985 to 2009. *J. Vet. Med. Sci.* 72: 1651–1653. [Medline] [CrossRef]

15. Pei, R. S., Zhou, F., Ji, B. P. and Xu, J. 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. *J. Food Sci.* 74: M379–M383. [Medline] [CrossRef]

16. Per, I., Pizzamiglio, V., Morfachini, M., Tesedeschi, M. and Piva, G. 2007. Lipid microencapsulation allows slow release of organic acids and natural identical flavors along the swine intestine. *J. Anim. Sci.* 85: 486–493. [Medline] [CrossRef]

17. Pringle, M., Landén, A., Unnerstad, H. E., Molander, B. and Bengtsson, B. 2012. Antimicrobial susceptibility of porcine *Brachyspira hydysenteriae* and *Brachyspira pilosicoli* isolated in Sweden between 1990 and 2010. *Acta Vet. Scand.* 54: 54. [Medline] [CrossRef]

18. Ricke, S. C. 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poult. Sci.* 82: 652–639. [Medline] [CrossRef]

19. Rubio, P. 2012. Spanish experiences with swine dysentery. Proceedings of the 4th European Symposium of Porcine Health Management, II.011, pp. 92–94.

20. Rugna, G., Bonilauri, P., Carr, E., Bergamini, F., Luppi, A., Gherpelli, Y., Magistrali, C. F., Nigrelli, A., Albornoi, G. L., Martelli, P., La, T., Hampson, D. J. and Merialdi, G. 2015. Sequence types and pleuromutilin susceptibility of *Brachyspira hydysenteriae* isolates from Italian pigs with swine dysentery: 2003-2012. *Vet. J.* 203: 115–119. [Medline] [CrossRef]

21. Skrivanová, E., Marounek, M., Dlouhá, G. and Kaňka, J. 2005. Susceptibility of Clostridium perfringens to C2-C18 fatty acids. *Lett. Appl. Microbiol.* 41: 77–81. [Medline] [CrossRef]

22. Suryanarayana, M. V. A. N., Suresh, J. and Rajasekhar, M. V. 2012. Organic acids in swine feeding – A Review. *Agric. Sci. Res. J.* 2: 523–533.

23. Trevisi, P., Merialdi, G., Mazzoni, M., Casini, L., Tittarelli, C., De Filippi, S., Miniieri, L., Lalatta-Costerbosa, G. and Bosi, P. 2007. Effect of dietary addition of thymol on growth, salivary and gastric function, immune response, and excretion of *Salmonella enterica* serovar Typhimurium, in weaning pigs challenged with this microbe strain. *Int. J. Anim. Sci.* 6: 374–376.

24. Upadhayya, A., Upadhyaya, I., Kollanoor-Johny, A. and Venkitanarayanan, K. 2014. Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. *Biomed. Res. Int.* 2014: 761741. [Medline] [CrossRef]

25. van Duijkeren, E., Greko, C., Pringle, M., Baptiste, K. E., Catry, B., Jukes, H., Moreno, M. A., Pomba, M. C. M. F., Pyörälä, S., Rantalà, M., Ružauskas, M., Sanders, P., Teale, C., Threlfall, E. J., Torren-Edo, J. and Törnke, K. 2014. Pleuromutilins: use in food-producing animals in the European Union, development of resistance and impact on human and animal health. *J. Antimicrob. Chemother.* 69: 2022–2031. [Medline] [CrossRef]

26. Verlinden, M., Boyen, F., Pasman, F., Garmyn, A., Haesebrouck, F. and Martel, A. 2011. Antimicrobial susceptibility pattern of *Brachyspira intermedia* isolates from European layers. *Microb. Drug Resist.* 17: 485–488. [Medline] [CrossRef]

27. Verlinden, M., Boyen, F., Pasman, F., Garmyn, A., Haesebrouck, F. and Martel, A. 2013. In vitro sensitivity of poultry *Brachyspira intermedia* isolates to essential oil components and in vivo reduction of *Brachyspira intermedia* in rearing pullets with cinnamaldehyde feed supplementation. *Poult. Sci.* 92: 1202–1207. [Medline] [CrossRef]

28. Windisch, W., Schiedel, K., Pitzner, C. and Kromsmaer, A. 2008. Use of phytotherapeutic products as feed additives for swine and poultry. *J. Anim. Sci.* 86 Suppl: E140–E148. [Medline] [CrossRef]