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In-vitro evaluation of antimicrobial activity of selected organic acids against *E. coli*, *Salmonella* and *Staphylococcus*

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Abstract
In recent years, there is an increasing trend towards the use of organic acids as growth promoters by feed manufacturers and animal producers to substitute antibiotics. For their rational use in animal production, accurate screening of organic acids for their antimicrobial effect is essential. Therefore, the objective of the present study is to determine *in-vitro* antimicrobial activity of selected organic acids (citric acid, fumaric acid and benzoic acid) against certain gram-positive and gram-negative bacteria (*E. coli, salmonella* and *Staphylococcus*). Fumaric acid and benzoic acid exhibited more antimicrobial activity with lower inhibitory concentrations against gram -ve bacteria (*E. coli* and *Salmonella*), as compared to citric acid. ABST (antibiotic sensitivity test) indicated that *staphylococcus* was more sensitive to organic acids with greater zone of inhibition. Both MIC and ABST indicated a synergistic effect when combination of organic acids (1:1:1 ratio) were used against *E. coli*. The results of the current study indicated that the selected organic acids exhibited promising *in-vitro* antimicrobial effects against both gram - ve and gram + ve bacteria.

Keywords: Organic acids, anti-microbial effects, MIC, ABST, G -ve and G +ve bacteria

1. Introduction

Exploring the compounds that may partially or completely replace antibiotics which can promote growth, improve feed efficiency, and reduce incidence of enteric diseases in livestock has become an important topic for researchers (Yang *et al.*, 2019) [13]. Organic acids are one among such alternatives, which are familiarly known as acidifiers with proven benefits. Organic acids are approved and recommended by federal drug administration as safe substances (GRAS – Generally recognized as safe) (FDA, 2013) [4]. They have been used in feed industry for more than 50 years and are mainly approved as preservatives (FEFANA, 2014) [5] due to their antimicrobial activity. Apart from their importance as preservative, organic acids enhance performance through modulation of gastrointestinal tract mucosa and microbiome.

The most commonly used organic acids include formic acid, citric acid, benzoic acid, fumaric acid and salts of short-chain fatty acids (Liu *et al.*, 2018) [8]. Each organic acid has a distinguished range of pH, antimicrobial potential, carbon chain length, structure, degree of saturation and pKa values. In general, antimicrobial activity is quoted as one of the primary mechanisms of action by which organic acids could improve animal health (Long *et al.*, 2018) [9]. As per the recent reports, combinations of organic acids have been shown to have synergistic benefits for animal gut health and performance compared to the individual products (Yang *et al.*, 2019; Long *et al.*, 2018; Zentek *et al.*, 2013) [13, 9, 14].

Although significant health benefits have been noted, little information is available on *in- vitro* antimicrobial activity of organic acids in terms of MIC and ABST. Therefore, the objective of the current study is to determine the *in-vitro* antimicrobial activity of selected organic acids against gram -ve and gram +ve bacteria (*E. coli, salmonella,* and *staphylococcus*).

2. Materials and Methods

2.1 Preparation and standardization of inoculum

The bacterial species were precultured in nutrient broth and incubated at 37 °C for 24 hours. After 24 hours of incubation, the turbidity of the inoculum was adjusted by comparing the tubes with turbidity of 0.5 Mc Farland standard (1.5×10^8cfu/ml) (CLSI, 2012).

2.2 Preparation of known concentrations of organic acids

The stock solutions of each organic acid were prepared by dissolving 100 mg in 1 ml of DMSO (dimethyl sulphoxide) to obtain a concentration of 100 mg/ml.

2.3 Procedure – MIC (Minimum Inhibitory Concentration)

MIC was performed using polystyrene, 96-well clear microtiter plates by micro broth-dilution method. The wells were filled with 100 µl of cation adjusted muller hinton broth. It was followed by addition of 100 µl of organic acid solution to the first well. The serial dilution was carried by transferring 100 µl from first well to next and so on, discard 100 µl from the last well. Finally, 10 µl of bacterial culture was added to each well. Positive control contains 100 µL of broth and 10 µl of bacterial culture, while negative control contains 100 µL of broth alone. After incubation for 24 hours at 37 °C, iodonitrotetrazoline chloride (0.2% INT) was added to all wells and further incubated at 37 °C for half an hour. Microbial growth was observed by change in color of iodonitrotetrazolium chloride (pinkish red) in the microplate wells.

2.4 Antibiotic Sensitivity Test (ABST) by agar well diffusion method

Antimicrobial activity of selected organic acids against the selected bacterial species was determined by agar well diffusion technique under aseptic conditions. 20ml of sterilized molten muller hinton agar was poured in to a sterile petri plate and bacterial inoculum was swabbed on respective petri plates with the aid of sterile cotton swab. By using aseptic sterile tip, wells were made in inoculate plates which were finally filled with 100 µL of organic acids. The plates were incubated at 37 °C for 24 - 48 hours After incubation, the zone of inhibition was measured and expressed in terms of milli meter (mm).

3. Results and Discussion

A number of studies have proposed that organic acids are an interesting alternative to antibiotics use in veterinary medicine due to their recognized antibacterial activity. However, the data obtained is difficult to compare due to the difference in various methods used for assessing antibacterial activity, different solvents used for preparing stock solution of test compounds, molecular structure of the test compound and purity of test compounds involved in the procedure (Rammohan *et al.*, 2019) [11]. Moreover, they were tested in various concentrations against different strains of the same species of micro-organisms.

The results of current study confirmed that there is a considerable antibacterial activity for selected organic acids against the Gram -ve and Gram +ve bacteria.

Citric acid shown the MIC values of citric acid ranged from 1.562 to 6.25mg/ml (Table 1). It inhibited *E. coli* at a concentration of 3.125 mg/ml. In contrast to the present findings, Nagoba *et al.* (2008) [10] and Gao *et al.* (2012) [6] reported that the MIC values of citric acid against E. *coli* was 1.5 and 1.66mg/ml, respectively. MIC of Fumaric acid against *E. coli* was lower than inhibition concentration required for *Salmonella* and *Staphylococcus* (Fig 1). In contrast to the present findings, He *et al.* (2011) [7] observed lower MIC of Fumaric acid than present findings against. The reason for the differences in the inhibitory effect might be attributed to differences in the method of assay, pH of the medium and difference in strains of bacteria.

In the present study, synergistic effect was observed for the organic acids when used in combination only against *E. coli*. These observations are consistent with the results reported by Anacarso *et al.* (2017) [1] in which 37 *E. coli* strains were highly susceptible to a blend containing monolaurin and butyric acid. The synergistic effect of organic acids varies depending on the specific combination, proportion of organic acids used and type of bacteria.

Fumaric acid and benzoic acid have better antimicrobial effect than citric acid against gram -ve bacteria. This might be attributed due to difference in the molecular weights of the selected organic acids. The presence of outer bilipid layer in gram -ve bacteria makes organic acids difficult to enter bacterial cell (Exner *et al.*, 2017) [3]. The molecular weights of fumaric acid and benzoic acid were low such that they can enter the bacterial cell easily when compared to citric acid.

The results of ABST (Table 2 & Fig 2) indicated that *staphylococcus* is more sensitive to organic acid than *E. coli* and *salmonella*. The reason might be due to the fact that absence of outer bilipid layer in gram +ve bacteria, so that organic acids can easily enter the bacterial cell and exert their antimicrobial effect (Exner *et al.*, 2017) [3].

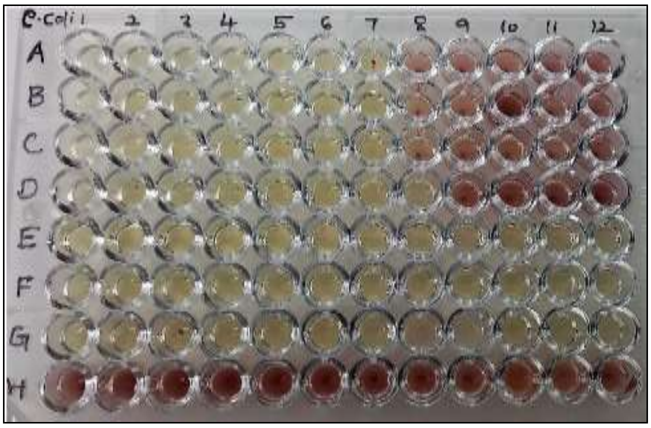
Comparison of the results with the available literature exhibited that the level of sensitivity of the bacterial species to organic acids is quite diverse and strongly depends not only on the bacterial species but also on the strains with in species under the study.

Table 1: MIC of selected organic acids against *E. coli*, *Salmonella* and *Staphylococcus*.

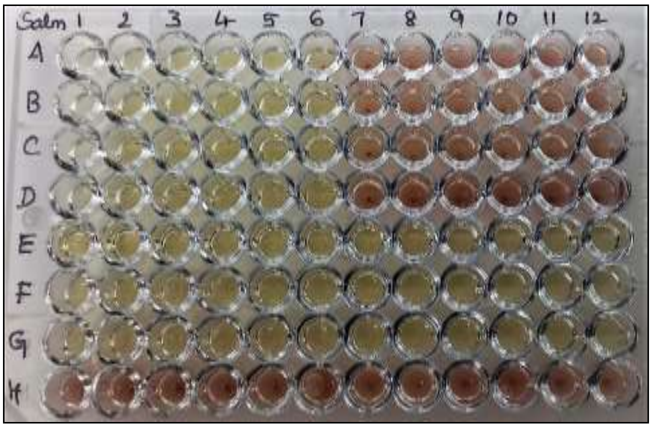
Organic acids	Minimum inhibitory concentration "mg/ml"		
	E. coli	***Salmonella***	***Staphylococcus***
Citric acid	3.125	6.25	1.562
Fumaric acid	1.562	3.125	3.125
Benzoic acid	1.562	3.125	3.125
Combination of acids (1:1:1)	0.781	3.125	3.125

Table 2: ABST of selected organic acids against *E. coli*, *salmonella* and *staphylococcus*.

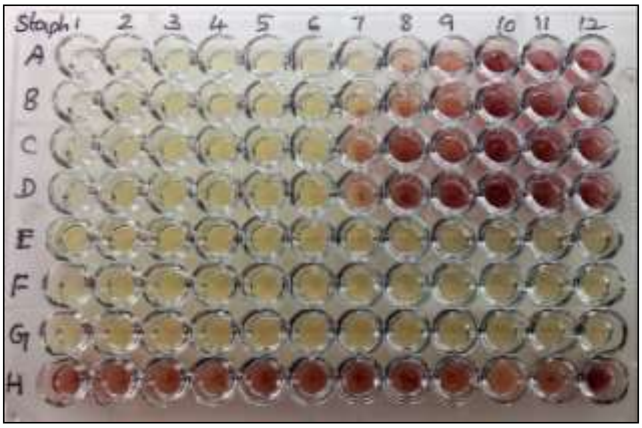
Organic Acids (100mg concentration)	Zone of inhibition in "mm"		
	E. coli	***Salmonella***	***Staphylococcus***
Citric acid	21	23	34
Fumaric acid	22	24	35
Benzoic acid	21	23	32
Combination of acids (1:1:1)	23	24	35



E. coli



Salmonella



Staphylococcus

Microtitre plates (Fig. 1) showing inhibition of *E. coli, Salmonella and Staphylococcus* growth on serial dilution of organic acids
In all the three microtitre plates,

- Row A, B, C and D : 100 µl of broth + 10 µl of selected bacterial culture (*E. coli, Salmonella* and *Staphylococcus*) + 100 µl of citric, fumaric, benzoic and combination of three organic acids serial dilution from 10mg to 0.0048mg, respectively.
- Row F: containing 100 µL of broth and 10 µl of DMSO reagent.
- Row G: negative control contains 100 µL of broth alone.
- Row H: Positive control containing 100 µL of broth and 10 µl of bacterial culture.

Fig 1: MIC of organic acids against *E. coli*, *salmonella* and *staphylococcus*

Ci: citric acid; B: benzoic acid; F: fumaric acid and
Co: combination of three organic acids

Fig 2: ABST of organic acids against *E. coli*, *salmonella* and *staphylococcus*

4. Conclusion

The present research confirmed the antibacterial activity of selected organic acids individually as well as in combination against *E. coli, salmonella* and *staphylococcus*. The results of MIC concluded that the fumaric acid and benzoic acid were more effective against gram -ve bacteria. The synergistic effect is observed (when organic acids are used in combination) only against *E. coli* but not on *salmonella* and *staphylococcus*. These compounds with promising *in-vitro* antimicrobial activities may present a feasible alternative to antibiotic growth promoters in animal feed. It is speculated that bacteria do not develop resistance to organic acids as they have for antibiotics. However, more research must be conducted to confirm this speculation. More *in-vivo* research in livestock animals is necessary to test the efficacy of these organic acids as alternatives to antibiotics in feed, to validate these results.

5. References

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