In vitro susceptibility of swine pathogens to feed additives and active ingredients with potential as antibiotic replacements.

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ABSTRACT

Aims

The rise in antibiotic resistance requires the reduction of antibiotic use in all sectors. In animal production, many commercial alternatives to antibiotics have been developed for incorporation into feeds, but a lack of evidence on their antibacterial activity limits confidence in their application. We aim to compare the antibacterial activity of feed additives and active ingredients to better understand their usefulness.

Methods and Results

The antibacterial activity of thirty-four active ingredients and feed additives, including medium and short chain organic acids and essential oils, were tested against pure cultures of five bacterial swine pathogens. Antibacterial activity was observed using an agar-plug diffusion method and quantified via broth-microdilution.

A diverse range of antibacterial activities were observed. The highest inhibitory activity against *S. aureus* and *S. suis* was exhibited by the C12 monoglyceride (0.49 mg ml^{-1}). The monoglyceride of C12 was more effective than C12:0 against *S. suis*, but neither C12:0 nor its monoglyceride showed efficacy against the gram-negative microorganisms tested. The most active against *E. coli* were the C6:0 MCOA and potassium diformate (1.95 mg ml^{-1}). For S. Typhimurium*;* potassium diformate, sodium diformate, and a blend of C8:0/C10:0 (each 1.96 mg ml^{-1}), and for *A. pleuropneumoniae;* eugenol (0.49 mg ml^{-1}), showed the most promising activity.

Conclusions

We identified broad-spectrum antibacterial activity, such as the C6:0 MFOA, and those with interesting narrow-spectrum activity, notably the killing of *S. suis* by C12 monoglyceride. We have identified additives which show the most promising bioactivity against specific pathogens.

Significance and Impact of the study

We broadly compare a large collection of feed additives and active ingredients for their antibacterial activity against a diverse panel of bacterial swine pathogens. This provides a solid base of evidence which can drive the development of feed supplementation strategies with the aim of reducing dependency on antibiotic use in swine production.

Key words: Swine pathogens, Pigs, Antimicrobial Feed additives, *Streptococcus suis, Escherichia coli, Salmonella* Typhimurium*, Staphylococcus aureus, Actinobacillus pleuropneumoniae*

INTRODUCTION

It is widely recognised that the use of antibiotics in agriculture, and especially in swine production must be reduced to slow the rates at which bacteria are developing antimicrobial resistance. Antimicrobial resistance (AMR) occurs through genetic mutation or by acquiring resistance genes from another bacterium, spreading both vertically, when new generations of bacteria inherit AMR genes, and horizontally, when bacteria share or exchange sections of genetic material with other bacteria, including bacteria of other species (Munita and Arias 2016). Environmentally, AMR spreads as the bacteria themselves move spatially, for example in sewage and waste. Resistance continues to emerge and is found in swine production systems at unacceptably high rates (Zhang et al. 2019; Holmer et al. 2019; Pollock et al. 2020).

Bacterial infections cause a large proportion of losses in pig production. Several bacterial species receive global attention, including *Salmonella* spp., *Escherichia coli, Streptococcus suis, Staphylococcus aureus* and *Actinobacillus pleuropneumoniae* (VanderWaal and Deen 2018). Several of these are zoonotic, and there is growing evidence and concern that antibiotic-resistant pathogenic bacteria can be transferred directly through the meat to cause critical, untreatable illness to consumers (EFSA 2020). S*almonella enterica* and pathogenic strains of *Escherichia coli are* members of the gram-negative *Enterobacteriaceae,* which, in addition to causing diarrhoea and gastrointestinal symptoms in pigs, are a common cause of food poisoning in humans. The organisms can be shed by asymptomatic carriers, are easily transmitted to other pigs, and ultimately transferred to retail pork (Zhao et al. 2001; Hansen et al. 2010). *Streptococcus suis*, a gram-positive bacterium, is a cause of septicaemia, meningitis, arthritis and death in pigs, and an emerging threat to humans, where it can also cause meningitis and septicaemia with serious complications (Goyette-Desjardins et al. 2014; Rayanakorn et al. 2018). *Staphylococcus aureus*, also a gram-positive bacterium can cause abscess, mastitis and other skin conditions in pigs. Antibiotic resistant strains of *S. aureus* can cause serious infection and death in humans. Livestock associated strains, such as ST398 clonal complex have been isolated from both pigs and livestock workers, suggesting that mammalian species could act as a reservoir for problematic antibiotic resistant infections in humans (Khanna et al. 2008, Witte et al. 2013). *Actinobacillus pleuropneumoniae*, a gram-negative facultative anaerobic bacterium, is a respiratory pathogen of pigs, leading to dyspnea, pneumoniae and other complications (Hričínová et al. 2010).

The UK's pig sector is still heavily dependent on prophylactic (preventative) and curative antibiotic use, despite progress in recent years to reduce antibiotic dependency. Antibiotics are routinely administered to groups of pigs in medicated feedingstuffs for a two-week period after weaning, often to prevent the general poor growth performance that accompanies subclinical levels of infection. The European Union's revised Medicated Feed Regulations, EU

2019/4, enters into force on January 28th, 2022, and marks an end to such prophylactic use of antibiotic without veterinary approval (Legislation.gov.uk. 2021). Antibiotics will no longer be permitted for prophylactic use except in very exceptional cases, and only to treat individual animals. However, the cost of endemic pig disease is estimated at around £190-250 million/annum in the UK (ProHealth FP7 consortium) and could increase if antibiotics are withdrawn without viable alternatives. It is recognised and accepted that antibiotic use in swine production must be substantially reduced. To reduce and replace antibiotics without negatively affecting animal welfare or production, safe and practical alternatives to in-feed medications are urgently needed. There has been a drive in the past decade to develop so-called alternatives to antibiotics including short-chain organic acids (SCOA), medium-chain organic acids (MCOA), monoesters of MCOA, other acids, essential oils and plant extracts (Diaz-Sanchez et al. 2015; Omonijo et al. 2018; Jackman et al. 2020). Many of these have direct antimicrobial effects; however, the published literature gives conflicting information about their efficacy. Lack of information and evidence is a key factor contributing to reluctance in their use. The current work was undertaken to evaluate and compare the *in vitro* antimicrobial activity of additives and active ingredients against a panel of bacterial pathogens of concern in swine, aiming to provide a solid base of evidence for strategies that can potentially replace antibiotics in swine production.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The activity of feed additives and active ingredients were tested for their bioactivity against pure cultures of relevant bacteria. *Staphylococcus aureus* ATCC25923, *Streptococcus suis* LMG 14181, *Escherichia coli* NCTC 10418, *Salmonella enterica* subsp. *enterica* serovar Typhimurium NCTC 74 and *Actinobacillus pleuropneumoniae* NCTC 11384 strains were used as representative swine pathogens. *S. aureus, E. coli* and *S.* Typhimurium were cultured aerobically on nutrient agar at 37 °C overnight. *S. suis* was cultured under microaerophilic conditions on Brain Heart Infusion (BHI) agar at 37 °C overnight. *A. pleuropneumoniae* was cultured under microaerophilic conditions on blood agar base supplemented with 7% defibrinated horse blood at 37 °C for approximately 48 hours. Microaerophilic conditions were established using the Anaerocult C mini system (Merck Millipore). Liquid cultures of bacteria were prepared in Mueller-Hinton II broth (*E. coli, S. aureus, S. suis,* and *S.* Typhimurium) or Mueller-Hinton fastidious broth medium with yeast extract (*A. pleuropneumoniae*), (Adams 2019), and incubated with shaking at 150 rpm overnight.

Compound Preparation

A total of 34 feed additives or active ingredients were tested and were grouped into four categories (medium chain organic acids, short chain organic acids, essential oils, & others) (Table 1). Compounds were stored at room temperature out of direct sunlight except for the blend of monoterpenes/ organic acids (sample no. 8) which was stored at 4 °C. Antimicrobial activity of each compound was tested by addition of each to broth or agar as required. pH measurements were made using a Hanna pH meter from 1 g of each compound diluted to 16 ml in Mueller-Hinton broth with 0.4 % polysorbate 80.

Agar Plug Diffusion

Antimicrobial activity of the test ingredients was determined using an agar plug diffusion assay (Balouiri et al. 2016), since this approach best accommodated the diverse range of samples. Overnight liquid cultures of bacteria were used to prepare confluent bacterial lawns on nutrient agar plates (*S. aureus*, *E. coli* and *S.* Typhimurium) or BHI agar plates (*S. suis*). Test samples were prepared by dissolving 1 g of each into 3 g molten BHI agar or nutrient agar, as appropriate generating a 25 % (w/w) starting suspension. Three holes were bored into each inoculated agar plate using a sterile cork borer, and 100 µl of each 25 % compound / agar suspension added to each well and allowed to solidify. Plates were incubated at 37 °C overnight.

Broth Microdilution Assays

Quantitative analysis of the inhibitory activity of the active ingredients against the panel of bacterial pathogens was carried out using triplicate broth microdilution assays. The method employed relevant CLSI recommendations (CLSI 2018). A stock solution of each test ingredient was prepared by adding 1 g of each to 16 g with Mueller-Hinton II broth with 0.4 % polysorbate 80, resulting in a 6.25 % starting suspension (62.5 mg ml^{-1}). This stock solution was used to prepare a 2-fold dilution series in 96-well microtiter plates, using Mueller-Hinton II broth with 0.4 % polysorbate 80 as the diluent, with a final volume of 100 µl per well. The dilution series was prepared in triplicate. To each well, 50 µl of Mueller-Hinton broth containing a bacterial suspension of the test organism, a 1/100 dilution prepared from a culture standardised to 0.5 McFarland (approximately 1.5 x 10^8 cfu per mL). Inoculated plates were incubated in sealed bags with a source of moisture overnight, at 37 ºC.

Following incubation, 0.015 % resazurin was added to each well and further incubated for 2 hours to generate a clearly visible colour change in the presence of viable bacteria (Elshikh et al. 2016). Minimum inhibitory concentrations were determined as the highest concentration of active test ingredient in which growth was not observed. For *A. pleuropneumoniae*, due to the colour of the Mueller-Hinton fastidious broth with yeast, determination of colour change with resazurin was not possible. For this organism, minimum bactericidal concentration was determined as the highest concentration of test ingredient in which bacterial growth was not observed following the transfer of 3 x 10 µl aliquots from test wells onto blood agar and overnight incubation.

RESULTS

Results of the broth microdilution assay are shown in Table 2. All triplicate samples gave identical readings (for the same sample). In general, the microdilution assay results were in good agreement with the results of the agar plug diffusion assay (Figure 1). Of the 34 samples tested, 11 showed no or little efficacy against any of the five bacteria. These were the alpha and beta pinenes (from two different sources), galangal oil, glycoside A and B, methoxybenzene, calcium and sodium butyrate and zinc oxide.

The agar plug diffusion assay results for the MCOA against *E. coli*, *S. suis*, *E. coli* and *S.* Typhimurium are presented (Figure 1a and Figure 1b). The zones of clearing surrounding wells filled with agar/MCOA combinations indicated that the antibacterial activity of each compound was different towards each species of bacteria. Both sources of C6:0 medium chain acids showed broad inhibitory activity, with distinct zones observed for each of the four pathogens tested by this method. For *E. coli*, *S. aureus* and *S.* Typhimurium, the size of the clearing zone decreased with increasing organic acid chain length; little activity was observed with C8:0, C10:0 or C12:0, although C12:0 did show inhibitory activity against *S. aureus*. Both composite blends of C8:0 and C10:0 appeared to show increased efficacy against the pathogens, compared to the individual organic acids (Figure 1b) with the blend containing the higher percentage of C8:0 (90%) showing higher efficacy against *S.* Typhimurium and *E. coli* than the blend containing the lower percentage (60%).

Results of the agar plug diffusion assay for the monoglycerides against the pathogens are given (Figure 1c). The monoglyceride of C6 appeared to show lower inhibitory activity against the bacteria tested, compared to the corresponding tri-esterified fatty acids. Conversely, the monoglyceride of C12 showed much higher efficacy against the gram-positive microorganisms *S. suis* and *S. aureus*, than the corresponding tri-esterified fatty acids. The C12 monoglyceride was up to 16 times more effective against *S. aureus,* and the difference was substantially

greater against *S. suis,* although there were differences between the sources. The monoglyceride of C9 showed strong inhibitory activity against the bacteria with the exception of *E. coli* against which it showed little efficacy.

Figure 2 and Figure 4 give the results of the agar plug diffusion assay for the short chain organic acids. Sodium and potassium diformate showed broad efficacy against the bacteria; additionally, sodium diformate, as well as benzoic acid, showed strong efficacy against *S. suis*. Results of the agar plug diffusion assay for the essential oils are given in Figure 3. Of the essential oils that showed efficacy, eugenol showed the strongest activity against the microorganisms tested while oregano oil was also efficacious against both the gram-positive and gram-negative microorganisms. Terpene showed good bioactivity against *S. suis* but only low efficacy against the other bacteria tested.

Comparing the series of active ingredients, the highest inhibitory efficacy against *S. aureus* and *S. suis* was achieved with the monoglycerides of C12 (0.49 mg ml^{-1}), followed by the blends of C8:0/C10:0, the corresponding monoglyceride blend, and the monoglycerides of C9. The highest inhibitory activity against *E. coli* was seen with C6:0 (from source 1; 1.95 mg ml^{-1}), and potassium diformate (1.95 mg ml^{-1}), while the highest activity against *S.* Typhimurium was seen with sodium and potassium diformate and the blend of C8:0 (90%)/ C10:0 (10%) (all 1.95 mg ml^{-1}). The strongest inhibitory activity against *A. pleuropneumoniae* was shown by eugenol (0.49 mg ml^{-1}), followed by oregano oil, both blends of C8:0/C10:0 and the corresponding monoglyceride blend, the C9 monoester, and one of the C6:0 sources.

DISCUSSION

The study achieved a systematic analysis of the antimicrobial activity of a range of different feed additives and active ingredients against a panel of pathogenic bacteria of significance in swine production. A key aspect of the development of the work was establishing a ‘catch-all’ testing regime that could incorporate the physiochemical range of the active ingredients, including powdery solids, waxy solids, and liquids of varying viscosity (Table 1). It proved challenging to establish common conditions in which each of these compounds could be included in the assay; solubility was variable, and for some compounds, saturated suspensions had to be used. The solubility, and ability to diffuse through agar, may have affected the results observed in the agar-well-diffusion assay. Observed differences in bioactivity of the same compound between the two assays may be explained by differences in the ability of these compounds to diffuse through agar-plugs, or their insolubility in the chosen solvent. In general, though, there was good agreement between the two different assays. A factor that would influence bioactivity is the pH of prepared solutions. We recorded

the pH of our starting solutions (Table 1), but did not control for pH in our experiments, deciding instead to test the additives as provided. We see a range of pH levels, expected for the diversity of active ingredients tested, but are therefore unable to differentiate between pH effects or other more specific modes of action. The data we provide is useful in identifying active ingredients and feed additives with bioactivity, but further studies would be required to establish precise mechanisms of action at different pH.

The diversity of bacterial pathogens included in our experiments, and variation in growth conditions, including agar preference, growth time and oxygen requirements, also presented challenges in developing optimal conditions for the tests. The inclusion of *Actinobacillus* species in the agar-well-diffusion method was not possible due to lysis of blood in the agar, which impacted on the ability to observe bacterial zones of clearing, again indicating the difficulties in generating a standard set of experimental conditions for comparison when such diverse organisms and compounds are included. Detailed analysis would require optimum assay conditions for each compound and pathogen pair to be established, and broader comparisons outside of these pairs would be challenging.

The lack of direct antimicrobial activity as measured *in vitro* for 10 of the compounds does not necessarily indicate a lack of activity in the live animal. Many of the active ingredients function at the gastrointestinal level and may have an indirect influence on microbial populations therein. Sodium and calcium butyrate showed no inhibitory activity in these tests but in piglets, butyrates have been demonstrated to increase intestinal villus height (Kotunia et al. 2004), increase mucosa thickness (Kien et al. 2007), and increase the number of parietal and enteroendocrine cells (Mazzoni et al. 2008). Zinc oxide also showed little activity in the assays yet is used routinely to prevent intestinal infection of young animals at weaning. While the mode of action is not clearly defined, it may participate in the stabilization of epithelial cell membrane (Srivastava et al. 1995) and has been shown to alter the enterobacterial population in the ileum of piglets (Vahjen et al. 2011; Starke et al. 2014). It has been proposed that the influence on the gastrointestinal microbiota may be through suppression of gram-positive commensal microorganisms rather than through suppression of gram-negative organisms (Hojberg et al. 2005). Lack of bactericidal activity does not necessarily imply that the compounds do not have an effect on the bacteria, as changes in gene expression in the surviving bacteria may be sufficient to reduce infection burden. Sub-lethal concentrations of fatty acids have been shown to alter gene expression and decrease the virulence and shedding of *S.* Typhimurium in pigs, thereby having an indirect effect on pathogenicity (Boyen et al. 2008).

Our observations did not support the proposal (Jackman et al. 2020) that the MCOAs have greater potency against gram-positive than gram-negative bacteria due to differences in cell

membrane architecture. Only C6:0 showed activity against gram-positive as well as gram-negative microorganisms, whereas C8:0. C10:0 and C12:0 inhibited only the gram-positive microorganisms, with C12:0 showing activity against *S. aureus* exclusively. It remains difficult to make broad conclusions based on the length of the fatty acid chains, as the activity is dependent on the microorganism, and in our opinion may reflect more complex species-specific differences that cannot be explained by cell membrane architecture alone. The markedly increased efficacy of the C8:0/ C10:0 blends against all of the microorganisms was likely to be a reflection of the individual organic acids having different efficacy depending on their source/ supplier rather than a synergistic effect. Our efforts to replicate the blend from the individual organic acids which originally showed rather low activity did not lead to increased efficacy. Results showing that the monoester of C12 was more effective against *S. suis* than the corresponding C12:0 was broadly supportive of previous work which demonstrated > 3 log CFU/ ml reduction against *S. aureus* and *Streptococcus pyogenes* in broth cultures (Schlievert and Peterson 2012) although it was not possible to make a direct comparison of dilutions from the current work. The difference in inhibitory efficacy between the C12:0 and the monoglyceride of C12 supports the understanding that the C12 monoglyceride is much more effective as an antimicrobial than the C12:0 fatty acid. It was clear from this work that the C12 monoglyceride was not effective against the gram-negative bacteria *S.* Typhimurium, E. *coli* and *A. pleuropneumoniae;* these findings were consistent with a lack of activity against *E. coli* and *Salmonella minnesota* (Schlievert et al. 1992). The specificity of the C12 monoglyceride to gram-positive bacteria has been previously established and is thought to involve the targeting of many bacterial surface signal transduction systems non-specifically through interaction with plasma membranes, as well as inhibition of exotoxin production by gram-positive bacteria at concentrations that do not inhibit bacterial growth (Schlievert et al. 1992; Projan et al. 1994; Vetter et al. 2005), properties in common with the antibiotic clindamycin, a protein synthesis inhibitor (Schlievert and Kelly 1984).

Both diformates showed a broad activity against the bacteria tested, additionally sodium, but not potassium diformate showed a high efficacy against *S. suis.* The direct antimicrobial mode of action of formic acid and its salts is thought to be common with other short and medium chain organic acids (as reviewed by Kovanda et al. 2019) and involves penetration of the undissociated molecule across the bacterial cell membrane, dissociation and reduction of cytoplasm pH and eventual disruption and death of the bacterial cell. The relatively narrow-spectrum activity of benzoic acid was unexpected. Benzoic acid is authorised in the European Union as a zootechnical additive (Regulation EU 2020/1031) and studies submitted during the approval process (EFSA, 2005) demonstrated a 93 % reduction in *E. coli* ($P<0.05$), 89 % reduction in total *Enterobacteriaceae spp* ($p=0.06$) and an 81 % reduction in coliforms ($p=0.06$)

in the caecum of pigs was seen. Such differences demonstrate the importance of following up in vitro assays with *in vivo* microbiology testing, as extrapolation should never be assumed. The minimum inhibitory values obtained against *A. pleuropneumoniae* with several of the compounds, especially eugenol are of particular interest as this microorganism is commonly resistant to antibiotics. In a study using 68 different isolates, 23 (33.8 %) were resistant to one or more antibiotics (Yoshimura et al. 2002).

Our results allow the compounds to be classified in terms of their inhibitory activity; such a classification is immediately useful to the swine production industry, as it allows decisions to be made to avoid unnecessary use of broad-spectrum antibacterial additives (which may also have unintended consequences for the microbiome of the pig). Identification of narrow spectrum feed additives is very promising as potential targeted interventions for the reduction of problematic pathogens. Notably, the C12 monoglycerides and benzoic acid were identified as having narrow-spectrum activity against *S. suis*. This finding is worthy of further *in vivo* studies to confirm whether their use leads to a concurrent reduction in *S. suis* carriage and subsequent disease. It remains to be determined whether the active ingredients that are proposed to replace antibiotics may themselves create a potential for the development of cross-resistance to other non-susceptible bacteria, or if sublethal concentrations may lead to tolerance. Such unintended consequences could have a detrimental impact on the development of cross-resistance to clinical antibiotics if used widely in pig feeds. It has been proposed that the MCOA have a relatively low propensity to create antimicrobial resistance due to their non-specific mode of action (Desbois and Smith 2010), and this point deserves further investigation.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Conceptualisation: CJ, NP. Data curation: CJ, CN. Formal analysis: CJ, NP, CN. Funding Acquisition: CJ, NP. Investigation: CJ, CN. Methodology: CJ, CN. Project Administration: CJ, CN, NP. Resources: CJ, NP. Supervision: CJ, NP. Validation: CJ, NP, CN. Visualisation: CJ, CN. Writing- Original Draft Preparation: CJ, CN, NP. Writing – Review & Editing: CJ, CN, NP. All authors read and approved the manuscript.

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Table 1: Additives and active ingredients tested in the study. The physical state of each additive and active ingredient was observed for the undiluted stock at room temperature, while pH was measured for a 1 in 16 dilution from the stock in Mueller-Hinton Broth.

No.	Active ingredient	Physical state (RT)	pH	Solubility in Mueller-Hinton broth
Short chain organic acids				
1	Calcium Butyrate	Solid	8.7	Dissolves completely, cloudy solution
2	Sodium Butyrate	Solid	9.8	Dissolves completely, clear solution
3	Sodium Diformate	Solid	3.5	Not completely soluble
4	Potassium Diformate	Solid	3.6	Not completely soluble
5	Monoglycerides of C3	Liquid	6.2	Soluble, clear solution
6	Monoglycerides of C4	Liquid	6.5	Soluble, cloudy solution.
7	Monoglycerides of C5	Liquid	6.3	Soluble but quickly settles
8	organic acid/ monoterpene	Liquid	4.5	Soluble, milky solution
Medium chain organic acids				
9	C6:0 (1)	Liquid	4.1	Soluble to cloudy solution, settles slightly eventually
10	C6:0 (2)	Liquid	4.0	Soluble, cloudy solution, some settling at upper
11	Monoglycerides of C6	Liquid	3.8	Soluble, clear solution
12	C8:0	Liquid	4.8	Soluble, milky solution
13	Blend C8:0 (90%) /C10:0	Liquid	7.0	Soluble, milky solution
14	Blend C8:0 (60%) / C10:0	Liquid	7.1	Soluble, milky solution
15	Monoglycerides of C8/C10	Liquid	6.5	Soluble, cloudy solution
16	Monoglycerides of C9	Liquid	5.9	Soluble, cloudy solution
17	C10:0	Solid	5.2	Soluble, milky solution
18	C12:0	Solid	6.3	Not completely soluble
19	Monoglycerides of C12 Dry (1)	Solid	7.1	Not completely soluble
20	Monoglycerides of C12 L (2)	Solid	5.9	Not completely soluble
21	Monoglycerides of C12 Dry (2)	Solid	7.1	Not completely soluble
Essential oils				
22	Alpha pinene (1)	Liquid	6.4	Initially dissolves to cloudy solution, eventually settles into layers
23	Alpha pinene (2)	Liquid	7.0	Soluble, cloudy solution
24	Beta pinene (1)	Liquid	7.1	Initially dissolves to cloudy solution, eventually settles into layers
25	Beta pinene (2)	Liquid	7.1	Soluble, cloudy solution, upper phase separates to thin layer
26	Eugenol	Liquid	7.0	Initially dissolves to cloudy solution, eventually settles into layers
27	Oregano oil	Liquid	7.2	Soluble, milky solution, thin upper layer separates
28	Galangal Oil	Liquid	6.9	Soluble cloudy solution
29	Terpene	Liquid	7.1	Soluble, cloudy solution, separates over time
30	Methoxybenzene	Liquid	7.0	Soluble, cloudy solution, separates over time
Other active ingredients				
31	Glycoside A	Liquid	6.2	Soluble, clear brown solution
32	Glycoside B	Solid	5.5	Soluble, dark brown, thick solution
33	Zinc oxide	Solid	8.3	Insoluble
34	Benzoic acid	Solid	3.9	Insoluble

476 **Table 2**: Minimum Inhibitory Concentrations of the additives and active ingredients

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		S. aureus	*S. suis*	*A. pleuro-pneumoniae*	*S.* Typhimurium	*E. coli*
		Minimum inhibitory concentration mg ml^{-1}				
Short Chain Organic Acids						
1	Calcium Butyrate	>31.25	>31.25	31.25	>31.25	31.25
2	Sodium Butyrate	>31.25	>31.25	>31.25	>31.25	>31.25
3	Potassium Diformate	1.95	1.95	1.95	1.95	1.95
4	Sodium Diformate	3.91	0.98	1.95	1.95	3.91
5	Monoglycerides of C3	>31.25	15.63	31.25	>31.25	>31.25
6	Monoglycerides of C4	31.25	31.25	15.63	15.63	31.25
7	Monoglycerides of C5	15.63	7.81	7.81	15.63	15.63
8	Monoterpene/ SCOA	7.81	7.81	7.81	7.81	15.63
Medium Chain Organic Acids						
9	C6:0 (1)	3.91	1.95	1.95	3.91	1.95
10	C6:0 (2)	3.91	1.95	0.98	3.91	3.91
11	Monoglycerides of C6	7.81	7.81	7.81	7.81	15.63
12	C8:0	3.91	3.91	1.95	7.81	7.81
13	C8:0 (90%)/ C10:0	0.98	0.98	0.98	1.95	3.91
14	C8:0 (60%)/ C10:0	0.98	0.49	0.98	3.91	15.63
15	Monoglycerides of C8/C10	0.98	0.98	0.98	3.91	15.623
16	Monoglycerides of C9	0.98	0.98	0.98	1.95	>31.25
17	C10:0	3.91	0.98	31.25	31.25	>31.25
18	C12:0	7.81	31.25	>31.25	>31.25	>31.25
19	Monoglycerides of C12 (1)	3.91	0.49	>31.25	>31.25	>31.25
20	Monoglycerides of C12 (2)	0.49	0.49	7.81	>31.25	>31.25
21	Monoglycerides of C12 (90)(2)	7.81	7.81	>31.25	>31.25	>31.25
Essential Oils						
22	Alpha Pinene (1)	>31.25	>31.25	>31.25	>31.25	31.25
23	Alpha Pinene (2)	>31.25	>31.25	>31.25	>31.25	>31.25
24	Beta Pinene (1)	>31.25	>31.25	>31.25	>31.25	31.25
25	Beta Pinene (2)	>31.25	>31.25	>31.25	>31.25	>31.25
26	Eugenol	3.91	0.98	0.49	3.91	3.91
27	Oregano Oil	7.81	1.95	0.98	3.91	3.91
28	Galangal Oil	31.25	>31.25	31.25	>31.25	>31.25
29	Terpene	7.81	1.95	15.63	7.81	7.81
30	Methoxybenzene	>31.25	>31.25	>31.25	>31.25	31.25
Other active ingredients						
31	Glycoside A	>31.25	>31.25	>31.25	>31.25	>31.25
32	Glycoside B	>31.25	>31.25	>31.25	>31.25	>31.25
33	Zinc Oxide	>31.25	>31.25	15.63	>31.25	>31.25
34	Benzoic Acid	15.63	0.98	3.91	15.63	15.63

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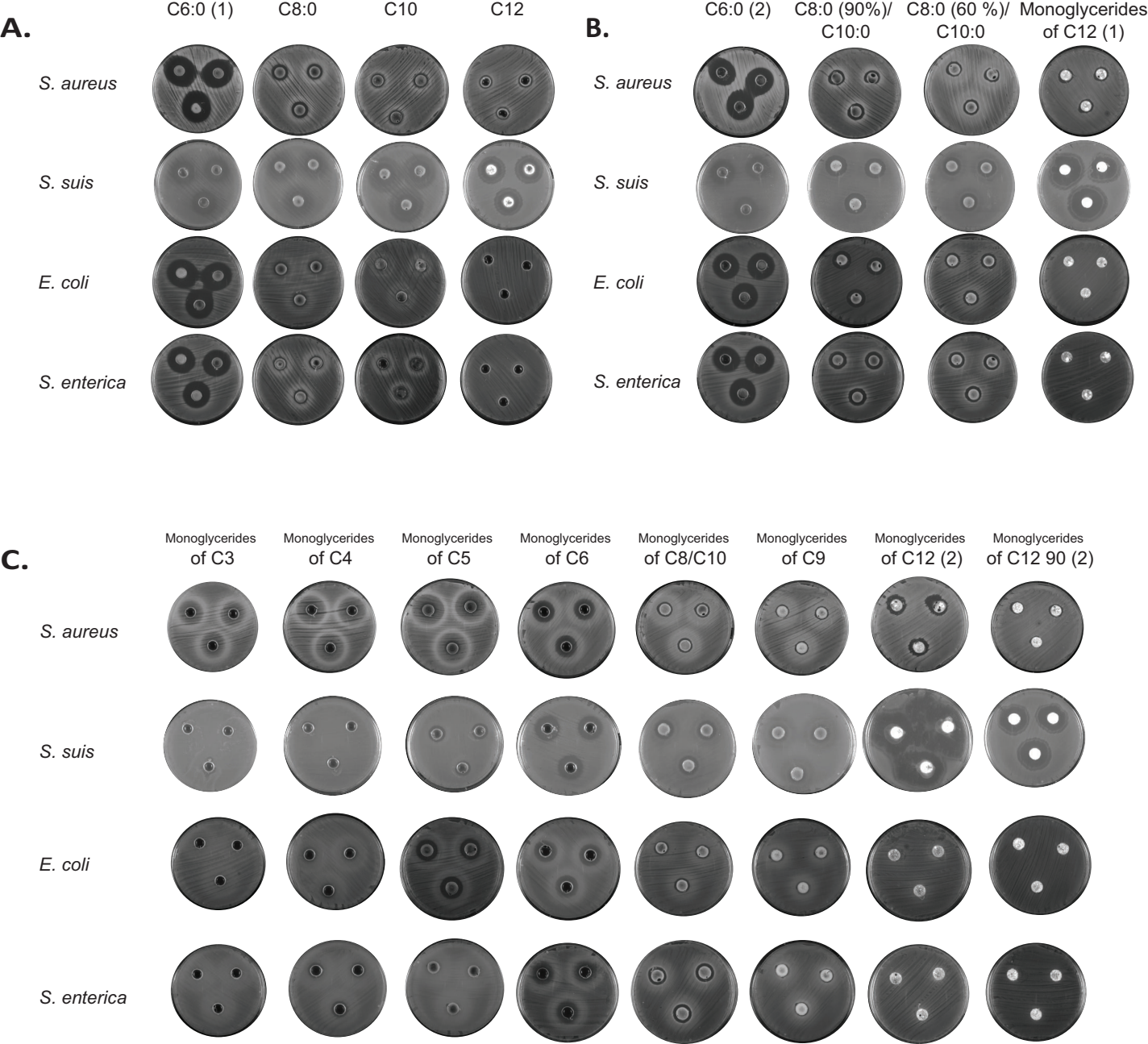
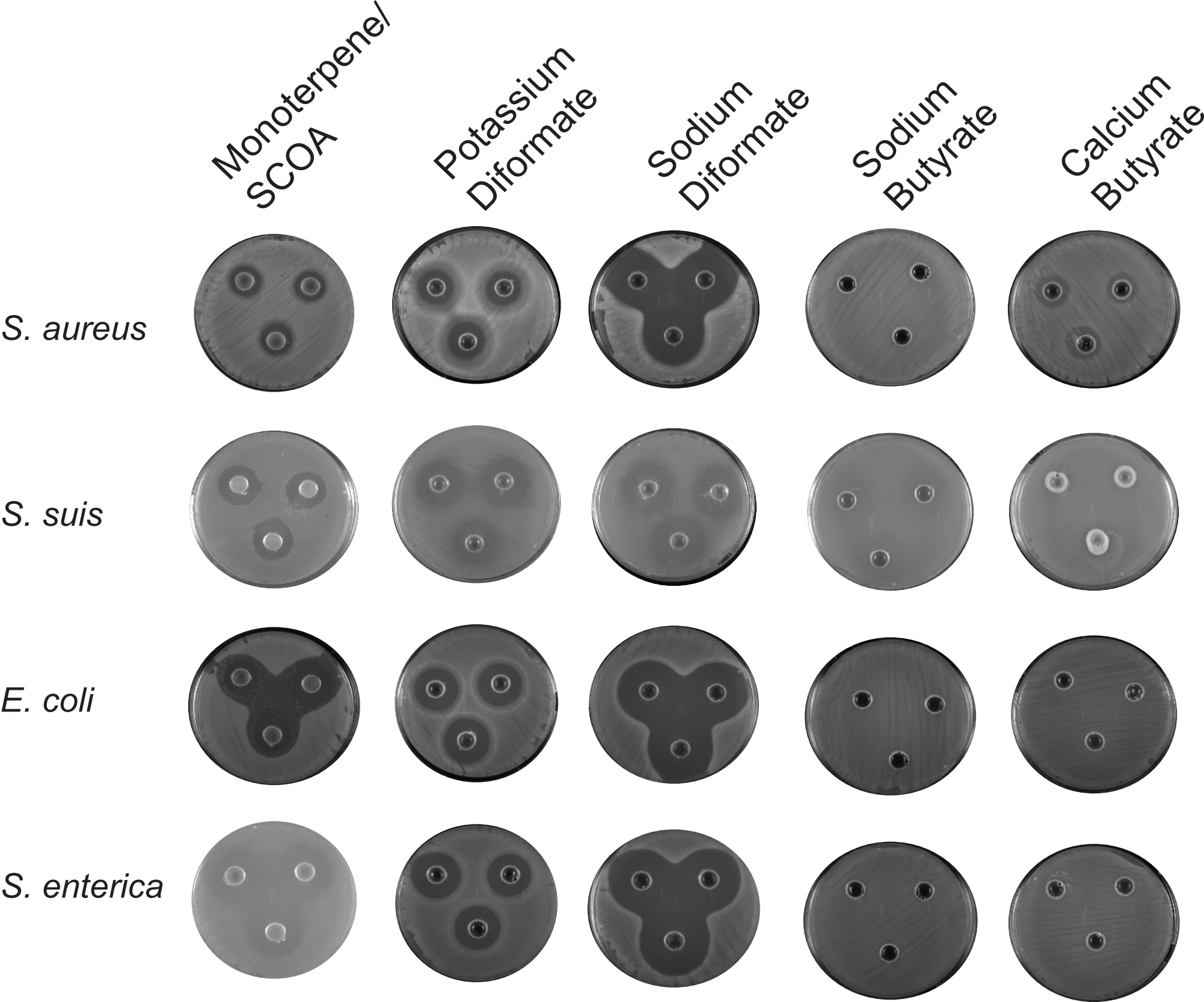
Figure 1. Inhibitory activity of Medium Chain Organic Acids. The inhibitory activity of medium chain organic acids against a panel of pathogens using an agar-plug diffusion assay is shown. Pathogen name is indicated for each row, and columns represent individual feed additives / active ingredients as labelled. Each individual image shows a technical replicate of activity on a lawn of the relevant bacterial species on each agar plate. Antibacterial activity is observed as clearing of the lawn surrounding the central wells. Feed additives from supplier 1, 2 and 3 are shown in figures 1a, 1b and 1c, respectively. Specific strain details can be found in the materials and methods.

Figure 2: Inhibitory activity of Short Chain Organic Acids. The inhibitory activity of short chain organic acids against a panel of bacterial pathogens using an agar-plug diffusion assay is shown. The arrangement of the figure is as described for Figure 1.

Figure 3. Inhibitory activity of Essential oils. The inhibitory activity of essential oils against a panel of bacterial pathogens using an agar-plug diffusion assay is shown. The arrangement of the figure is as described for Figure 1.

Figure 4. Inhibitory activity of other active ingredients. The inhibitory activity other active ingredients against a panel of bacterial pathogens using an agar-plug diffusion assay is shown. The arrangement of the figure is as described for Figure 1.


A.
C6:0 (1)
C8:0
C10
C12
S. aureus
S. suis
E. coli
S. enterica
B.
C6:0 (2)
C8:0 (90%)/ C10:0
C8:0 (60 %)/ C10:0
Monoglycerides of C12 (1)
S. aureus
S. suis
E. coli
S. enterica
C.
Monoglycerides of C3
Monoglycerides of C4
Monoglycerides of C5
Monoglycerides of C6
Monoglycerides of C8/C10
Monoglycerides of C9
Monoglycerides of C12 (2)
Monoglycerides of C12 90 (2)
S. aureus
S. suis
E. coli
S. enterica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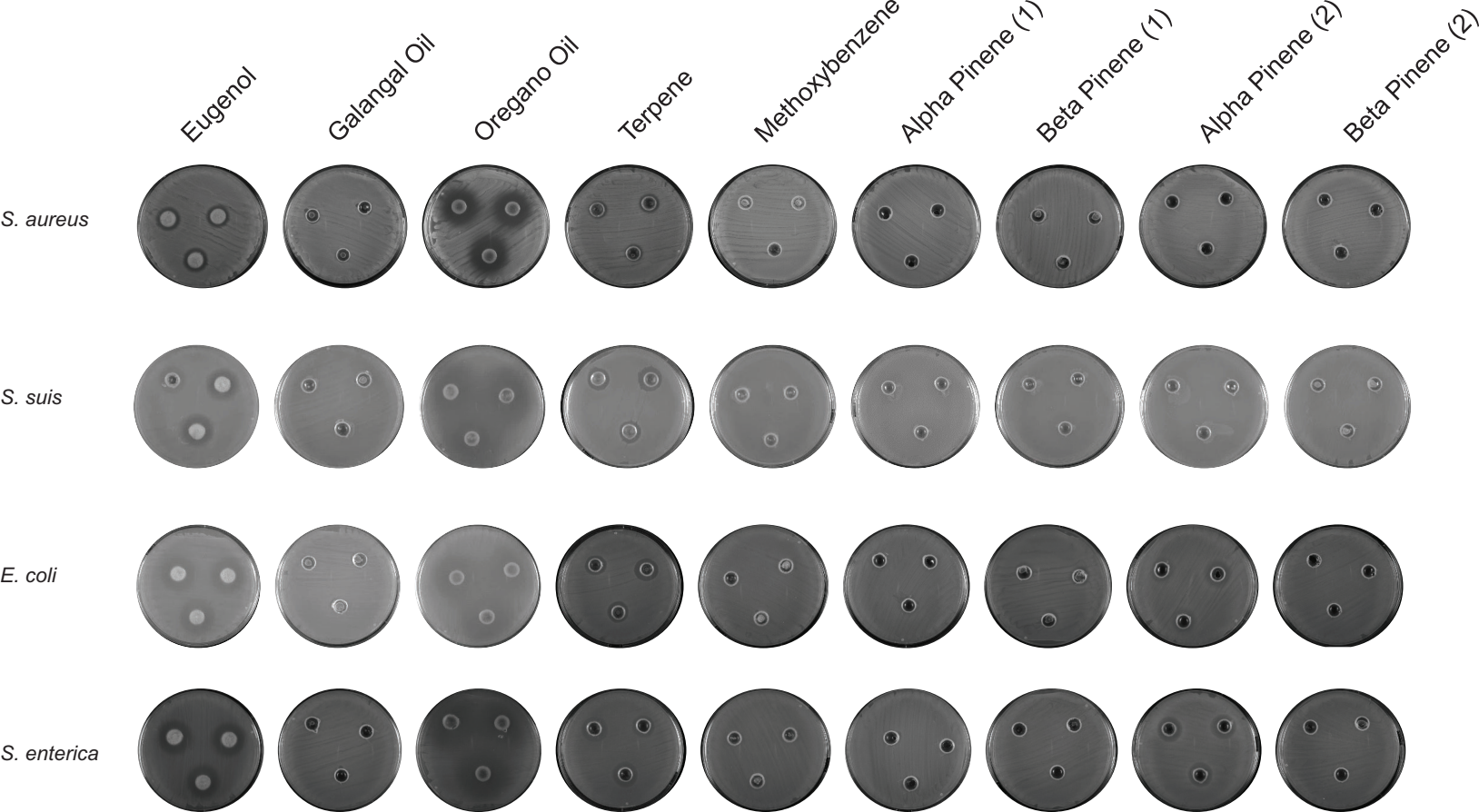
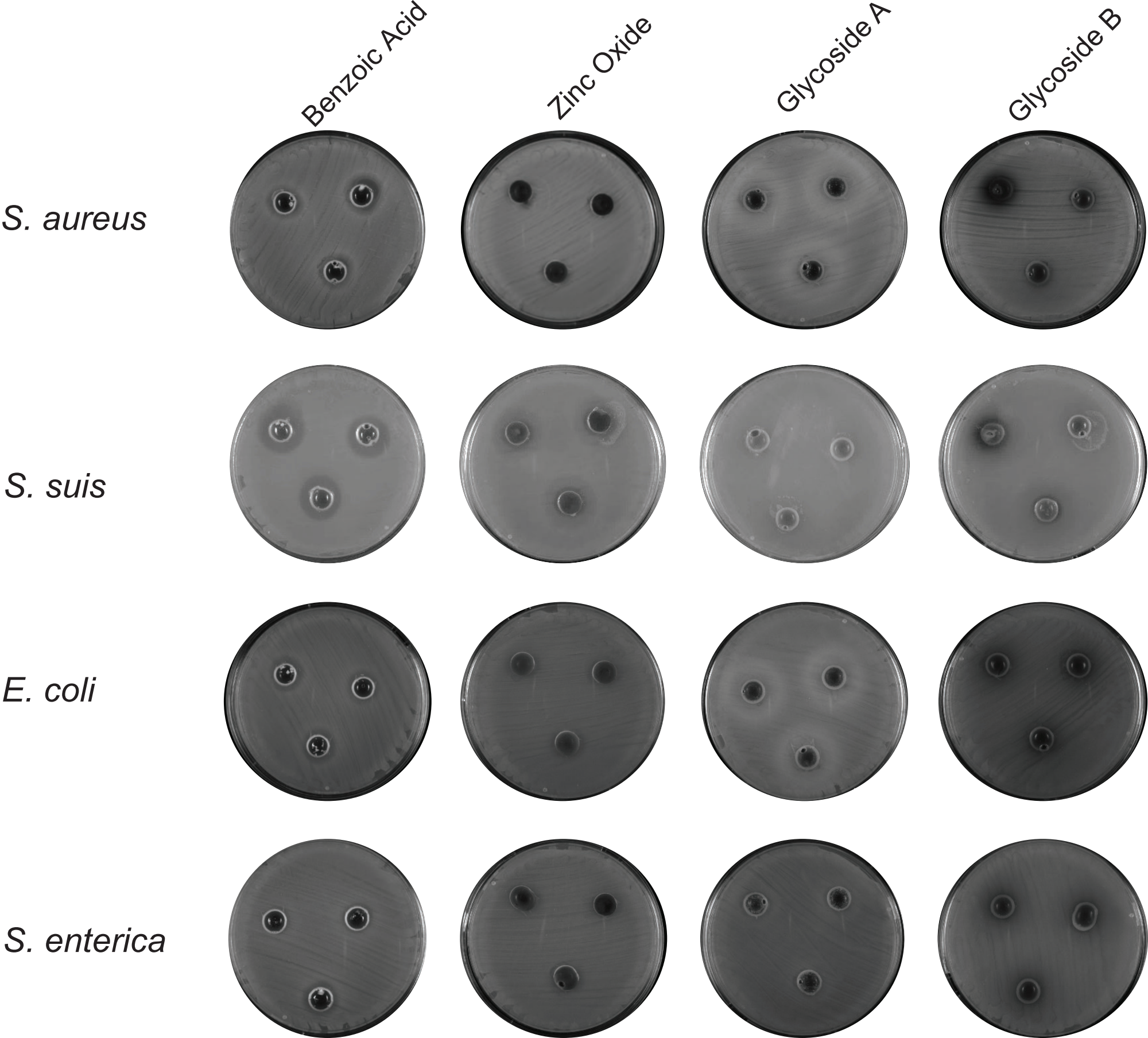
Potassium Diformate
Sodium Diformate
Sodium Butyrate
Calcium Butyrate
S. aureus
S. suis
E. coli
S. enterica


Eugenol
Galangal Oil
Oregano Oil
Terpene
Methoxybenzene
Alpha Pinene (1)
Beta Pinene (1)
Alpha Pinene (2)
Beta Pinene (2)
S. aureus
S. suis
E. coli
S. enterica


Benzoic Acid
Zinc Oxide
Glycoside A
Glycoside B
S. aureus
S. suis
E. coli
S. enterica