K-STATE Research and Extension

Swine Day 2018

Determining the Minimum Inhibitory Concentration of Medium Chain Fatty Acids for Generic *Escherichia coli*, Enterotoxigenic *Escherichia coli*, *Salmonella* Typhimurium, and *Campylobacter coli*[1]

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Summary

Research has demonstrated that medium chain fatty acids (MCFA) can serve as reduction strategies for bacterial and viral pathogens in animal feed and ingredients. However, it is unknown how the type or level of MCFA impact bacteria growth. This can be tested through a minimum inhibitory concentration (MIC) benchtop assay, which identifies the lowest concentration of a chemical that prevents visible growth of a bacterium. The objective of this study was to 1) determine the MCFA MIC of C6:0, C8:0, C10:0, and C12:0 for generic *Escherichia coli*, Enterotoxigenic *Escherichia coli*, *Salmonella* Typhimurium, *Campylobacter coli*, and *Clostridium perfringens*; 2) determine the MIC of commercial based MCFA products against the same bacteria; and 3) determine the effect of 2 commercial based MCFA products on the quantification of Enterotoxigenic *Escherichia coli*. For Exp. 1 and 2, MIC were determined by modified microbroth dilution method using a 96 well microtiter plate with a concentration of 10^5 CFU/mL for each bacterial strain. For Exp. 3, the two products selected for quantification were mixed with a complete swine diet and inoculated with two concentrations (10^6 or 10^2 CFU/g of feed) of a Nal^R strain of Enterotoxigenic *Escherichia coli* (ETEC) for bacterial enumeration. From Exp. 1, the MIC of MCFA varied among bacteria species. The lowest MIC of the MCFA was 0.43% of a 1:1:1 blend of C6:0, C8:0, and C10:0 for *Campylobacter coli*, 0.25% C12:0 for *Clostridium perfringens*, 0.60% 1:1:1 blend for generic *Escherichia coli*, 0.53% C6:0 for ETEC, and 0.40% C6:0 for *Salmonella* Typhimurium. In Exp. 2, products containing high concentrations of C6:0 or C8:0 had lower MIC in gram negative bacteria. In Exp. 3, feed containing either of the commercial based MCFA products reduced (linear, $P < 0.05$) quantifiable ETEC. Overall, the inhibitory efficacy of MCFA varies among bacteria species. This suggests that MCFA mixtures may provide a wider spectrum of bacterial control. As commercial

[1]Appreciation is expressed to the National Pork Board for financial support.

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products containing MCFA become available for livestock, it is important to consider the interaction between MCFA chain length and concentration on the potential to effectively mitigate various feed-based bacteria.

Introduction

Medium chain fatty acids (MCFA) have been demonstrated to significantly reduce problematic bacterial and viral contamination in animals, animal feed, and feed ingredients.[3,4,5] Compared to other feed additives, MCFA are unique in their potential mode of action. It is thought that the MCFA carry bacteriostatic and bactericidal properties by causing a destabilization of the bacterial double phospholipid bilayer membrane and causing leakage of intracellular content.[3] It is also thought that the MCFA can acidify the cell by liberating H^+ ions, leading to cell death.[3] More recently, a 2% inclusion of a 1:1:1 ratio of C6:0, C8:0, and C10:0 reduced *Salmonella enterica* subsp. enterica serovar Typhimurium from 2.35 to 0.66 log CFU/g within 1 day.[4] The MCFA were also very effective on the initial inoculation day compared to the inoculated feed matrices containing no mitigation additives (2.35 vs. 5.45 log CFU/g, respectively).[4] However, there is limited information regarding which specific MCFA is the most effective, whether combinations of different MCFA exhibit additive effects, and what the optimal level of MCFA is that will impact various bacteria associated with animal production. This can be determined utilizing a minimum inhibitory concentration (MIC) benchtop assay, which identifies the lowest concentration of a treatment that prevents visible growth of a bacterium. Therefore, the objective of this study was to determine the minimum inhibitory concentration of specific MCFA and commercial products for *Campylobacter coli*, *Clostridium perfringens*, generic *Escherichia coli*, Enterotoxigenic *Escherichia coli*, and *Salmonella* Typhimurium as well as their potential application in feed as a reduction strategy.

Procedures

Bacterial Inoculum

Bacterial strains of generic *Escherichia coli* (*E. coli*) ATCC 25922, Enterotoxigenic *Escherichia coli* (ETEC) 3030-2, and *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) ATCC 14028 were grown using Luria Bertani, *Campylobacter coli* (*C. coli*) 7A #2016-1 using Mueller-Hinton, and *Clostridium perfringens* (*C. perfringens*) 4026 using anaerobic Brain Heart Infusion broth medium at 37°C for 24 h. For *E. coli*, ETEC, *S.* Typhimurium, and *C. coli*, 1 mL of bacterial inoculum was serially diluted using 9 mL of phosphate-buffered saline to achieve one concentration (10^5 CFU/mL) for each bacterial strain. For *Clostridium perfringens*, the bacterial concentration was adjusted to 0.5 McFarland Standards using fresh Brain Heart Infusion broth medium per Clinical and Laboratory Standards Institute (CLSI) recommendations.[6]

[3]Kim and Rhee. 2013. Marked synergistic bactericidal effects and mode of action of medium chain fatty acids in combination with organic acids in Escherichia coli O157:H7. Appl Environ Microbiol. 79:6552–6560.

[4]Cochrane et al., 2016. Evaluating chemical mitigation of *Salmonella* Typhimurium in animal feed ingredients. J Food Prot. 79(4):672-676.

[5]Dee et al., 2016. Modeling the transboundary risk of feed ingredients contaminated with porcine epidemic diarrhea virus. BMC Vet Res. 12:51-63.

[6]Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard, 4th ed. 2013. Document VET01-A4. CLSI, Wayne, PA.

Experiment 1: MIC Determination of MCFA

For *E. coli*, ETEC, *S.* Typhimurium, and *C. coli* the compounds tested were C6:0, C8:0, C10:0, and a 1:1:1 blend of C6:0, C8:0, and C10:0. For *C. perfringens*, the compounds tested were C6:0, C8:0, C10:0, and C12:0[a].

The MIC were determined by the micro-broth dilution method as per CLSI guidelines[8] in *E. coli*, ETEC, *S.* Typhimurium, and *C. coli* from 0.1% until an MIC was established, with a maximum tested level of 1.0%. The MIC was also determined using the same method for *C. perfringens*, with a maximum tested level of 2.0%. There were three replications per product and bacteria combination.

Experiment 2: MCFA Profiles and MIC Determination of Commercially-Based Products

The fatty acid profile of 21 commercially-based products was analyzed, with an emphasis on the MCFA concentration. The 24 products were, 1) Product A[b]; 2) Product B[c]; 3) Product C[b]; 4) Product D[d]; 5) Product E[d]; 6) Product F[d]; 7) Product G[d]; 8) Product H[e]; 9) Product I[f]; 10) Product J[f]; 11) Product K[g]; 12) Product L[h]; 13) Product M[h]; 14) Product N[f]; 15) Product O[f]; 16) Product P[f]; 17) Product Q[f]; 18) Product R[f]; 19) Coconut oil[g]; 20) Palm oil[g]; and 21) Palm kernel oil[g]. Samples were analyzed according to procedures outlined by Sukhija and Palmquist.[7] From this analysis, products A, B, G, H, and a commodity fat source (coconut oil) were selected as having representative MCFA profiles for use in MIC assays. The profiles were selected based on products having the highest concentrations of C6:0 and C8:0 within the fatty acid profile, and coconut oil because of its natural source of MCFA and medium chain triglycerides. The MIC were determined as described in Exp. 1 in *E. coli*, ETEC, *S.* Typhimurium, and *C. coli* from 0.1% until an MIC was established, with a maximum tested level of 5.0%. There were three replications per product and bacteria combination.

Experiment 3: Quantification of Enterotoxigenic Escherichia coli-inoculated Feed After Treatment with Two Commercially-Based MCFA-Containing Products

Based on their lower MIC compared to other products tested in Exp. 2, Products A and B were selected as treatments to determine their reduction capacity in swine feed inoculated with ETEC. The strain of ETEC was first made resistant to 50 µl/mL nalidixic acid (Nal^R) antibiotic before being used for inoculation. A complete swine diet was either left un-inoculated and untreated, or mixed with 0.00, 0.25, 0.50, 1.00, or 2.00% Product A or B and inoculated with ETEC. For inoculation, 1 g of each feed sample was mixed with 1 mL of Nal^R ETEC at one of two concentrations (10^6 or 10^2 CFU/g of feed) of bacteria. The higher concentration was utilized for quantification of ETEC and the lower for detection. The 10 treatments were: 1) control feed with no bacteria; 2) control feed inoculated with bacteria and no addition of an additive; 3) 0.25% Product A; 4) 0.5%, Product A; 5) 1.0%, Product A; 6) 2% Product A; 7) 0.5% Product B; 8) 1.0% Product B; 9) 2.0% Product B; and 10) 4.0% Product B. The levels for each product were selected based on the results of Exp. 2. Product A was tested at a lower

[7]Sukhija and Palmquest. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J Agric Food Chem. 36:1202-1206.

concentration in the feed because of the lower MIC value established in Exp. 2. Product B was then tested at higher concentrations because of the higher MIC value that was established in Exp. 2. It was also determined that treatment 1 was confirmed to be negative of ETEC and was not included in the statistical model.

Samples were incubated at 37°C for 24 h. Then, 1 g of the incubated feed containing bacterial inoculum was suspended in 9 mL of PBS, serially diluted, and plated onto MacConkey agar containing nalidixic acid. The plates were incubated at 37°C for 24 h for bacterial enumeration using a standard plate count for viable cells. There were three replications per product and bacteria combination.

Statistical Analysis

Data from each MIC experiment were analyzed as a completely randomized design using PROC GLIMMIX in SAS version 9.4 (SAS Inst. Inc., Cary, NC, USA) to evaluate the effect of each treatment within each bacterium. If the MIC value was greater than the detection limit of the analysis, the next logical concentration (increase in 0.1% addition) was utilized for the statistical analysis. For Exp. 3, the PROC GLIMMIX procedure of SAS was utilized to evaluate linear and quadratic contrasts of increasing product levels. The coefficients for the unequally spaced linear and quadratic contrasts utilized in Exp. 3 were derived using the PROC IML procedure in SAS. In all experiments, results for treatment criteria were considered significant at $P \leq 0.05$.

Results and Discussion

Experiment 1

The MIC of each MCFA in *C. coli*, *C. perfringens, E. coli*, ETEC, and *S.* Typhimurium, are presented in Table 1. The MIC for *C. coli* was lower ($P < 0.05$) in C6:0, C8:0, or the MCFA blend than in C10:0. In *C. perfringens*, the longer chain fatty acids were more effective with C12:0 and C10:0 providing the lowest ($P < 0.05$) MIC results with C12:0 being the most effective ($P < 0.05$) overall. Within generic *E. coli*, the 1:1:1 MCFA blend of C6:0, C8:0, and C10:0 provided the lowest ($P < 0.05$) MIC value followed by C6:0 and C8:0. Within ETEC, C6:0 had a lower ($P < 0.05$) MIC than C8:0, which was still lower ($P < 0.05$) than either C10:0 or the MCFA blend, which were greater than the maximum tested value of 1%. In *Salmonella* Typhimurium, C6:0 resulted in an MIC similar ($P > 0.05$) to C8:0. However, C6:0 did differ ($P < 0.05$) from the blend. Again, no MIC was determined for C10:0 within *S.* Typhimurium.

Experiment 2

The fatty acid profile varied widely in the 21 commercially-based products (Table 2). Based on these analyses, Product A, B, F, G, and coconut oil were selected as candidate products for MIC determination in gram negative bacteria due to their high concentrations of C6:0 and C8:0. In *C. coli*, the MIC for Product B was lower ($P < 0.05$) than either Product F or G, with Product A intermediate (Table 3). Product A and B had lower ($P < 0.05$) MIC in generic *E. coli*, ETEC, and *Salmonella* Typhimurium than other tested products. The MIC for coconut oil was not detected in any bacteria as it was greater than the maximum tested level of 5.0%.

Experiment 3

Due to their efficacy in the MIC determination, products A and B were selected as treatments to determine their effect on detectable or quantifiable ETEC in feed. In the higher concentration of bacteria, Product A resulted in a linear decrease (linear, $P < 0.05$) in the number of quantifiable bacteria (Table 4). For Product B, as the level increased, the number of quantifiable bacteria decreased (quadratic, $P < 0.05$). In the lower concentration of bacteria, Product A again resulted in a decrease (linear, $P < 0.05$) in the number of quantifiable bacteria (Table 5). However, in Product B, no linear or quadratic response was observed ($P > 0.10$).

In summary, MCFA mixtures may provide a wider spectrum of bacterial control. As commercial products containing MCFA become available for livestock, it is important to consider the interaction between MCFA chain length and concentration on the potential to effectively mitigate various feed-based bacteria.

Table 1. Minimum inhibitory concentration of medium chain fatty acids in generic *Escherichia coli*, Enterotoxigenic *Escherichia coli* (ETEC), *Salmonella enterica* serotype Typhimurium, and *Clostridium perfringens*[1]

Item	MIC, %	SEM	*P*-Value
Campylobacter coli		0.047	0.0004
C6:0	0.50[b]		
C8:0	0.47[b]		
C10:0	0.90[a]		
1:1:1 Blend	0.43[b]		
Clostridium perfringens		0.030	< 0.0001
C6:0	1.65[a]		
C8:0	0.85[b]		
C10:0	0.70[c]		
C12:0	0.25[d]		
Generic *E. coli*		0.014	<.0001
C6:0	0.70[a]		
C8:0	0.85[b]		
C10:0[2]	> 1.00[c]		
1:1:1 Blend	0.60[d]		
Enterotoxigenic *E. coli*		0.024	<.0001
C6:0	0.53[c]		
C8:0	0.67[b]		
C10:0[2]	> 1.00[a]		
1:1:1 Blend[2]	> 1.00[a]		
Salmonella Typhimurium		0.050	<.0001
C6:0	0.40[c]		
C8:0	0.50[cb]		
C10:0[2]	> 1.00[a]		
1:1:1 Blend	0.60[b]		

[1]Minimum inhibitory concentration for C6:0, C8:0, C10:0, and a 1:1:1 blend of C6:0, C8:0, and C10:0 were tested in *E. coli*, ETEC, *S.* Typhimurium, and *C. coli* using a 96 well microtiter plate with a concentration of 10^5 CFU/mL for each bacterial strain. For *C. perfringens*, the compounds tested were C6:0, C8:0, C10:0, and C12:0 utilizing a 96 well microtiter plate with a concentration of 0.5 McFarland Standards for each well. Each value is represented by an N=3.

[2]Minimum inhibitory concentration was above the tested detection limit and therefore the next logical inclusion level (increase in 0.1% inclusion) was utilized for the statistical analysis.

[abcd] Means within a bacterial species lacking a common superscript differ ($P < 0.05$).

Table 2. Medium chain fatty acid profiles for the tested products (mg/g)

Item	Total analyzed fatty acids	C6:0	C8:0	C10:0	C12:0
Product A[1]	294.58	29.53	123.20	101.43	40.23
Product B[2]	1092.66	43.12	610.28	436.50	2.15
Product C[1]	123.07	12.35	51.42	42.28	16.85
Product D[3]	303.36	8.43	103.64	88.92	86.81
Product E[3]	369.33	9.02	123.38	105.61	111.06
Product F[3]	603.77	27.37	248.7	206.41	120.18
Product G[3]	494.34	0.98	227.13	188.00	74.50
Product H[4]	362.92	0.09	1.32	1.16	359.47
Product I[5]	349.54	2.19	159.32	131.10	56.71
Product J[5]	101.32	0.00	41.42	34.03	25.70
Product K[5]	402.37	0.20	128.21	99.30	122.71
Product L[7]	983.16	0.02	0.02	0.04	0.19
Product M[7]	520.80	3.78	40.87	31.21	227.83
Product N[5]	158.76	1.8	69.72	57.91	19.36
Product O[5]	145.57	1.74	68.08	56.43	18.56
Product P[5]	317.48	4.78	151.41	129.46	31.33
Product Q[5]	2.78	0.00	0.02	2.60	0.00
Product R[5]	314.01	0.69	101.44	83.01	90.15
Coconut oil[6]	894.09	6.82	72.07	53.74	409.62
Palm oil[6]	894.34	0.00	0.51	0.22	2.35
Palm kernel oil[6]	918.84	2.83	37.86	33.21	418.05

[1]Nuscience Group, Ghent (Drongen), Belgium.
[2]Kemin Industries, Des Moines, IA, USA.
[3]PMI Nutritional Additives, Arden Hills, MN, USA.
[4]Framelco, Raamsdonksveer, Netherlands.
[5]Nutreco, Amersfoort, Netherlands.
[6]ADM, Chicago, IL, USA.
[7]Cargill, Minneapolis, MN, USA.

Table 3. Minimum inhibitory concentration of commercially-based medium chain fatty acid based products in generic *Escherichia coli*, Enterotoxigenic *Escherichia coli* (ETEC), and *Salmonella enterica* serotype Typhimurium[1]

Item	MIC, %	SEM	*P*-Value
Campylobacter coli		0.629	0.0026
Product A[2]	1.20[cd]		
Product B[3]	0.33[d]		
Product F[2]	2.75[bc]		
Product G[2]	3.33[ab]		
Coconut oil[4,5]	> 5.0[a]		
Generic *E. coli*		0.424	<.0001
Product A[2]	0.37[c]		
Product B[3]	1.20[c]		
Product F[2]	3.33[b]		
Product G[2]	4.17[ab]		
Coconut oil[4,5]	> 5.0[a]		
Enterotoxigenic *E. coli*		0.309	< .0001
Product A[2]	0.33[c]		
Product B[3]	1.30[c]		
Product F[2]	3.83[b]		
Product G[2]	4.33[ab]		
Coconut oil[4,5]	> 5.0[a]		
Salmonella Typhimurium		0.308	<.0001
Product A[2]	0.47[c]		
Product B[3]	1.30[c]		
Product F[2]	3.83[b]		
Product G[2]	4.33[ab]		
Coconut oil[4,5]	> 5.0[a]		

[1]Minimum inhibitory concentration for products (Product A, B, F, G, and coconut oil were tested in *E. coli*, ETEC, *S.* Typhimurium, and *C. coli* using a 96 well microtiter plate with a concentration of 10^5 CFU/mL for each bacterial strain. Each value is represented by an N=3.
[2]Nuscience Group, Ghent (Drongen), Belgium.
[3]Kemin Industries, Des Moines, IA, USA.
[4]ADM, Chicago, IL, USA.
[5]Minimum inhibitory concentration was above the tested detection limit and therefore the next logical inclusion level (increase in 0.1% inclusion) was utilized for the statistical analysis.
[abcd] Means within a bacteria species lacking a common superscript differ ($P < 0.05$).

Table 4. Effects of commercially-based products containing medium chain fatty acids on the growth of 10^6 CFU/g feed Enterotoxigenic *Escherichia coli* (ETEC)[1]

Item	Log CFU/g	SEM	*P*-Value Linear	*P*-Value Quadratic
Product A[2]		0.011	<.0001	0.9641
0.00%	5.44			
0.25%	5.37			
0.50%	5.24			
1.00%	5.15			
2.00%	4.81			
Product B[3]		0.017	<.0001	<.0001
0.00%	5.44			
0.50%	5.19			
1.00%	5.14			
2.00%	4.71			
4.00%	3.49			

[1]Products A and B were tested in a concentration of 10^6 CFU/g of feed ETEC in a complete swine diet in order to determine the growth of that bacteria using MacConkey agar containing nalidixic acid for bacterial enumeration.
[2]Nuscience Group, Ghent (Drongen), Belgium.
[3]Kemin Industries, Des Moines, IA, USA.

Table 5. Effects of commercially-based products containing medium chain fatty acids on the growth of 10^2 CFU/g feed Enterotoxigenic *Escherichia coli* (ETEC)[1]

Item	Log CFU/g	SEM	*P*-Value Linear	*P*-Value Quadratic
Product A[2]		0.007	0.0060	0.1180
0.00%	2.95			
0.25%	2.93			
0.50%	2.93			
1.00%	2.95			
2.00%	2.91			
Product B[3]		0.012	0.1041	0.1579
0.00%	2.95			
0.50%	2.90			
1.00%	2.93			
2.00%	2.91			
4.00%	2.91			

[1]Products A and B were tested in a concentration of 10^2 CFU/g of feed ETEC in a complete swine diet in order to determine the growth of that bacteria using MacConkey agar containing nalidixic acid for bacterial enumeration.
[2]Nuscience Group, Ghent (Drongen), Belgium.
[3]Kemin Industries, Des Moines, IA, USA.