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Interactions of Organic Acids with *Staphylococcus aureus* and MRSA Strains from Swine Mandibular Lymph Node Tissue, Commercial Pork Sausage Meat and Feces

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Abstract: *Staphylococcus aureus* is a Gram-positive bacterium affecting human health, and a major cause of skin infections, endocarditis, meningitis, and sepsis. Methicillin-resistant *S. aureus* (MRSA) is a worldwide health concern, occurs in food animals, is consistently found in swine, and improved strategies are needed to ensure the removal of MRSA from food products. A total of 164 *S. aureus* strains were isolated from swine mandibular lymph node tissue, commercial pork sausage meat, and feces. These strains were tested for methicillin-resistance, and 7 of the strains isolated from the mandibular lymph node tissue and pork sausage meat were resistant to cefoxitin and oxacillin, and tested positive for staph specific rRNA and for the *mecA* gene and are therefore, MRSA strains. An intracellular MRSA contamination of 8.2% within swine lymph node tissue and 5.8% MRSA contamination in pork sausage meat was demonstrated. Lymph node tissue may be utilized in producing pork sausage; therefore, the prevalence of MRSA in final pork products may not only be caused by surface contamination, but by internal tissue infection. The strains were tested for susceptibility to six organic acids (OAs) citric, L-lactic, butyric, acetic, propionic, and formic acid. The pH was determined at each of the minimum inhibitory concentrations (MICs) observed for the *S. aureus* strains. The Henderson-Hasselbalch equation was used to calculate the ratio of the undissociated to dissociated OA concentrations, and the molar concentrations of each were calculated from the total OA present and the ratio. Inhibition of *S. aureus* did not correlate with pH or the undissociated OA concentrations, but it did correlate with the dissociated OA concentrations. A dissociated OA concentration of 21 mM was successful for inhibiting the *S. aureus* strains tested. Studies must be conducted *in vivo* to confirm this concentration value. Acetic, butyric, formic, and propionic acid were the most effective OAs tested against *S. aureus*.

Keywords: Acetic Acid, Citric Acid, Formic Acid, L-Lactic Acid, Molar Minimum inhibitory Concentrations (MIC$_M$s), Organic Acids, Propionic Acid, *Staphylococcus aureus*, Susceptibility, Swine

1. Introduction

Staphylococcus aureus is a Gram-positive bacterium and one of the most important pathogens that affects human health [1, 2]. *S. aureus* is a major cause of both hospital and community acquired diseases of the skin, endocarditis, meningitis, and sepsis [3, 4], and

is involved in the toxic shock syndrome [5, 6]. The Centers for Disease Control (CDC) has recognized *S. aureus* as one of the top five germs that cause foodborne illness in the United States, and since it is commonly carried on the skin or in the nose of healthy people, when the situation is right *S. aureus* can cause infections in the blood stream and in major organs [7]. *S. aureus* can become

resistant to the antibiotic methicillin, resulting in methicillin-resistant *S. aureus* (MRSA), and MRSA also may confer resistance to other common antibiotics like amoxicillin, oxacillin, and penicillin, rendering the MRSA isolate more difficult to treat [8, 9]. Therefore, MRSA is regarded as a worldwide health concern [10]. There have been a number of reports worldwide of MRSA occurring in food animals since about 2004 [9–14].

S. aureus is known to commonly colonize pigs [9], and pig herds can be a reservoir for MRSA [10, 12, 13, 15]. MRSA is well known for being an important medical issue, and swine can be reservoirs for MRSA that can infect both swine workers and consumers through food products containing MRSA [16]. The prevalence of livestock-associated MRSA is known to be influenced by the use of disinfectants and feeding zinc [17, 18]. Previous to 1995, MRSA infections primarily occurred in hospitalized patients; however, since that time MRSA infections have been reported in non-hospitalized people [19]. *S. aureus* carriage in swine can result in occupational exposure, including exposure to antimicrobial resistant strains [19, 20]. In fact, occupational exposure to swine is a significant risk factor for contracting *S. aureus* infections [14, 21–25]. People with occupational exposure to swine production facilities may be up to six times more likely to be a carrier of MRSA than people without this exposure [9, 21]. Workers may be exposed by direct contact with swine, or by airborne animal dander and decomposing waste [20, 26], and by aerosols as a result of the animals' normal activities [27]. These exposure risks may be enhanced by environmental dispersion of *S. aureus* and MRSA [28–30] and by leakage of stored waste or by applications of swine waste for crop fertilization [31–33]. Also, there is a doubling of the risk associated for contacting MRSA when a residential property is within 1 mile of a large swine feeding operation [15].

When pigs are colonized with *S. aureus* and MRSA and brought to the abattoir, they will bring that contamination to the abattoir and the processing plant [19]. Studies have shown that the prevalence of MRSA throughout different stages of processing from the carcass to the final cuts of meat show a decrease in MRSA along the process line [34, 35]. The studies revealed that even with reductions in MRSA along the production line, a carriage of 11.3 to 64.7% measured at the point of stunning the animals results in a 2.2 to 6.6% prevalence of MRSA in the final product. The consumption of contaminated food causing MRSA colonization in humans has been observed [36, 37].

Developing new and improved strategies to control or eliminate foodborne pathogens in our food supply from farm-to-fork is a continuous research need required to help prevent illness from new or reemerging pathogens [38, 39]. In the United States, animal food processing plants utilize organic acids (OAs) to remove bacteria from the surfaces of hides and meats, and these treatments are simple, cost efficient, fast, and shown to have good efficacy [40]. Some of the OAs used are the following: propionic [41–43], lactic [41–49], formic [41], citric [43, 46], and acetic acid [41–43, 46, 48, 49], with lactic and acetic acid being the most often used [48, 49]. The bacteria on a carcass not removed by the OA wash may later be found in the processed meat. Therefore, it is of importance to focus on improving the organic acid carcass wash step through a better understanding of the organic acid inhibition of bacteria.

Bacterial inhibition caused by OAs has been assumed by most researchers to be a result of the pH [50] or dependent on the undissociated form of the OAs [51–58]. However, to our knowledge there is no definitive study that shows that the undissociated form of the acid is the active OA species in the inhibition process, and the specific mechanism (s) causing bacterial inhibition by OAs are unknown [50, 59–61]. It has been demonstrated that the activities of OAs are altered with the level of pH, the concentration of OA, and the type of OA used [59]. Also, it is known that the ability to utilize an OA as an energy source can drastically affect the bacterium's susceptibility to that OA [62]. Our previous studies with Gram-negative pathogenic bacteria [62–67] and a Gram-positive pathogenic bacterium [68] showed no correlation between the molar minimum inhibitory concentrations (MIC$_M$S) of the bacteria and the pH at the MICs or the undissociated OA levels at the MICs. The bacterial MIC$_M$S were correlated with the molar concentrations of the dissociated OAs. Molar MICs were used throughout the studies because using molarity demonstrated an accurate comparison of MICs with OAs having different molecular weights. Other researchers have shown that a dissociated OA can cause the disintegration of a bacterial LPS layer [69].

In this work we studied the inhibition interactions of 164 *S. aureus* strains isolated from swine mandibular lymph node tissue, commercial pork sausage meat and feces against six organic acids. Studies to determine the susceptibility of these *S. aureus* isolates were conducted against the six OAs, citric, butyric, acetic, formic, L-lactic and propionic acid. The pH was determined at the MICs of the bacteria against each OA. Utilizing the determined pH for each bacteria-OA combination, the concentrations of both undissociated and dissociated OA species were calculated at the bacterial MICs.

2. Materials and Methods

2.1. Staphylococcus aureus Strains

S. aureus strains were isolated from 220 frozen 2017 swine mandibular lymph node tissue samples held at −72°C (*n*=49 strains), from 56 frozen 2019 blended commercial pork sausage meat samples held at −72°C (*n*=52 strains) and from 266 frozen 2006 swine feces samples held at −72°C (*n*=63 strains) for a total of 164 *S. aureus* strains. *S. aureus* cultures were grown for 24 h at 37°C on Trypticase Soy Agar w/5% Sheep Blood (TSA II™) (BD BBL™ Stacker™ Plate, Becton, Dickinson and Company, Sparks, MD, USA).

2.2. Chemicals and Materials

Butyric, citric, formic and propionic acid came from Sigma-Aldrich (Milwaukee, WI, USA). Acetic acid was procured from EM Science (Gibbstown, NY, USA). L-Lactic acid was procured from Alfa Aesar (Ward Hill, MA, USA). Reverse osmosis water was produced on a reverse osmosis system (Millipore Corp., Bedford, MA, USA), and solutions of OAs were sterilized using 0.2 × 25 mm syringe filters (No. 431224, Corning Inc., Corning, NY, USA).

2.3. Bacterial Culture and Isolation

2.3.1. Feces

One-g of swine feces was added to 9-mL of m Staphylococcus Broth (BD Difco™, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 37°C for 18–24 h. Following incubation, a 10 µL loop was used to streak the mixture onto CHROMagar™ Staph aureus Agar (CHROMagar, Paris - France). The agar plates were incubated at 37°C for 18–24 h. Typical mauve colored colonies were selected and re-streaked onto CHROMagar™ Staph aureus Agar and incubated at 37°C for 18–24 h. After incubation, those colonies were re-streaked onto BBL™ Mannitol Salt Agar (Becton, Dickinson and Company) for secondary confirmation. Typical yellow colonies were selected and streaked onto TSA II™ and incubated at 37°C for 18–24 h. Hemolytic reactions were noted and identification testing was performed using ID 32 STAPH or RAPIDEC® Staph (bioMérieux, Inc, Durham, NC, USA). Confirmed *S. aureus* colonies were frozen in CryoCare™ Bacteria Preservers (Key Scientific Products, Inc, Stamford, TX, USA) until antibiotic and OA susceptibility testing were performed.

2.3.2. Ground Pork Sausage

Approximately 35-g of ground pork meat was placed into a Whirl-Pak® filter bag (Nasco, Fort Atkinson, WI, USA) and 80-mL of m Staphylococcus Broth was added. The sample bags were homogenized for 30 s in a stomacher machine and then incubated at 37°C for 18–24 hr. A 10 µL sample was removed and streaked onto CHROMagar™ Staph aureus Agar and further processed exactly as stated above for the feces samples.

2.3.3. Swine Lymph Node and Tissue

Approximately 30-g of swine mandibular lymph node with the attached tissue was removed from the carcasses by hand, placed in a Whirl-Pak bag and frozen at –72°C until further processing was performed. The lymph node/tissue was thawed at room temperature then surface sterilized by submersion in boiling water for 3 to 5 s. After boiling, the lymph node/tissue was placed into a WHIRL-PAK® filter bag and pulverized with a rubber mallet. Eighty-mL of m Staphylococcus Broth was added and processed as stated above for the ground pork meat.

2.4. Determination of MRSA by Traditional Cefoxitin and Oxacillin Susceptibility Tests

Frozen *S. aureus* isolates were plated onto TSA II™ agar and incubated at 37°C for 18–24 h. Well-isolated colonies from the overnight plate were used to obtain a 0.5 McFarland equivalent in 0.85% saline. Following the manufacturer's recommendations for Etest® (bioMérieux SA, Marcy-I'Étoile – France), Mueller Hinton agar (MHA) was used as the medium for testing cefoxitin (FX), and MHA + 2% NaCl was used for testing oxacillin (OX) Etest® gradient strips. The isolates on MHA and MHA + 2% NaCl were incubated at 35°C for 18–24 h and 24 h, respectively. An isolate was considered resistant at >4 µg/mL and ≥4 µg/mL for FX and OX, respectively. Any isolate resistant to FX was subsequently tested with OX. The quality controls used were *Staphylococcus aureus* ATCC® 29213™ and ATCC® 43300™.

2.5. Determination of MRSA by Polymerase Chain Reaction (PCR) Methods

Pure Staphylococcus isolates were plated and grown overnight on TSA with 5% sheep blood (BVA Scientific, San Antonio, TX, USA), at 37°C. The Staphylococcus DNA templates were obtained by heat treatment. Five colonies from an overnight incubated plate were suspended in 500 µL of biotek water (Thermo Fisher Scientific, Carlsbad, CA, USA), and were heated to 100°C for 10 min. This was followed by a brief centrifugation for 1 min. at 5,000 rpm in an Eppendorf microfuge (Eppendorf North America, Enfield, CT, USA) as previously described [70].

Each 50 µL PCR reaction mixture contained 5.0 µL of a DNA template, 1 µL of each primer (shown in Table 1) diluted to 10 pM as stock [71], 25 µL of Thermo Scientific Phusion high-Fidelity DNA polymerase master mix (Fisher Scientific, 300 Industry Dr., Pittsburgh, PA, USA), and 18 µL of biotek water. The PCR reactions were run on an Eppendorf flexlid nexus gradient Mastercycler (Sigma-Aldrich, St. Louis, MO, USA).

All reactions were heated to 94°C for 1.0 min., followed by 35 cycles of PCR with a denaturation temperature of 94°C (1.0 min.), a primer annealing temperature of 55°C for 1.0 min., and an extension temperature of 72°C for 1.0 min. The final extension was at 72°C for 7 min. Ten microliters of each PCR reaction mixture were combined with 6X loading buffer and loaded into a 1% agarose gel. The gel system was run at 100 volts using a Bio-Rad Powerpac 200 (Bio-Rad Laboratories, Hercules, CA, USA) for 2 hr. The gel was then visualized in an AlphaImager® HP gel imaging system from Alpha Innotech (San Leandro, CA, USA).

Table 1. Oligonucleotide sequences of PCR primers.

Gene		Primers[a]
clfA	5′	GCAAAATCCAGCACAACAGGAAACGA
	3′	CTTGATCTCCAGCCATAATTGGTGG
	Length (bp)	638
mecA	5′	TCCAGGAATGCAGAAAGACCAAAGC
	3′	GACACGATAGCCATCTTCATGTTGG
	Length (bp)	499
staphylococcal 16S rRNA	5′	CCTATAAGACTGGGATAACTTCGGG
	3′	CTTTGAGTTTCAACCTTGCGGTCG
	Length (bp)	791

[a] Primers for the genes *clfA*, *mecA*, and staphylococcal rRNA are from Mason et al. [71] and are written 5′ to 3′ as synthesized.

2.6. Bacterial Susceptibility Testing with Organic Acids

The *S. aureus* MICs obtained against the six OAs, citric, butyric, acetic, L-lactic, propionic, and formic acid were determined using broth microdilution methods similar to those provided by the Clinical and Laboratory Standards Institute (CLSI) [72]. The MICs were determined to be the lowest concentration of an OA that resulted in no visible growth of *S. aureus* [73]. These susceptibility studies were conducted similarly to the susceptibility studies we carried out with other Gram-negative and Gram-positive pathogenic bacteria [63–66, 68]. Briefly, 50 μL of Mueller-Hinton broth (Becton, Dickinson and Company, Sparks, MD, USA) was added to wells #2 through #12 of a 96-well U-bottom Greiner bio-one microplate (#82050-626, VWR, Houston, TX, USA). The OA solutions (50 μL) were then added to well #1 and well #2 of the microplate. The OA solution in well #2 was diluted 1:2 across the microplate through well #11, and well #12 was used for the positive control [66]. *S. aureus* colonies were selected from TSA II™ and transferred to a 5 mL Sensititre® demineralized water tube to mimic a 0.5 McFarland standard using a Sensititre® Nephelometer® (Remel, Lenexa, KS, USA). Since each well of the 96-well plate contains 50 μL of an OA solution, 200-μL of the 0.5 McFarland *S. aureus* mixture was added to a tube containing 11 mL Mueller-Hinton broth w/TES (Remel, Lenexa, KS, USA) resulting in an inoculum of about 1×10^6 CFU/mL for each bacteria tested, and 50 μL of this bacterial solution was added to each well in the 96 well microplate. Each plate was then covered with the adhesive film, SealPlate® (EXCEL Scientific, Inc., Victorville, CA, USA) and incubated for 20 h at 37°C. Bacterial growth in the wells was determined by visual observation using a Sensititre® SensiTouch® (Remel, Lenexa, KS, USA). *Staphylococcus aureus* ATCC 29213 was the control organism used for OA susceptibility testing.

The OA concentrations evaluated were the following: citric acid, 32 – 32,768 μg/mL; L-lactic acid, 32 – 32,768 μg/mL; butyric acid, 32 – 32,768 μg/mL; acetic acid, 32 – 32,768 μg/mL; propionic acid, 32 – 32,768 μg/mL; and formic acid, 16 – 16,384 μg/mL.

2.7. Determination of pH at the S. aureus *MICs Against the OAs Using a 96-well Microplate*

The pH was measured as described in a previous paper [66]. Briefly, the pH was measured in the wells of the 96-well microplate containing OA solutions at the *S. aureus* MICs against all OAs, in triplicate. Solutions from 16-wells (100 μL/well) were combined at each *S. aureus* MIC for all six OAs resulting in a total volume of 1600 μL at each individual MIC and each combined set of wells per MIC were transferred to a 5 mL sterile microtube (Argos Technologies, Inc., Vernon Hills, IL, USA). A ROSS Ultra glass combination electrode with the Orion benchtop pH meter (Thermo Fisher Scientific, Chelmsford, MA, USA) was used to measure the pH of each microtube sample. The pH means and standard deviations were calculated and are shown in graphs presenting pH data.

2.8. Calculation of the Ratios of the Undissociated to Dissociated OAs

The Henderson-Hasselbalch equation can be used to calculate the ratio of the undissociated/dissociated OA concentration for a specific OA when the pH and pK_a of the OA is known. The general Henderson-Hasselbalch equation is shown in (1) [74]:

$$pH = pK_a + \log([A^-]/[HA]) \tag{1}$$

where the $-\log_{10}$ of the specific OA dissociation constant $(K_a) = pK_a$, the molar concentration of the conjugate base (the dissociated OA) = $[A^-]$, and the molar concentration of the undissociated OA = [HA] [74]. When the equation is rearranged as shown in (2), it calculates the ratio of the undissociated to dissociated OA [63].

$$\text{ratio} = [HA]/[A^-] = 1/10^{pH-pKa} \tag{2}$$

The ratio of each OA at the MICs was calculated given the use of published pK_a's for each OA and the measured pH at each of the MICs. The pK_a for L-lactic, citric, butyric, acetic, propionic, and formic acid is 3.86, 3.14, 4.82, 4.75, 4.87, and 3.75, respectively. Citric acid does have 3 pK_as, 3.14, 4.77, and 6.39; the first $pK_a = 3.14$ was used for the calculations since this work is only concerned with the dissociation of any one OH group, it is not dependent on multiple OH groups. Since the molar concentrations of the OAs are exactly known at the MICs, then each calculated undissociated/dissociated OA ratio obtained from the Henderson-Hasselbalch equation at the *S. aureus* MICs will allow the calculation of the molar concentrations of the undissociated and dissociated OAs at the MICs [62–68].

2.9. Statistics

The central tendency was determined for the MICs and MIC_Ms of the 164 *S. aureus* strains against the 6 OAs for all swine isolates. The central tendency also was determined for the MICs and MIC_Ms of the 52 *S. aureus* strains against the OAs isolated from only the pork sausage meat.

3. Results

3.1. MRSA Strains Found in the 164 Strains Tested

Seven strains (4.3%) were determined to be MRSA out of the 164 total *S. aureus* strains collected in this study. Table 2 shows the number and percent of *S. aureus* and MRSA strains obtained from each swine source.

3.2. Susceptibility Studies of S. aureus *Strains Against Six OAs*

3.2.1. Susceptibility Results for the Combined 164 S. aureus *Strains*

The total MIC and MIC_M data for the 164 *S. aureus* strains isolated from the 2006 swine feces, 2017 swine lymph node

tissue, and 2019 pork sausage meat against six OAs are shown in Table 3. Citric acid and L-lactic acid (85.28 and 181.88 mM), respectively, had the highest OA molar concentrations required to inhibit *S. aureus*. Acetic acid and butyric acid (68.21 and 46.49 mM), respectively, had the next highest OA

molar concentrations necessary for *S. aureus* inhibition, and formic acid and propionic acid (22.25 and 27.65 mM), respectively, had the lowest OA molar concentrations required to inhibit the *S. aureus* strains tested.

Table 2. Seven MRSA strains were determined out of the total 164 strains isolated from the three swine sources, 2017 lymph node tissue, 2019 pork sausage meat, and 2002 feces.

2017 LNT[a]				2019 PSM[b]				2002 Feces
FOX[c]	OXA[d]	rRNA[e]	*mecA*	FOX	OXA	rRNA	*mecA*	FOX
21L-DZ[f]	21L-DZ	+	+	D15-α	D15-α	+	+	None
38L-DZ	38L-DZ	+	+	D16-DZ	D16-DZ	+	+	
147L-DZ	147L-DZ	+	+	D16a-α	D16a-α	+	+	
150L-DZ	150L-DZ	+	+					
220 (22.3%)[g]				56 (92.9%)				266 (23.7%)
4/49 (8.2%)[h]				3/52 (5.8%)				0/63 (0%)

[a]LNT=mandibular lymph node tissue; [b]PSM=pork sausage meat; [c]FOX=cefoxitin; [d]OXA=oxacillin; [e]Staph specific rRNA. [f]Strain designation for FOX and OXA resistant strains; [g]Total number of samples and (% S. aureus positives); [h]Number of MRSA positive strains per number of S. aureus strains and (% of MRSA strains).

Table 3. The MIC[a] and MIC$_M$[b] data measured for the 164 Staphylococcus aureus *strains isolated from 2017 swine lymph node tissue, 2019 pork sausage meat and 2006 feces against the six OAs tested.*

Organic Acids	MICs (µg/mL)	MIC$_M$s (mM)	*S. aureus* strains (No.)
Acetic acid	4096	68.21	1
	2048	34.1	111
	1024	17.05	52
Butyric acid	4096	46.49	5
	2048	23.25	157
	512	5.81	1
	256	2.91	1
Citric acid	16384	85.28	4
	8192	42.64	13
	4096	21.32	94
	2048	10.66	51
	128	0.665	2
Formic acid	1024	22.25	164
L-Lactic acid	16384	181.88	1
	8192	90.94	7
	4096	45.47	102
	2048	22.74	54
Propionic acid	2048	27.65	108
	1024	13.82	56

[a]MIC=Minimum inhibitory concentration, [b]MIC$_M$=Molar MIC.

Table 4 shows the central tendency of the MIC and MIC$_M$ data listed in Table 3 for 164 *S. aureus* strains isolated from the total number of strains from the 2006 swine feces, 2017 swine lymph node tissue, and 2019 pork sausage meat against six organic acids. The typical values observed for a probability distribution are referred to as central tendency. The largest

median values were observed for L-lactic acid and acetic acid, 45.47 and 34.10 mM, respectively. The largest range values were for L-lactic, citric and acetic acid of 181.88, 85.28 and 68.21 mM, respectively, while the largest 90th percentile values were for L-lactic and citric acid of 45.47 and 42.64 mM, respectively.

Table 4. Central tendency of the MIC[a] and MIC$_M$[b] data for the 164 Staphylococcus aureus *strains isolated from the combined total strains of the 2006 swine feces, 2017 swine lymph node tissue, and 2019 pork sausage meat against the six OAs tested.*

Organic Acid	Median	Mode	Range	90th Percentile
Acetic Acid				
MIC (µg/mL)	2048	2048	1024–4096	2048
MIC$_M$ (mM)	34.1	34.1	17.05–68.21	34.1
Butyric Acid				
MIC (µg/mL)	2048	2048	256–4096	2048
MIC$_M$ (mM)	23.25	23.25	2.91–46.49	23.25
Citric Acid				
MIC (µg/mL)	4096	4096	128–16384	8192
MIC$_M$ (mM)	21.32	21.32	0.665–85.28	42.64

Organic Acid	Median	Mode	Range	90th Percentile
Formic Acid				
MIC (µg/mL)	1024	1024	1024	1024
MIC$_M$ (mM)	22.25	22.25	22.25	22.25
L-Lactic Acid				
MIC (µg/mL)	4096	4096	2048–16384	4096
MIC$_M$ (mM)	45.47	45.47	22.74–181.88	45.47
Propionic Acid				
MIC (µg/mL)	2048	2048	1024–2048	2048
MIC$_M$ (mM)	27.65	27.65	13.82–27.65	27.65

[a]MIC=Minimum inhibitory concentration. [b]MIC$_M$=Molar MIC.

3.2.2. Results for the 52 S. aureus *Strains Isolated from the 2019 Pork Sausage Meat*

The MIC and MIC$_M$ data for 52 *S. aureus* strains isolated from 2019 pork sausage meat against the six OAs are shown in Table 5. Butyric and L-lactic acid, 46.49 and 45.47 mM, respectively, had the highest OA molar concentrations for inhibition of *S. aureus*. Acetic and propionic acid, 34.10 and 27.65 mM, respectively, had the next highest OA molar concentrations for *S. aureus* inhibition, and citric and formic acid, 21.32 and 22.25 mM, respectively, had the lowest OA molar concentrations.

Table 6 shows the central tendency of the MIC and MIC$_M$ data listed in Table 5 for the 52 *S. aureus* strains isolated from the 2019 pork sausage meat tested against six organic acids. The largest molar median values were observed for butyric, L-lactic, citric, and formic acid, at 23.25, 22.74, and 22.25 mM, respectively. The largest range values were observed for butyric and L-lactic acid, 46.5 and 45.47 mM, respectively, while the largest 90th percentile value for a *S. aureus* MIC$_M$ was for L-lactic acid, 45.47 mM.

Table 7 and Table 8 show the MICs and MIC$_M$S and Table 9 and Table 10 show the central tendency of the MICs and

MIC$_M$s for the 2006 feces and 2017 swine mandibular lymph node tissue, respectively.

3.3. The pH for the 164 S. aureus *Strains at Their MICs Against the Six OAs*

The pH values obtained for all *S. aureus* strains (n=164) were grouped for each OA, resulting in six groups. These experimentally determined pH values at the MICs of the 164 *S. aureus* strains were plotted against the MIC$_M$S for each of the OAs (Figure 1). In Figure 1 the number of strains at each MIC are shown next to the data points, and the pH values are the average of triplicate samples with the standard deviation (SD) shown at each data point. Acetic acid inhibits 100% of the *S. aureus* strains at a pH of 4.4, butyric and formic acid inhibits 100% of the strains at a pH of 4.64 and 4.66, respectively, while propionic acid inhibits 100% of the strains at the pH of 4.94. Therefore, there is 0.54 pH unit difference in these inhibition values. However, L-lactic and citric acid inhibit 100% of the *S. aureus* strains at the pH of 3.59 and 3.07, respectively. The difference in these two sets of OAs is approximately Δ=1.33 pH unit.

Table 5. *The MIC[a] and MIC$_M$[b] data for the 52* Staphylococcus aureus *strains isolated from the 2019 swine pork sausage meat against the six OAs tested.*

Organic Acids	MICs (µg/mL)	MIC$_M$S (mM)	*S. aureus* strains (No.)
Acetic acid	2048	34.1	3
	1024	17.05	49
Butyric acid	4096	46.49	1
	2048	23.25	51
Citric acid	4096	21.32	8
	2048	10.66	44
Formic acid	1024	22.25	52
L-Lactic acid	4096	45.47	19
	2048	22.74	33
Propionic acid	2048	27.65	18
	1024	13.82	34

[a]MIC=Minimum inhibitory concentration. [b]MIC$_M$=Molar MIC.

Table 6. *Central tendency of the MIC[a] and MIC$_M$[b] data for the 52* Staphylococcus aureus *strains isolated from the 2019 pork sausage meat against the six OAs tested.*

Organic Acid	Median	Mode	Range	90th Percentile
Acetic Acid				
MIC (µg/mL)	1024	1024	1024–2048	1024
MIC$_M$ (mM)	17.05	17.05	17.05–34.1	17.05
Butyric Acid				
MIC (µg/mL)	2048	2048	2048–4096	2048
MIC$_M$ (mM)	23.25	23.25	23.25–46.5	23.25
Citric Acid				
MIC (µg/mL)	2048	2048	2048–4096	4096
MIC$_M$ (mM)	10.66	10.66	10.66–21.32	21.32

Organic Acid	Median	Mode	Range	90th Percentile
Formic Acid				
MIC (µg/mL)	1024	1024	1024	1024
MIC$_M$ (mM)	22.25	22.25	22.25	22.25
L-Lactic Acid				
MIC (µg/mL)	2048	2048	2048–4096	4096
MIC$_M$ (mM)	22.74	22.74	22.74–45.47	45.47
Propionic Acid				
MIC (µg/mL)	1024	1024	1024–2048	2048
MIC$_M$ (mM)	13.82	13.82	13.82–27.65	27.65

[a]MIC=Minimum inhibitory concentration. [b]MIC$_M$=Molar MIC.

Table 7. *The* Staphylococcus aureus *MICs[a] and MIC$_M$s[b] for 63 strains isolated from 2006 swine feces against six organic acids.*

Organic Acids	MICs (µg/mL)	MIC$_M$s (mM)	*S. aureus* strains (No.)
Acetic acid	4096	68.21	1
	2048	34.10	59
	1024	17.05	3
Butyric acid	2048	23.25	61
	512	5.81	1
	256	2.91	1
Citric acid	16384	85.28	3
	8192	42.64	4
	4096	21.32	51
	2048	10.66	3
	128	0.665	2
Formic acid	1024	22.25	63
L-Lactic acid	16384	181.88	1
	8192	90.94	3
	4096	45.47	47
	2048	22.74	12
Propionic acid	2048	27.65	44
	1024	13.82	19

[a]MICs=Minimum inhibitory concentrations. [b]MIC$_M$s=Molar MICs.

Table 8. *The* Staphylococcus aureus *MICs[a] and MIC$_M$s[b] for 49 strains isolated from 2017 swine mandibular lymph node tissue against six organic acids.*

Organic Acids	MICs (µg/mL)	MIC$_M$s (mM)	*S. aureus* strains (No.)
Acetic acid	2048	34.10	49
Butyric acid	4096	46.50	4
	2048	23.25	45
Citric acid	16384	85.28	1
	8192	42.64	9
	4096	21.32	35
	2048	10.66	4
Formic acid	1024	22.25	49
L-Lactic acid	8192	90.94	4
	4096	45.47	36
	2048	22.74	9
Propionic acid	2048	27.65	46
	1024	13.82	3

[a]MICs=Minimum inhibitory concentrations. [b]MIC$_M$s=Molar MICs.

Table 9. *Central tendency of the* Staphylococcus aureus *MICs[a] and MIC$_M$s[a] for 63 strains isolated from the 2006 swine feces against six organic acids.*

Organic Acid	Median	Mode	Range	90th Percentile
Acetic Acid				
MIC (µg/mL)	2048	2048	1024–4096	2048
MIC$_M$ (mM)	34.1	34.1	17.05–68.21	34.1
Butyric Acid				
MIC (µg/mL)	2048	2048	256–2048	2048
MIC$_M$ (mM)	23.25	23.25	2.91–23.25	23.25
Citric Acid				
MIC (µg/mL)	4096	4096	128–16384	8192
MIC$_M$ (mM)	21.32	21.32	0.665–85.28	42.64
Formic Acid				

Organic Acid	Median	Mode	Range	90th Percentile
MIC (μg/mL)	1024	1024	1024	1024
MIC$_M$ (mM)	22.25	22.25	22.25	22.25
L-Lactic Acid				
MIC (μg/mL)	4096	4096	2048–16384	4096
MIC$_M$ (mM)	45.47	45.47	22.74–181.88	45.47
Propionic Acid				
MIC (μg/mL)	2048	2048	1024–2048	2048
MIC$_M$ (mM)	27.65	27.65	13.82–27.65	27.65

[a]MICs=Minimum inhibitory concentrations. [b]MIC$_M$s=Molar MICs.

Table 10. *Central tendency of the* Staphylococcus aureus *MICs[a] and MIC$_M$s[b] for 49 strains isolated from the 2017 swine mandibular lymph node tissue against six organic acids.*

Organic Acid	Median	Mode	Range	90th Percentile
Acetic Acid				
MIC (μg/mL)	2048	2048	4096	2048
MIC$_M$ (mM)	34.1	34.1	34.1	34.1
Butyric Acid				
MIC (μg/mL)	2048	2048	2048–4096	2048
MIC$_M$ (mM)	23.25	23.25	23.25–46.5	23.25
Citric Acid				
MIC (μg/mL)	4096	4096	2048–16384	8192
MIC$_M$ (mM)	21.32	21.32	10.66–85.28	42.64
Formic Acid				
MIC (μg/mL)	1024	1024	1024	1024
MIC$_M$ (mM)	22.25	22.25	22.25	22.25
L-Lactic Acid				
MIC (μg/mL)	4096	4096	2048–8192	4096
MIC$_M$ (mM)	45.47	45.47	22.74–90.94	45.47
Propionic Acid				
MIC (μg/mL)	2048	2048	1024–2048	2048
MIC$_M$ (mM)	27.65	27.65	13.82–27.65	27.65

[a]MICs=Minimum inhibitory concentrations. [b]MIC$_M$s=Molar MICs.

The pH vs MIC$_M$s of the *S. aureus* strains isolated from 2006 swine feces are shown in Figure 2, from 2017 mandibular lymph node tissue are shown in Figure 3, and from 2019 pork sausage meat are shown in Figure 4.

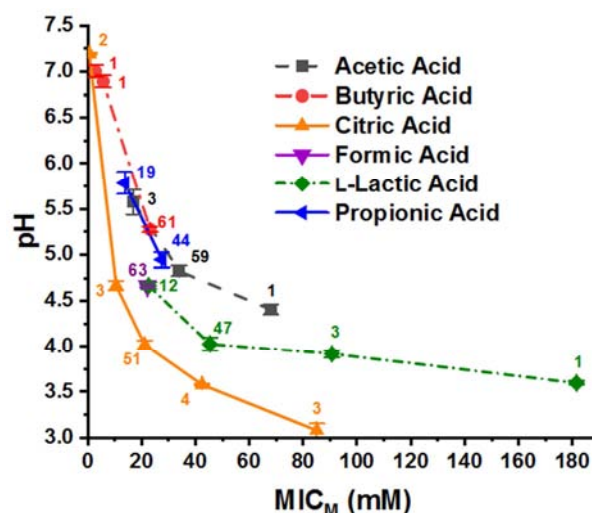**Figure 1.** *pH vs. MIC$_M$ for 164 total strains of* Staphylococcus aureus *isolated from swine.*

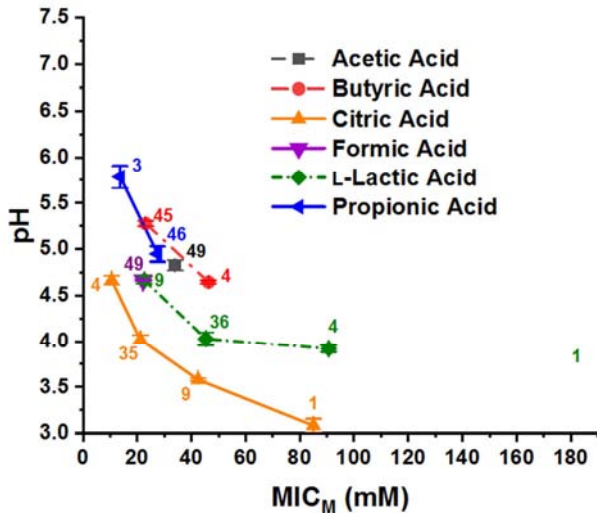**Figure 2.** *pH vs. MIC$_M$ for 63 strains of* Staphylococcus aureus *isolated from 2006 swine feces.*

Figure 3. *pH vs. MIC_M for 49 strains of* Staphylococcus aureus *isolated from 2017 swine mandibular lymph node tissue.*
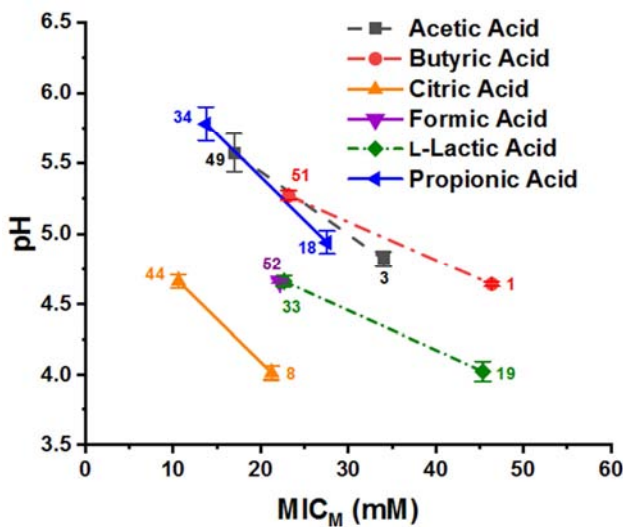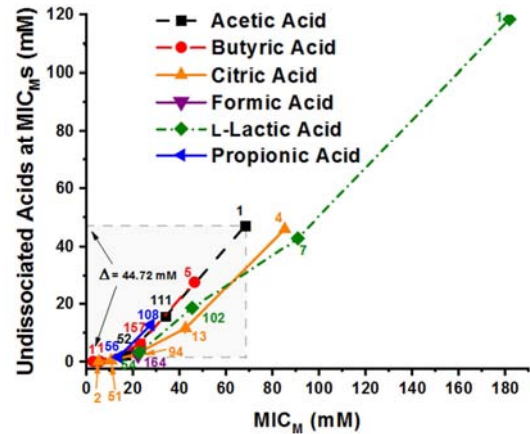

Figure 5. *The concentration of undissociated OAs at the MIC_Ms for 164 total isolates of* Staphylococcus aureus *from swine. 100% of all isolates were inhibited by acetic, butyric, formic, and propionic acids over a concentration range of $\Delta=44.72$ mM; acetic acid at 47.16 mM – formic acid at 2.44 mM.*
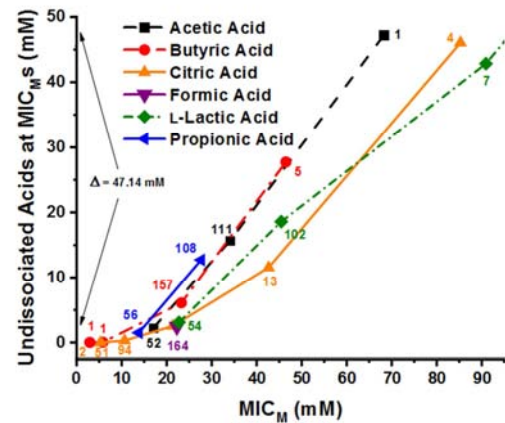

Figure 4. *pH vs. MIC_M for 52 strains of* Staphylococcus aureus *isolated from 2019 pork sausage batter.*

Figure 6. *The concentration of undissociated OAs at the MIC_Ms for 164 total isolates of* Staphylococcus aureus *from swine. 100% of all isolates were inhibited by acidic, butyric, formic, and propionic acid over a concentration range of $\Delta=44.72$ mM; acetic acid at 47.16 mM – formic acid at 2.438 mM. Expanded version of Figure 5 for visual purposes.*
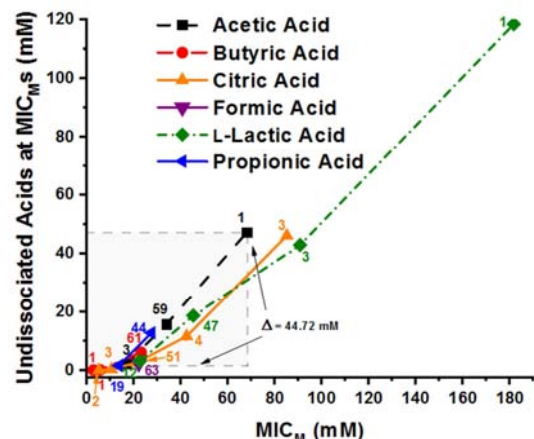
3.4. The Calculated Molar Undissociated OA Concentrations at the S. aureus MIC_Ms

The molar concentrations of undissociated OAs were calculated at the MIC_Ms for the six OAs, citric, L-lactic, butyric, acetic, propionic, and formic acid using the Henderson-Hasselbalch equation and the plot is depicted in Figure 5. The number of *S. aureus* strains at each MIC is shown next to the data points. The levels of the undissociated OAs calculated at the MICs for 100% of the *S. aureus* strains against citric, L-lactic, butyric, and acetic acid are 46.07, 118.33, 27.74, and 47.16 mM, respectively. But, the undissociated propionic and formic acid levels required for inhibition of 100% of the same strains were 12.71 and 2.44 mM, respectively. The difference between the undissociated acetic and undissociated formic acid levels needed for inhibition of 100% of the strains was $\Delta=44.72$ mM, shown by the light grey box in Figure 5.

Figure 7. *The concentration of undissociated OAs at the MIC_Ms for 63 strains of* Staphylococcus aureus *isolated from 2006 swine feces.*

An expanded version of Figure 5 can be found in Figure 6. The concentrations of the undissociated OAs vs MIC_Ms of the

S. aureus strains isolated from 2006 feces are shown in Figure 7, from 2017 mandibular lymph node tissue are shown in Figure 8, and from 2019 pork sausage are shown in Figure 9.
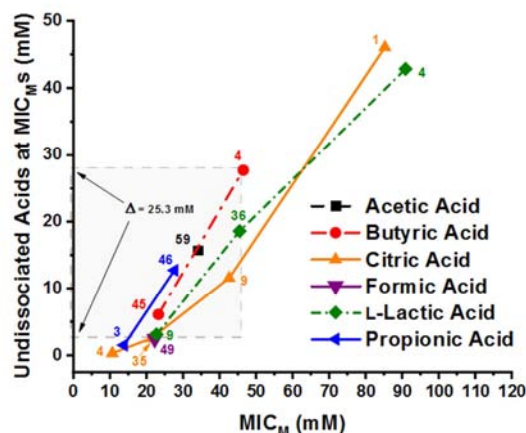

Figure 8. *The concentration of undissociated OAs at the MIC$_M$s for 49 strains of* Staphylococcus aureus *isolated from 2017 swine mandibular lymph node tissue.*
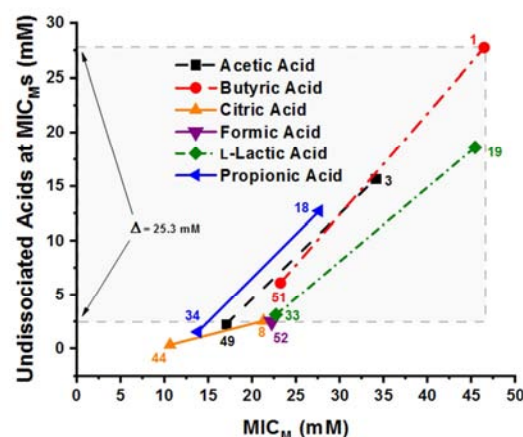

Figure 9. *The concentration of undissociated OAs at the MIC$_M$s for 52 strains of* Staphylococcus aureus isolated from 2019 pork sausage meat.

Figure 10. *pH vs. the undissociated OA concentrations at the MIC$_M$s for 164 total* Staphylococcus aureus *strains from swine. Butyric acid (pH 5.27) (97%) – citric acid (pH 3.57) (97.6%)=Δ 1.7 pH units. Butyric acid (pH 4.64) (100%) – citric acid (pH 3.07) (100%)=Δ 1.57 pH units.*

The pH versus the molar concentrations of undissociated OAs at the MIC$_M$s of the 164 *S. aureus* strains is depicted in

Figure 10. The number of *S. aureus* strains at each MIC is shown next to the data points. The difference in pH between inhibition of 100% of the *S. aureus* strains by propionic and acetic acid is Δ=0.54 pH unit, and the difference in undissociated OA between these two data points for propionic and acetic acid is Δ=34.45 mM. The difference between inhibition of 100% of the *S. aureus* strains by propionic and L-lactic acid is Δ=1.35 pH units, and the difference between inhibition of 100% of the *S. aureus* strains by propionic and citric acid is Δ=1.87 pH units.

3.5. The Calculated Molar Dissociated OA Concentrations at the S. aureus MIC$_M$s

The molar concentrations of the dissociated OAs, citric, L-lactic, butyric, acetic, propionic, and formic acid were calculated with the Henderson-Hasselbalch equation using the determined pH data and known concentrations of OAs at the MICs of the 164 *S. aureus* strains and plotted as depicted in Figure 11. The number of *S. aureus* strains at each MIC is shown next to the data points. The maximum molar concentration of dissociated OAs needed to inhibit 100% of the *S. aureus* strains against butyric, acetic, propionic, and formic acid are shown within the grey-shaded rectangle. The two acids that encompass the grey-shaded rectangle are acetic and propionic acid, with a difference in concentration of Δ=6.11 mM dissociated OA. 100% of the *S. aureus* strains inhibited by dissociated propionic acid is achieved at 14.94 mM and the molar concentration of dissociated acetic acid required to inhibit 100% of the bacteria was at 21.05 mM. The citric and L-lactic acid concentrations are affected by *S. aureus* utilization of these acids.
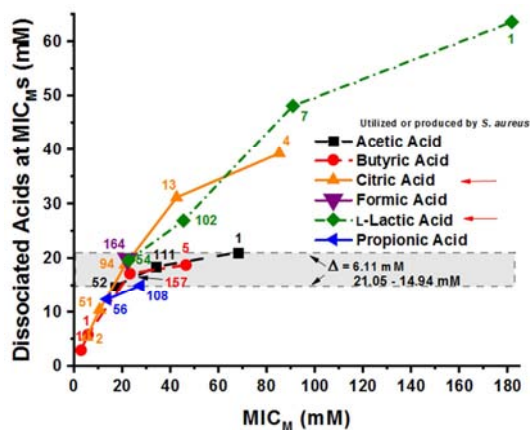

Figure 11. *The concentration of dissociated organic acids at the MIC$_M$s for 164 total isolates of* Staphylococcus aureus *from swine. 100% of all isolates were inhibited by acetic, butyric, formic, and propionic acids with a difference in concentration of Δ=6.11 mM; 21.05 mM acetic – 14.94 mM propionic acid.*

An expanded version of Figure 11 can be seen in Figure 12. The concentrations of the dissociated OAs vs MIC$_M$s of the *S. aureus* strains isolated from 2006 swine feces are shown in Figure 13, from 2017 swine mandibular lymph node tissue are shown in Figure 14, and from 2019 pork sausage meat are shown in Figure 15.
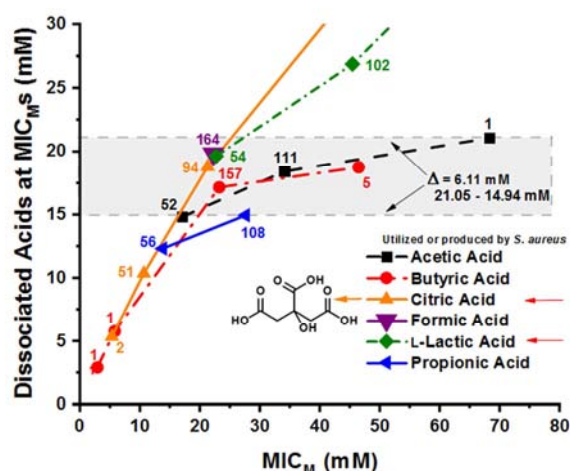
Figure 12. *The concentration of dissociated organic acids at the MIC$_M$s for 164 total isolates of* Staphylococcus aureus *from swine. 100% of all isolates were inhibited by acetic, butyric, formic, and propionic acid with a difference in concentration of Δ=6.11 mM; 21.05 mM acetic acid – 14.94 mM propionic acid. This is an expanded version of Figure 11 for visual reasons.*
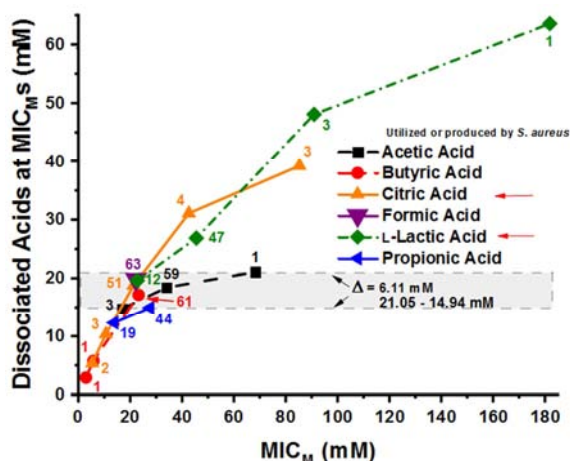

Figure 13. *The concentration of dissociated organic acids at the MIC$_M$s for 63 strains of* Staphylococcus aureus *isolated from 2006 swine feces.*
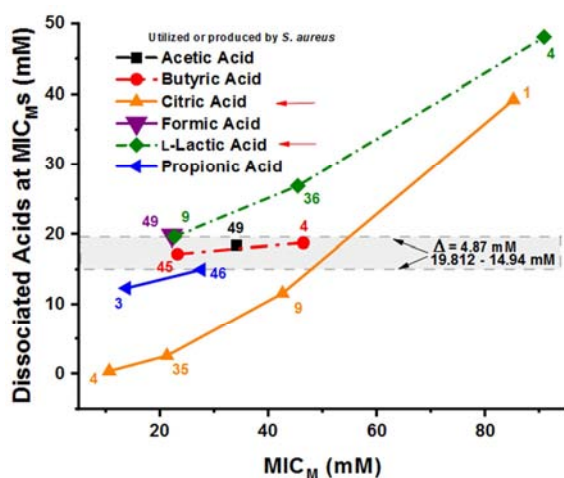

Figure 14. *The concentration of dissociated organic acids at the MIC$_M$s for 49 strains of* Staphylococcus aureus *isolated from 2017 swine mandibular lymph node tissue.*
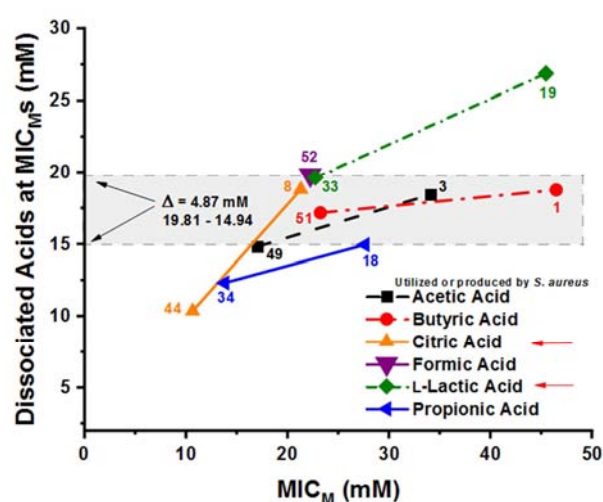

Figure 15. *The concentration of dissociated organic acids at the MIC$_M$s for 52 strains of* Staphylococcus aureus *isolated from 2019 pork sausage meat.*

The molar concentrations of the dissociated OAs, citric, L-lactic, butyric, acetic, propionic, and formic acid versus the pH determined at the MICs of the *S. aureus* strains are plotted in Figure 16. The number of *S. aureus* strains at each MIC is shown next to the data points. The *S. aureus* strains are all inhibited by the dissociated OAs butyric, formic, propionic, and acetic acid at or below the concentration of dissociated acetic acid (21.05 mM) indicated by the narrow light grey vertical box. These four OAs inhibit 100% of the *S. aureus* strains over the range of 14.94–21.05 mM OA. Whereas, 97.6% of the strains are inhibited by citric acid (narrow light orange vertical box) at a dissociated acid concentration of 31.09 mM, and 99.4% of the strains are inhibited by L-lactic acid (narrow light green vertical box) at a dissociated acid concentration of 48.08 mM.

4. Discussion

Staphylococcus aureus is commonly known as both a commensal and a pathogen in animals and humans [10]. Pigs are commonly known to be colonized by *S. aureus* bacteria [9], which can result in a reservoir of MRSA at the pig farm [10, 15]. Therefore, workers that have occupational exposure to swine production facilities are at risk of being a carrier of MRSA [20]. When pigs are transported to the abattoir for processing, they bring colonized *S. aureus* and MRSA with them [19]. Various methods have been used to remove *S. aureus* and other bacterial contamination from swine carcasses, like dehairing [10, 75], singeing [10], scalding water [76, 77], OA carcass washes [42, 46], and cut meat OA surface washes [41]. But even with all the applied methods available to decontaminate swine carcasses and meat cuts to remove contaminating bacteria, final pork products still end up with *S. aureus* and MRSA contamination [34, 35, 78].
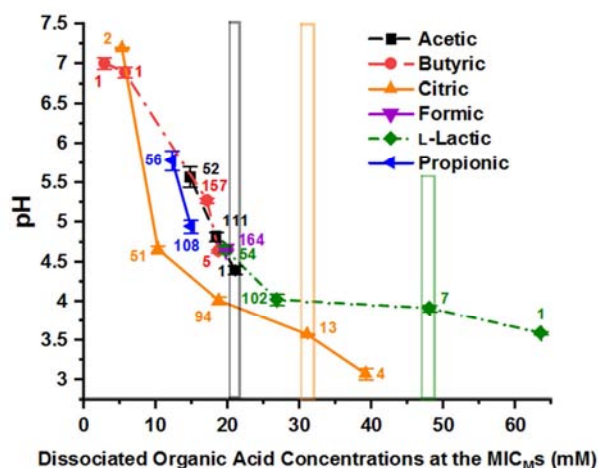
Figure 16. *pH vs. dissociated organic acid concentrations at the MIC$_M$s for 164 total* Staphylococcus aureus *strains isolated from swine. The grey box shows the concentration of dissociated acetic acid that inhibits 100% of the isolates. The light orange box shows the concentration of dissociated citric acid that inhibits 97.6% of the isolates. The light green box shows the concentration of lactic acid the inhibits 99.4% of the isolates.*

Many foodborne pathogens can adapt to varying pH environments and to numerous decontamination strategies based on pH [79]. We have isolated 164 *S. aureus* strains from swine mandibular lymph node tissue, commercial pork sausage meat and swine feces, and the susceptibility of these strains was evaluated against six OAs, citric, L-lactic, butyric, acetic, propionic, and formic acid to determine the inhibition mode of action of the OAs. Similar MICs were observed for the strains isolated from pork sausage meat as for those isolated from other swine sources, except for the MICs against citric and L-lactic acid. The MICs for these two OAs are higher for the strains isolated from mandibular lymph node tissue and swine feces than for the strains from pork sausage meat.

4.1. *S. aureus* Was Found Intracellularly in Swine Lymph Node Tissue

Upon evaluation of the 164 *S. aureus* strains with cefoxitin and oxacillin antibiotics and PCR studies, seven strains were determined to be MRSA. The feces strains contained *S. aureus* strains, but none were MRSA. *S. aureus* strains were found in both the mandibular lymph node tissue and pork sausage meat, and both sources contained MRSA strains at 4/49 (8.2%) and 3/52 (5.8%), respectively. The pork sausage meat contamination may well be from cross-contaminated meat cuts. However, the lymph node tissue was specifically removed from the swine carcasses by hand and later immersed in boiling water to surface sterilize the tissue. Therefore, one would expect the hand removed tissue to have very low numbers of bacteria including MRSA, but that was not the case. These results indicate that *S. aureus* bacteria had invaded the tissue and were within the tissue of the swine lymph nodes (intracellular). Mann and coworkers have shown a large array of bacteria in swine lymph nodes, including *Salmonella* and *Staphylococcus* among others [80, 81]. These lymph nodes may remain on the carcasses following slaughter and end up in

ground meat and sausage products [82]. Studies conducted to determine the percentage of MRSA in final pork products consistently demonstrate a prevalence of 2.2–6.6% MRSA [34, 35, 79]. Based on the findings of our study showing 8.2% intracellular MRSA contamination in swine lymph node tissue, it is likely that the prevalence of MRSA determined in final pork products is not primarily from surface contamination but rather from bacterial intracellular invasion.

4.2. pH Differences at the *S. aureus* MIC$_M$s Tested Against Solutions Containing Different OAs

A difference of 0.55 pH unit was observed when inhibiting 100% of the strains by propionic, butyric, formic, and acetic acid. A difference of 1.87 pH unit was observed when inhibiting 100% of the strains by all OAs, propionic – citric acid, 4.94 – 3.07 pH, respectively. If only pH is assumed to be the important attribute of OAs for inhibiting bacteria, then acid-resistant pathogens [79, 83–85] may be a real threat in OA acidified foods [55]. However, acidified food products without heat treatments have been safely made for many years [86]. Also, if pH were the primary cause of *S aureus* inhibition, then it would be expected that MICs for the same bacteria against different OAs would be observed at very similar pH values, but that is not what we observed. Our laboratory has previously published the differences in pH between the inhibition of Gram-positive and Gram-negative bacterial species by OAs. The change in pH for 100% of vancomycin-resistant *Enterococcus faecium* (VRE) strains against all six OAs was 1.1 pH units [68]. Ninety-eight percent of 175 *Pseudomonas aeruginosa* strains were inhibited by different OAs by a difference of 0.98 pH unit [64]. Ninety-eight percent of 344 *Escherichia coli* O157:H7 strains were inhibited by three different OAs by a difference of 0.56 pH unit [63]. All 138 non-O157 Shiga toxin-producing *E. coli* strains were inhibited by four OAs by a difference of 0.99 pH unit [65]. Ninety-five percent of 145 *Salmonella* strains were inhibited by four different OAs by a difference of 1.1 pH unit [66]. One hundred percent of 111 *Campylobacter coli* strains were inhibited by all six OAs by a difference of 1.76 pH units [62]. Ninety-seven to ninety-nine percent of 96 *C. jejuni* strains were inhibited by all six OAs with a difference of an average of 1.34 pH units [67]. In other studies, it was shown that two different acids, acetic acid and HCL, inhibited *E. coli* O157:H7 at different pH values, 5.5 and 4.5 pH, respectively, for a difference of Δ=1 pH unit apart [87]. The inhibition of *S. aureus* in this study as well as the previous studies demonstrate that bacterial inhibition is not dependent on pH, as other researchers have hypothesized [88]. Therefore, bacterial inhibition by OAs must then be attributable to some other characteristic of OAs [67, 89].

4.3. The Relationship of Undissociated OA Concentrations to the MIC$_M$ Concentrations and pH

S. aureus strains (163 of 164) were inhibited by the six undissociated OAs, citric, butyric, acetic, formic, L-lactic and propionic acid between the concentrations of undissociated

acetic and undissociated formic acid at 47.16 and 2.44 mM, respectively. This is a difference in undissociated OA levels of Δ=44.72 mM required for inhibition of the same strains by all OAs. The inhibition of most of the strains by some OAs, butyric, citric, and formic acid occurred at very dilute OA concentrations, 6.09, 2.53, and 2.44 mM, respectively, if indeed the undissociated OAs were causing inhibition in *S. aureus*. There appears to be no correlation between the undissociated OA levels with respect to the MIC$_M$s of the 164 *S. aureus* strains. This result agrees with our earlier published studies of a Gram-positive and Gram-negative bacteria. Inhibition of 50 Gram-positive VREs required a difference of Δ=35.21 mM undissociated OAs, and all strains would have been inhibited by undissociated citric and formic acid at a concentration of 1.9 and 2.49 mM, respectively [68]. One-hundred percent of 175 *P. aeruginosa* strains were inhibited by undissociated citric and acetic acid concentrations at 2.53 and 21.65 mM, respectively, for a Δ=19.12 mM OAs [64]. Undissociated citric and acetic acid at 2.86 and 50.63 mM, respectively, inhibited 98.3% of 344 *E. coli* O157:H7 strains for a difference of Δ=47.77mM OAs [63]. One-hundred percent of 138 non-O157 STEC strains were inhibited by undissociated citric and acetic acid at 2.2 and 49.11 mM, respectively, for a difference of Δ=46.91 mM OAs [65]. One-hundred percent of 145 *Salmonella* strains were inhibited by undissociated citric and acetic acid at 2.29 and 19.0 mM, respectively, for a difference of Δ=16.71 mM OAs [66]. Undissociated citric and acetic acid at 0.024 and 39.93 mM, respectively, would inhibit 100% of 111 *C. coli* strains for a difference of Δ=39.91 mM OAs [62], and 100% of all 96 *C. jejuni* strains would be inhibited by the six undissociated OAs, citric, butyric, acetic, formic, L-lactic and propionic acid with citric and L-lactic acid concentrations at 0.932 and 51.71 mM, respectfully, for a difference of Δ=50.78 mM OAs [67]. The difference in the undissociated OAs required to inhibit the *S. aureus* strains tested in this study is similar to the concentrations of undissociated OAs we have seen in other studies of pathogens; having variable and high levels of undissociated OAs.

We compared the undissociated OA concentrations to the pH at the MIC$_M$s to obtain a further understanding of the undissociated OA results. When viewing the pH at the *S. aureus* MIC$_M$s for the 164 strains, 90% of the strains are suggested to be inhibited by an undissociated citric acid concentration at extremely low levels. All of the strains would be inhibited by undissociated acid at a pH nearly midway between the pH values from other OAs that inhibit the same strains, and the concentration of undissociated formic acid is also extremely low; whereas, other OAs like acetic and butyric required higher levels of undissociated acid, and citric and L-lactic acid could have much higher undissociated acid levels. From this perspective there is no consistent values of undissociated acids or pH levels that correspond with the inhibition of *S aureus* strains.

4.4. The Relationship of Dissociated OA Concentrations to the MIC$_M$ Concentrations and pH

One-hundred percent of the 164 *S. aureus* strains were inhibited by the four dissociated OAs, acetic, butyric, formic, and propionic acid within a narrow concentration range of the dissociated OAs, 21.05 mM acetic acid – 14.94 mM propionic acid, for a difference of Δ=6.11 mM dissociated OAs. The other two OAs, citric and L-lactic acids are utilized by *S. aureus*. The results of this study are quite similar to those obtained for another Gram-positive pathogen, VRE [68]. In that study all 50 VRE strains were inhibited by the same six OAs used in this study with a narrow band of dissociated OAs, Δ=3.1 mM. Similarly, other Gram-negative pathogenic bacteria behaved in a like manner when challenged with OAs. Ninety-eight percent of 175 *P. aeruginosa* strains were inhibited by a narrow band of two dissociated OAs bracketed by dissociated citric acid (10.24 mM) and dissociated acetic acid (9.98 mM) for a difference of Δ=0.26 mM [64]. 98.3% of 344 *E. coli* O157:H7 strains were inhibited by a narrow band of dissociated OAs bracketed by dissociated propionic (13.82 mM) and L-lactic acid (19.12 mM) for a difference of Δ=5.54 mM dissociated OAs [63]. One-hundred percent of 138 non-O157 STEC strains were inhibited by a narrow band of dissociated OAs bracketed by dissociated L-lactic (12.93 mM) and dissociated citric acid (19.12 mM) having a concentration difference of Δ=6.19 mM dissociated OAs [65]. One-hundred percent of 145 *Salmonella* strains were inhibited by a narrow band of dissociated OAs bracketed by dissociated propionic (13.67 mM) and dissociated citric acid (19.03 mM) having a concentration difference of Δ=5.36 mM dissociated OAs [66]. One-hundred percent of 111 *C. coli* strains were inhibited by dissociated butyric (22.56 mM) and dissociated L-lactic acid (21.17 mM) having a concentration difference of Δ=1.39 mM dissociated OAs [62]. Ninety-seven to 100% of 96 *C. jejuni* strains were inhibited by a narrow band of dissociated OAs bracketed by dissociated butyric (22.72 mM) and dissociated acetic acid (24.86 mM) having a concentration difference of Δ=4.47 mM dissociated OAs [67]. In all these cases only a well-defined narrow range of dissociated OA concentrations were required to inhibited 100% of these pathogenic bacteria. The dissociated OAs appear to be consistently able to inhibit bacteria over a concentration range from 12.45 mM to 21.05 mM dissociated OAs.

Alternatively, we also can consider the pH observed and the concentrations of dissociated OA levels at the MICs. Here we observed that the pH varied widely over the *S. aureus* MICs for both citric and L-lactic acid, with pH values of from 3.04 to 7.19, respectively, and dissociated OA concentrations of 39.21 mM for citric and 63.55 mM for L-lactic acid to inhibit all strains. Again, these two OAs are utilized by *S. aureus* which causes high levels of these two OAs required to inhibit all 164 strains tested. But a value of only 21.05 mM dissociated acetic acid inhibited 100% of all 164 strains tested by the other four OAs. Three of the OAs, acetic, butyric and formic acid also inhibited these same 164 strains at the 21.05 mM level. Acetic, butyric, formic and propionic acid inhibited 164 strains of *S. aureus* between the pH of 4.4 and 4.94, and between concentrations of dissociated OAs of 14.94 and 21.05 mM. Consistent and efficient inhibition of *S. aureus* strains was observed in our studies by the four dissociated non-utilized

OAs at a maximum concentration of 21.05 mM.

We observed moderately high levels of citric and L-lactic acid that were required to inhibit *S. aureus* compared to the other four OAs tested. Identification of *S. aureus* by a biochemical test demonstrated that citrate is utilized by the bacterium [90]. The coagulation of plasma by *S. aureus* was shown to be caused by the utilization of citrate by the bacterium [91]. Multiple transport systems have evolved in *S. aureus* to assure the supply of iron from host sources [92]. Citrate is utilized by *S. aureus* in the production of two iron siderophores, staphyloferrin A (SA) and staphyloferrin B (SB) [93, 94]. SA contains two molecules of citrate [95, 96], and SB contains a single molecule of citrate [97]. *S. aureus* can efficiently utilize L-lactate for growth [98–101]. L-Lactate can be effectively converted by *S. aureus* to acetate, and *S. aureus* can simultaneously use both L-lactate and glucose for growth [99]. When glucose becomes limiting, *S. aureus* can maintain exponential growth by switching to total aerobic utilization of L-lactate [99].

Studies aimed at comparing the potency of acetic and citric acid against a *S. aureus* contaminated mayonnaise-based salad demonstrated that acetic acid was more effective at inhibiting *S. aureus* than citric acid [102]. Also, a challenge test study to determine the combination of chemical preservatives and acidification that would be best for storage of orange fleshed sweet potato puree found that citric acid by itself was much poorer to use as a preservative than a combination of chemicals [103]. Therefore, based on our studies and the literature we disagree with the authors of a paper that suggested that "citric acid offers a novel prophylactic approach for controlling MRSA and VISA infections of wounds from orthopaedic patients" [104], and conclude that citric acid should definitely not be used to treat patients with *S. aureus* infections.

In order for *S. aureus* to infect areas of the human body it must counter act host innate immunity molecules [105], and the nitric oxide (NO*) radical is a common broad-spectrum antimicrobial toxic agent used by a host to remove pathogenic microorganisms [98, 106, 107]. *S. aureus* is resistant to the NO* radical [97, 101, 107], because on long term exposures to NO* it will primarily utilize L-lactate [98]. When *S. aureus* encounters NO* it shifts to a new metabolic state and excretes L-lactate [107]. Also, the main reason *S. aureus* is found in skin infections and in the nasal cavity is because human sweat can contain as much as ~50 mM L-lactate which aids in colonization [108]. Also, nasal secretions are known to contain large concentrations of L-lactate, and *S. aureus* is known to commonly colonize the nasal passages which provides *S. aureus* a comfortable environment [109, 110]. Toxic shock syndrome (TSS) is commonly caused by *S. aureus* infection, but rarely occurs and the details leading from colonization to infection are not clear [111]. Only a small proportion of *S. aureus* strains carry the *tst* gene that encodes for the TSS toxin-1, which is basically responsible for the syndrome [112]. Lactic acid bacteria commonly colonize the vagina as well as other areas of humans, and produce lactic acid [113]. Over about the last 20 years the use of lactic acid

producing bacteria has emerged as a common probiotic for the vaginal ecosystem [114]. The practice of using a probiotic that produces lactic acid in hopes of removing *S. aureus* does not appear appropriate and may be a catalyst for the low number of cases of toxic shock syndrome that regularly appear [111].

Based upon the above information concomitant with our data, L-lactic acid producing bacteria should not be used in areas where *S. aureus* is known to colonize, and citric acid and L-lactic acid should not be used in OA carcass washes or meat sprays to remove bacteria if *S. aureus* is one of the bacterial contaminates of concern.

5. Conclusions

During these studies we observed an 8.2% MRSA intracellular contamination of the swine mandibular lymph node tissue, demonstrating that *S. aureus* strains had invaded the tissues and were not just surface contamination, and this tissue may be used in pork sausage production. The pH values observed at the MIC_{MS} demonstrated that pH is not consistent with bacterial inhibition. The wide range of concentration values associated with the undissociated OAs show the variability in the undissociated OA concentrations from high to very low concentrations, and these low concentrations are unlikely to inhibit *S. aureus*. The high concentrations of citric and L-lactic acid observed for inhibition of *S. aureus* are a result of the bacterium utilizing citrate and L-lactate for siderophore production and bacterial growth, respectively. The use of L-lactic acid for increased growth of *S. aureus* explains why the human skin and nasal cavities are good sites for colonization and infection by *S. aureus*, and lactic acid may influence the toxic shock syndrome. These two OAs, citric and L-lactic acid, would not be good candidates to use for OA carcass washes or for OA meat sprays for removing bacteria since these OAs would be expected to help revive *S. aureus*. Whereas, all 164 *S. aureus* strains tested were inhibited by the non-utilized OAs at a concentration of 21.05 mM dissociated OAs. Therefore, in this study as well as in previous studies with other pathogenic bacteria, the dissociated OAs showed good inhibition properties against pathogenic bacteria. A dissociated OA concentration of approximately 21 mM of acetic, butyric, formic, and propionic acid could potentially be used to remove *S. aureus* from contaminated carcasses, meats, and possibly other surfaces. At lower dissociated OA concentrations, *S. aureus* strains would be expected to escape inhibition. Of the six OAs tested in this study, acetic, butyric, formic, and propionic acid are the most efficient at inhibiting *S. aureus*.

article are those of the authors and do not necessarily reflect the official policy of the U.S. Department of Agriculture or the U.S. Government.

The authors declare that they have no competing interests.

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