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In Vitro Activity and Atom Pair Fingerprint Analysis of Potent Hits from Malaria Box against *Staphylococcus aureus* Isolated from Cows with Clinical Mastitis

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Abstract: *Staphylococcus aureus* (*S. aureus*) is one of the most prevalent contagious bacterial pathogens causing clinical bovine mastitis. In the present study, plate (A), which consists of 80 compounds (40 drug-like and 40 probe-like), was assessed from the Malaria Box against the in vitro growth of *S. aureus* isolated from cows naturally infected with *S. aureus*. Anti-*Staphylococcus aureus* medications that are newer and more potent than those that have been used previously were discovered. Bioinformatics analysis for the potent identified hits was performed. Three compounds—two probe-like and one drug-like—showed strong inhibitory action against *S. aureus* growth in vitro. Among these hits, MMV665941 exhibited the highest minimum inhibitory concentration value of 0.0078. Combination therapy was carried out using the most potent anti-*S. aureus* medication, MMV665941, and the traditionally used anti-*S. aureus* drug, amoxicillin/clavulanate (Augmentin 625[®]). The atom pair fingerprint (APfp) analysis revealed that MMV665941 and Augmentin showed maximum structural similarity. Based on the results obtained, it can be concluded that the Medicines for Malaria Venture Box has a wealth of potential anti-*S. aureus* candidates.

Keywords: Staphylococcus aureus; MMV665941; MMV001038; MMV666021

1. Introduction

Bovine mastitis, also known as an intra-mammary infection, is a mammary gland inflammation brought on by bacterial invasion and infection of the gland's secretory tissue [1-3]. The disease is considered one of the most prevalent, expensive, and widely applicable diseases in the dairy sector worldwide [4-6]. Each year, 30–50% of cows suffer from bovine mastitis worldwide [7–9]. Mastitis is a significant problem impacting animal welfare, productivity, and food safety and is thought to be the primary cause of dairy cow culling, in addition to the monetary losses brought on by decreased milk quality and yield, higher personnel costs, veterinary care, and medication [10]. Generally, cows with mastitis exhibit a wide range of symptoms, including lethargy, anorexia, increased body temperature, udder swelling, heat and pain, and milk with unusual appearance [10]. According to the severity of the inflammation, bovine mastitis can be divided into three categories: subclinical, chronic, and clinical [4]. Many risk factors have been linked to the incidence of clinical mastitis; in studies examining these risk factors, the sampling unit can range from the quarter level to the herd level [9,10]. The variance in the incidence of clinical mastitis in various quarters of an identical animal can be attributed to risk factors that are specific to that quarter [9,10]. The infection is mainly brought on by S. aureus.

S. aureus is one of the most significant zoonotic bacterial diseases which affects both humans and a variety of animals, particularly dairy cows [10]. Indeed, *S. aureus* has a smart



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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). pathogenesis, including facultative intracellular parasitism, increasingly serious antimicrobial resistance, and biofilm formation, making it challenging to treat using conventional therapy [4]. Additionally, S. aureus does not respond well to antibiotic therapy and can cause long-lasting infections that can persist through the lactation and into subsequent lactations [11-13]. As a result, it is important to find and employ innovative medications that have specific targets. Modern medicine is in danger due to the spread of microorganisms that are resistant to antibiotics, as well as from the overuse and abuse of antibiotics. To address this, the Medicines for Malaria Venture (MMV), a public–private partnership, recently developed the Malaria Box, a set of substances split into different compounds that are drug-like and different compounds that are probe-like [6,14,15]. The compounds with physicochemical characteristics that are appropriate for oral absorption are known as drug-like compounds and were subjected to substructure filters [16–18], as well as REOS (Rapid Elimination Of Swill) and the PAINS (Pan Assay Interference Compounds) filters [19], to remove known toxicophores. The hits classified as probe-like were those that did not pass through any of these filters [19,20]. This collection was selected from 19,000 structurally unique molecules that had previously shown activity against the erythrocytic stage of *Plasmodium* (*P.)* falciparum in three large phenotypic high-throughput screening (HTS) campaigns reported by GlaxoSmithKline (GSK) [21], St. Jude Children's Research Hospital [22], and the Genomics Institute of the Novartis Research Foundation (GNF) [23]. The Malaria Box is designed to be a starting point for drug discovery and the identification of drug targets and pathways for *P. falciparum* and other medically important pathogens [20,24]. All of these compounds are commercially available, which adds further merit to this huge library [20,25]. Most previous studies evaluated different candidates against laboratory S. aureus strains. There is paucity in the studies evaluating the selected drugs in terms of bacteria isolated from field samples. Using a broth microdilution experiment on a microtiter plate, it was possible to analyze the impact of bacterial strains on the efficacy of the tested medicine and assess MIC. The powerful hit against the bacterium strain would have the greatest value. Therefore, in the current investigation, we assessed the Malaria Box compounds' in vitro growth-inhibiting ability against S. aureus isolated from cows with clinical mastitis as a preliminary step within the drug discovery process. This study opens the way for the completion other phases of drug discovery procedures, including pre-clinical phases, clinical phases, and regulatory approval.

2. Materials and Methods

2.1. Chemical Reagents

Amoxicillin/clavulanate (Augmentin $625^{\text{(B)}}$) is a broad-spectrum antibacterial drug that was used as a control drug. Augmentin was prepared as a 100 Mm stock solution and, prior to use, MMV compounds were made as a 100 mM stock solution and kept at -30 °C. The Malaria Box compounds were sent frozen in 20 µL of a 10 mM DMSO (Sigma-Aldrich, Tokyo, Japan) solution and were provided in V-shaped 96-well plates. As supporting information for the Malaria Box, plate mapping and complete data with the original GSK/St Jude/Novartis compound number, ID, set, molecular weight, canonical SMILES, source, and biological data are provided as supporting information for Malaria Box [26–28]. Additionally available [28–30] is a list of vendors that provided the compounds for the Malaria Box, along with the number of compounds from each vendor and their website address.

2.2. Sampling and Isolation

A total of 200 milk samples were collected from cows affected by clinical mastitis in three different dairy farms in Dakahlia Governorate, Egypt. Every sample was obtained in an aseptic manner and placed in sterile falcon tubes before being sent for analysis to the Laboratory of Bacteriology, Immunology, and Mycology, Faculty of Veterinary Medicine, Mansoura University, Egypt. Adequate biosecurity and biosafety measures were taken. Hands were washed before and after specimen collection. In line with standard precautions, appropriate personal protective equipment was worn when collecting or handling specimens. Specimens were collected in sterile containers with close-fitting lids to avoid contamination and spoilage.

The isolation of *S. aureus* strains was carried out as follows: 100 μ L of phosphate buffered saline (PBS)-diluted milk samples was spread onto Baird-Parker agar (Becton Dickinson and Co., Sparks, MD, USA) plates and incubated at 37 °C for 48 h. Resulting colonies were then subjected to Gram staining and standard biochemical tests, including catalase, oxidase, DNase, and coagulase tests [24,25]. For catalase and coagulase tests, 3% hydrogen peroxide and fresh rabbit plasma were used, respectively. Presumptive *S. aureus* isolates were further scrutinized on Mannitol Salt Agar (Acumedia Manufacturers Inc., Lansing, MI, USA) plates. All isolates were stored in 30% glycerol solution at -20 °C for further investigation.

2.3. Molecular Characterization of S. aureus

The uniplex PCR test for *S. aureus* was carried out by amplification of the *nuc* gene using oligonucleotide primers (Metabion, Planegg, Germany) *nuc*-F (5GTGCTGGCATAT-GTATGGCAATTG3) and *nuc*-R (5CTGAATCAGCGTTGTCTTCGCTCCAA3) [26,27]. A thermocycler (Biometra, Göttingen, Germany) was used to perform PCR amplifications. The reaction mixture (25 μ L) contained 3 μ L of genomic DNA, 12.5 μ L of 2X PCR Master Mix (Takara code number RR310A), and 1 μ L of each of the two primers, with the remaining volume comprising nuclease-free water. The cycling conditions were initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and final elongation at 72 °C for 7 min. The amplified PCR products were separated by electrophoresis in 1.5% agarose gel (Lonza, Walkersville, MD, USA) at 100 V for 30 min, stained with ethidium bromide, and then viewed and recorded using a UV trans-illuminator (Biometra, Göttingen, Germany).

2.4. Antimicrobial Susceptibility Assay

The broth microdilution assay was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) standards for evolution MMV hits against *S. aureus* in vitro. The assay was performed for the MVV compounds provided by the MMV. After 24 h incubation of the bacterial cells with different dilutions of Malaria Box compounds at 37 °C, the lowest dilution columns with no bacterial growth were reported as the minimum inhibitory concentration (MIC) value. All assays were performed at least twice in triplicate. Using the broth microdilution assay, the 80 compounds (40 drug-like and 40 probe-like) from the Malaria Box were found to have inhibitory effects on *S. aureus* growth. *S. aureus* was isolated in pure cultures and the final inoculum size for broth dilution was 5×10^5 colony-forming units (CFU).

2.5. Structural Similarity Measurements

The degree of structural similarity was determined between the drug-like MMV compounds and Augmentin 625[®], a medication that is frequently used to treat *S. aureus*. The chemical ID acquired from PubChem for each compound was used to calculate the atom pair fingerprint (APfp). The CIDs were then used to calculate the APfp of all compounds using ChemMine Tools software [13,14]. ChemmineR software was used to perform hierarchical clustering analysis (HCA) on the APfp [15].

2.6. Microdilution Assays and Minimum Inhibitory Concentration (MIC)

Augmentin $625^{\text{(B)}}$ was prepared as 0.016989 gm dissolved 100 µL DMSO. Then, 20 µL of liquid drug was diluted by 180 µL of sterile broth to obtain a final concentration of 1 mM of the liquid drug. The 96-well plates (Nunc, Roskilde, Denmark) were prepared by dispensing 25 µL of sterile broth into each well. In order to determine the MIC values of the positive controls, an experiment was performed every time. The MIC was ascertained using the broth microdilution method in accordance with the Clinical and Laboratory Standards

Institute's (CLSI, Clinical and Laboratory Standards Institute, 2018) recommendations. For the preparation of bacterial inoculum, 1 mL of 24 h culture was suspended in 9 mL of sterile broth. The bacterial suspension was further diluted through five-fold broth dilution, and the last one was used in the experiment, with 25 μ L added to each well of the micro-titer plate and prepared freshly every time [30]. A total of 25 μ L of the liquid drug and 25 μ L of the sterile broth were added and mixed in the first well, which contained the highest concentration of drug. Then, 25 μ L of the solution was used for downstream serial dilutions in ten consecutive wells, each well already containing 25 μ L of sterile broth. The last 25 μ L was discarded. Then, 25 μ L of diluted bacteria was added to each well so the final volume in each well was 50 μ L. The microplates were incubated in an incubator for 24 h.

The Malaria Box compounds were first screened at a concentration of 1 mM. For every experiment, there was a negative control (50 μ L of sterile Tryptone Soya Broth) and positive control (25 μ L of sterile Tryptone Soya Broth, 25 μ L diluted bacteria).

In order to calculate the MIC values of the positive controls for each experiment, 96-well plates (U shapes) were generally created by dispensing 25 μ L of sterile broth into each well. The microplates spent 24 h in an incubator. The MIC values for the trials were calculated in triplicate, just like they were for the positive controls.

2.7. In Vitro Drug Combination Test

Combination therapy involving the MMV compound that exhibited the highest MIC value, MMV665941, and the traditionally used anti-*S. aureus* drug at a concentration of 1mM was used. Every time an experiment began, 12.5 μ L of the liquid drug, 12.5 μ L of Augmentin, and 25 μ L of sterile broth were added and mixed in the first well, which had the highest concentration of the drug.

2.8. Determination of Fractional Inhibitory Concentration (FIC)

The effect of the combination consisting of MMV66594 and the traditionally used anti-*S. aureus* drug was investigated by determining the \sum FIC. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)⁶⁵ described the method for quantifying MIC results in terms of the FIC index, which is defined as the sum of the FIC values of two drugs in combination [16]. An example of the method used to calculate the

SFIC is as follows:

For two anti-bacterial substances (A and B) both alone and in combination:

$$(FIC_{(A)} = \frac{MIC \text{ A in presence of } B}{MIC \text{ A}}, FIC_{(B)} = \frac{MIC \text{ B in presence of } A}{MIC \text{ B}}, and \sum FIC = FIC_{(A)} + FIC_{(B)})$$

2.9. Statistical Analysis

A two-way ANOVA test was used to identify the significant differences between the analyzed groups using GraphPad Prism (version 5.0 for Windows; GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was defined as a *p*-value of less than 0.05.

3. Results

3.1. Detection of S. aureus

On Baird-Parker Agar, *S. aureus* isolates formed black colonies surrounded by clear zones, while on Mannitol Salt Agar they formed yellow colonies and on Tryptose Soya Agar they formed golden yellow colonies (Figure S1). *S. aureus* exhibited as Gram-positive spherical cocci clustered in clusters resembling grapes under the microscope (Figure S2). Out of the 200 milk samples, 132 (66%) isolates were detected as *S. aureus* strains based on growth pattern, gram staining, and biochemical tests, which was confirmed by the PCR-targeting *nuc* gene which was identified in all suspected isolates, yielding 270 bp PCR products (Figure 1).



Figure 1. Agarose gel electrophoresis showing amplification of the *S. aureus nuc* gene (270 bp). Lane 1: 100 bp ladder; lanes 2–4, 5, 6, 8–13: positive samples; lane 14: negative control.

3.2. MMV665941, a Potent Hit against S. aureus

In the present study, 80 compounds from the Malaria Box (40 drug-like and 40 probelike) were tested for their ability to inhibit the growth of *S. aureus*, and it was found that MMV665941 exhibits a potent hit against *S. aureus* isolated from cows with clinical mastitis (Tables 1–4). In detail, MMV665915, MMV665876, MMV666596, MMV009063, MMV396 797, MMV006087, MMV011256, MMV396680, MMV011259, MMV396679, MMV666601, MMV396693, MMV006558, MMV020548, MMV008294, MMV666607, MMV085203, and MMV008956 exhibited an MIC of 0.5. Interestingly, MMV665941 showed the lowest MIC value (0.0078), followed by MMV66621 and MMV001038 with MIC values of 0.125 (Tables 1 and 3 and Figure 2). Other screened MMV compounds showed MIC values of 0.25, similar to the traditionally used drug Augmentin, which exhibited an MIC value of 0.25.

Table 1. Characterization and MIC values of Malaria Box compounds number 2 to 21 from plate (A) evaluated for *S. aureus*.

Compound No.	Compound ID ^a	Set *	MW * (g/mol)	AlogP *	MIC ^b
A-2	MMV665915	Drug-like	420.47	3.27	0.50
A-3	MMV666023	Probe-like	453.53	8.50	0.25
A-4	MMV665876	Drug-like	326.43	4.29	0.50
A-5	MMV019406	Probe-like	367.52	4.76	0.25
A-6	MMV019871	Drug-like	329.37	2.42	0.25
A-7	MMV666596	Probe-like	468.09	8.66	0.5
A-8	MMV006309	Probe-like	292.33	3.77	0.25
A-9	MMV000642	Probe-like	468.99	5.09	0.25
A-10	MMV396672	Drug-like	396.46	4.77	0.25
A-11	MMV001038	Drug-like	308.37	3.13	0.125
B-2	MMV665916	Drug-like	353.37	1.65	0.25
В-3	MMV666101	Probe-like	631.71	6.99	0.25
B-4	MMV665841	Probe-like	273.32	3.13	0.25
B-5	MMV007695	Probe-like	657.75	7.24	0.25
B-6	MMV020788	Probe-like	271.39	3.60	0.25
B-7	MMV009063	Drug-like	322.44	4.48	0.50
B-8	MMV007384	Probe-like	460.52	6.65	0.25
B-9	MMV006429	Drug-like	409.50	3.58	0.25

Table 1. Cont.

Compound No.	Compound ID ^a	Set *	MW * (g/mol)	AlogP *	MIC ^b
B-10	MMV007116	Drug-like	245.27	2.92	0.25
B-11	MMV396703	Drug-like	317.81	3.35	0.25

^a Compounds are identified by their MMV identifier codes. ^b The growth inhibitions ascertained by the microdilution assay were used to calculate the minimum inhibitory concentration (MIC) for each drug. The means of three separate experiments are presented. * The set, AlogP, and molecular weight (MW) data are included in the Malaria Box supporting documentation.

Table 2. Characterization and MIC values of Malaria Box compounds number 22 to 41 from plate (A) evaluated for *S. aureus*.

Compound No.	Compound ID ^a	Set *	MW * (g/mol)	AlogP *	MIC ^b
C-2	MMV665785	Probe-like	261.40	4.38	0.25
C-3	MMV665805	Drug-like	367.39	4.06	0.25
C-4	MMV020492	Drug-like	309.81	3.50	0.25
C-5	MMV006937	Drug-like	279.33	4.35	0.25
C-6	MMV666693	Drug-like	309.31	3.58	0.25
C-7	MMV007686	Probe-like	334.38	3.51	0.25
C-8	MMV019066	Drug-like	359.39	1.60	0.25
C-9	MMV000662	Drug-like	434.55	4.43	0.25
C-10	MMV007839	Drug-like	312.18	2.61	0.25
C-11	MMV396680	Probe-like	476.90	5.66	0.50
D-2	MMV665782	Drug-like	324.41	2.62	0.25
D-3	MMV665874	Drug-like	341.71	4.81	0.25
D-4	MMV011567	Drug-like	389.78	3.52	0.25
D-5	MMV006278	Drug-like	200.27	3.11	0.25
D-6	MMV085583	Probe-like	592.64	592.64 7.34	
D-7	MMV020885	Probe-like	510.42	6.83	0.25
D-8	MMV006457	Probe-like	293.31	3.93	0.25
D-9	MMV020549	Drug-like	398.50	4.63	0.25
D-10	MMV011259	Drug-like	321.30	4.63	0.50
D-11	MMV396679	Probe-like	492.90	5.16	0.50

^a Compounds are identified by their MMV identifier codes. ^b The growth inhibitions ascertained by the microdilution assay were used to calculate the minimum inhibitory concentration (MIC) for each drug. The means of three separate experiments are presented. * The set, AlogP, and molecular weight (MW) data are included in the Malaria Box supporting documentation.

Table 3. Characterization and MIC values of Malaria Box compounds number 42 to 61 from plate (A) evaluated for *S. aureus*.

Compound No.	Compound ID ^a	Set *	MW * (g/mol)	AlogP *	MIC ^b
E-2	MMV665941	Probe-like	389.53	4.76	0.0078
E-3	MMV665878	Drug-like	367.39	2.72	0.25
E-4	MMV000448	Probe-like	279.37	3.36	0.25
E-5	MMV666691	Probe-like	340.39	3.75	0.25
E-6	MMV006455	Drug-like	370.48	3.97	0.25
E-7	MMV666600	Probe-like	459.92	6.82	0.25

Compound No.	Compound ID ^a	Set *	MW * (g/mol)	AlogP *	MIC ^b
E-8	MMV006558	Probe-like	287.78	4.91	0.5
E-9	MMV020548	Drug-like	413.51	3.77	0.5
E-10	MMV011256	Drug-like	327.69	4.32	0.5
E-11	MMV396678	Probe-like	462.87	5.18	0.25
F-2	MMV666021	Probe-like	272.30	4.17	0.125
F-3	MMV665820	Drug-like	293.53	3.13	0.25
F-4	MMV085203	Probe-like	362.42	3.72	0.50
F-5	MMV008956	Drug-like	377.86	4.13	0.50
F-6	MMV007907	Drug-like	267.34	3.45	0.25
F-7	MMV666601	Probe-like	637.71	7.03	0.25

278.34

327.42

309.32

277.79

5.29

3.38

1.54

3.33

0.25

0.25

0.50

0.50

Table 3. Cont.

E-8 E-9 E-10 E-11 F-2 F-3 F-4 F-5 F-6 F-7 F-8

F-9

F-10

F-11

^a Compounds are identified by their MMV identifier codes. ^b The growth inhibitions ascertained by the microdi-lution assay were used to calculate the minimum inhibitory concentration (MIC) for each drug. The means of three separate experiments are presented. * The set, AlogP, and molecular weight (MW) data are included in the Malaria Box supporting documentation.

Probe-like

Drug-like

Drug-like

Drug-like

MMV000570

MMV001246

MMV396797

MMV006087

Compound No.	Compound ID ^a	Set *	MW * (g/mol)	AlogP *	MIC ^b
G-2	MMV666062	Probe-like	571.66	7.03	0.25
G-3	MMV665827	Probe-like	346.39	3.36	0.25
G-4	MMV666688	Probe-like	476.03	7.22	0.25
G-5	MMV008416	Probe-like	228.33	4.02	0.25
G-6	MMV006172	Probe-like	368.47	5.02	0.25
G-7	MMV007160	Probe-like	621.76	8.04	0.25
G-8	MMV006427	Drug-like	463.95	4.49	0.25
G-9	MMV019258	Drug-like	397.49	4.16	0.25
G-10	MMV011099	Drug-like	275.30	3.52	0.25
G-11	MMV019110	Drug-like	250.29	3.10	0.25
H-2	MMV665977	Probe-like	456.50	4.32	0.25
H-3	MMV665831	Probe-like	426.52	4.33	0.25
H-4	MMV006203	Probe-like	319.44	4.07	0.25
H-5	MMV020500	Drug-like	249.73	2.15	0.25
H-6	MMV006861	Probe-like	355.43	4.32	0.25
H-7	MMV008294	Probe-like	380.43	4.71	0.50
H-8	MMV020439	Drug-like	459.60	4.45	0.25
H-9	MMV666607	Probe-like	281.26	2.76	0.50

Table 4. Characterization and MIC values of Malaria Box compounds number 62 to 81 from plate (A) evaluated for S. aureus.

Compound No.	Compound ID ^a	Set *	MW * (g/mol)	AlogP *	MIC ^b
H-10	MMV008138	Drug-like	361.22	1.77	0.50
H-11	MMV396693	Probe-like	254.30	2.81	0.50

^a Compounds are identified by their MMV identifier codes. ^b The growth inhibitions ascertained by the microdilution assay were used to calculate the minimum inhibitory concentration (MIC) for each drug. The means of three separate experiments are presented. * The set, AlogP, and molecular weight (MW) data are included in the Malaria Box supporting documentation.

2-({2-[(3,4-

C.

MMV666021:

indeno[1,2-c] pyridazin-5-one

PubChem CID: 2767330 MW: 272.30068

A.

MMV665941: Crystal violet cation PubChem CID: 103714763 MW: 346.91578

B. MMV001038: Dimethylphenyl)amino]-4quinazolinyl)amino)ethanol hydrochloride PubChem CID: 2226126. MW: 308.3776

D. Augmentin (Amoxicillin/clavulanate)

3-(4-methylphenyl)-5H-



Pubchem CID: 23665637 MW: 602.65528



Figure 2. Chemical structures of the identified potent MMV anti-*S. aureus* compounds and the commonly used drug Augmentin: (**A**) MMV665941, (**B**) MMV001038, (**C**) MMV666021, (**D**) Augmentin. The structures are imported from PubChem (https://pubchem.ncbi.nlm.nih.gov/ (accessed on 11 October 2023)).

3.3. Bioinformatic and Combination Analysis

In the current study, the molecular weight between the potent hits of the MMV and the commonly used anti-staphylococcus drug were calculated. MMV665941 (CID: 68050), MMV001038 (CID: 2226126), MMV666021 (CID: 2767330), and Augmentin (Amoxicillin/clavulanate) (CID: 23665637) exhibited very similar molecular weights with an AP Tanimoto value of 0.10 (Table 5 and Figure S3). The PubChem fingerprint for similarity workbench revealed that N-Methylaniline and 1-Methylethyl)benzene (propan-2-yl) benzene were the differences between MMV665941 and Augmentin (Amoxicillin/clavulanate), respectively (Figure 3). In general, the distances shown in a distance matrix are proportional to each other. If the distance between A and B is twice the distance between A and C, that means that B is twice as far from A as it is from C. By contrast, in a dissimilarity matrix, the values may only reflect relative differences. The distances matrix between the selected compounds is shown in Table 6. Collectively, such findings highlight the possible potential anti-staphylococcus efficacy of MMV665941 and Augmentin (Amoxicillin/clavulanate) when administrated as a combination therapy. Following that, MMV665941 and Augmentin (Amoxicillin/clavulanate) were chosen to test their inhibitory effects when used in combination against in vitro growth of *S. aureus* isolated from bovine milk with clinical mastitis. Unfortunately, when MMV665941 was combined with Augmentin (Amoxicillin/clavulanate), all combination ratios showed antagonistic inhibitory effects (≥ 2) against the in vitro growth of *S. aureus*.

Compounds	AP Tanimoto	MCS Tanimoto	MCS Size	MCS Min	MCS Max	Chemical Structure
MMV001038 + Augmentin	0.121951	0.1250	7	0.3043	0.1750	CHa
MMV666021 + Augmentin	0.0987203	0.1296	7	0.3333	0.1750	CH3
MMV665941 + Augmentin	0.0855148	0.1481	8	0.3636	0.2000	° → N N N CH3
MMV665941 + MMV666021	0.0450237	0.1316	5	0.2381	0.2273	њссњ
MMV665941 + MMV001038	0.102506	0.1250	5	0.2273	0.2174	њссн ₂
MMV666021 + MMV001038	0.357771	0.2222	8	0.3810	0.3478	NH

 Table 5. Bioinformatic similarity analysis between two compounds.



Figure 3. Hierarchical clustering analysis between MMV665941, MMV666021, and MMV001038. The chemical ID for each compound acquired from PubChem was used to calculate APfp. The CIDs were then used to calculate the atom pair fingerprint (APfp) of all compounds using ChemMine Tools software. ChemmineR software 3.44 was used to perform hierarchical clustering analysis on the APfp. Compound CID: 2767330 = MMV666021; 2226126 = MMV001038; 103714763 = MMV665941; 23665637 = Augmentin.

	MMV001038	MMV665941	MMV666021	Augmentin
MMV001038	0	0.90	0.64	0.88
MMV666021	0.64	0.95	0	0.90
MMV665941	0.90	0	0.95	0.91
Augmentin	0.88	0.91	0.90	0

 Table 6. Distance matrix between identified potent compounds.

4. Discussion

The Malaria Box was created to find new medications to fight different apicomplexan parasites and is a rich source of commercially accessible compounds that represent families of structures found in phenotypic screenings of pharmaceutical and academic libraries against P. falciparum [8,28]. The 400-compound collection known as the Malaria Box has been broken down into 200 distinct drug-like compounds and 200 distinct probe-like compounds; toxophores are included in the drug-like compounds category. Additional hits fall under the category of probe-like hits. A comprehensive dataset encompassing sixteen protozoa, seven helminths, nine bacterial and mycobacterial species, and the NCI60 human cancer cell line was released along with the in vitro inhibitory effects of Malaria Box compounds [8]. We were motivated to conduct a large-scale in vitro screening of MMV compounds (n = 80) against S. aureus in the current study due to the encouraging candidates found from the extensive screening of Malaria Box compounds and the zoonotic significance of S. aureus. In this study, we have screened the antibacterial activity of the Malaria Box compounds against *S. aureus* using broth microdilution assays in microtiter plates in which we put 25 μ L of broth in each well and then 25 μ L of the drug via serial dilution, with 25 μ L of bacterial inoculum then added in each well. The final inoculum size for broth dilution was 5×10^5 colony-forming units (cfu), which was then incubated for 24 h.

Amoxicillin/clavulanate (Augmentin $625^{\text{(B)}}$) was included as the internal control in every microtiter plate, which showed activity against *S. aureus* with an MIC value of 0.25 mg/L. On the other hand, three compounds (MMV665941, MMV666021, and MMV001038) showed excellent activity against *S. aureus* with MIC values of 0.0078 mg/L, 0.125 mg/L, and 0.125 mg/L. Combination therapy was employed to combine the most potent anti-*S. aureus* drug, the MMV665941 compound, with a traditional anti-*S. aureus* drug, and according to fractional inhibitory concentration (FIC), there is antagonism between both of them.

Methylrosaniline, tris [4-(dimethylamino)phenyl]methanol, and the crystal violet carbinol base are among the names for MMV665941, a triarylmethane dye with a variety of medicinal applications, including antibacterial, antifungal, antiseptic, antihelminthic, antitrypanosomal, anti-angiogenic, and anti-tumor applications [28]. A significant protozoal illness in oysters, Perkinsus marinus, was also found to be susceptible to MMV665941's effectiveness; however, not all strains of the parasite showed the same level of resistance [31]. It has been demonstrated that Brugia malayi and B. pahangi, Cryptosporidium, Tritrichomonas fetus trophozoites, and Trypanosoma cruzi are all vulnerable to this chemical [32–35]. MMV665941 and gentian violet share structural similarities. Gentian violet is used as a mold inhibitor in feed components and has also been used in human medicine to treat a range of bacterial infections [36]. However, it is strictly forbidden to use gentian violet in any food animal in the United States [37]. A previous study [28] demonstrated the CC_{50} of MMV665941 to be equal to 353.55 nM when evaluated in a culture against human fibroblast (MRC-5) cells. Of note, there have been few grounds given for gentian violet prohibition, other than the FDA's statement that the impact of drug residues on human health has not been sufficiently studied [37].

Typically, the LD_{50} of the drug under test in the chosen animal model is used to calculate the administrative dose in an in vivo study [38,39]. Even the same medication,

administered via various routes, showed varying LD₅₀ values in the same experimental animal model [38]. Among the identified potent MMV hits in the present study, namely MMV665941, the only available information for its administrative safe dose is by the oral route in mice [40]. However, future studies need to identify the best administration method (intramuscular, intravenous, subcutaneous, or intramammary) of the identified potent anti-*S.aureus* MMV compounds and determine the withdrawal period for each compound from the treated animals.

The decreasing quantity of viable antimicrobial medications poses a challenge to modern medicine, as does the emergence of drug-resistant bacteria and the abuse and overuse of antibiotics. In light of the way that pathogen-eliminating medicines exert strong selective pressure on the development of antimicrobial resistance, it is imperative to explore novel therapeutic approaches that can decelerate the formation of resistance. MMV665941, MMV666021, and MMV001038 showed excellent activity against S. aureus in the present study. It is known that the drug discovery process involves many different stages and series of actions. Typically, it can be divided into four main stages: early drug discovery, the pre-clinical phase, clinical phases, and regulatory approval. Identifying and characterizing molecules with the potential to safely modulate disease with the goal of bringing medicines that can improve the lives of patients represents the main aim of the drug discovery process. In the present study, the earlier phases of this process were achieved. Further experiments into antibiofilm activity and intracellular killing tests for the identified potent MMV compound are required to be performed in the future. Completing other phases in an experimental animal model in the future may suggest the usage of MMV665941, MMV666021, and MMV001038 in the treatment of previously untreatable S. aureus.

This study is considered the starting point for the evaluation of antimalarial compounds against the growth of *S. aureus*. Other studies are required to complete the screening of other plates in the Malaria Box against *S. aureus*. Furthermore, given that this work is thought to be the first to compare MMV compounds against bacteria collected from field samples, future studies are required to evaluate the identified potent compounds in an experimental animal model involving animals infected with *S. aureus*.

5. Conclusions

Finding new, more potent medications than those currently in use was made possible through the screening of Malaria Box compounds against the in vitro growth of *S. aureus*. Attractively, three novel potent anti-*S. aureus* compounds were identified: MMV665941, MMV666021, and MMV001038. One hit, MMV665941, exhibited the highest MIC value. The most effective anti-*S. aureus* drug, MMV compound MMV665941, and a conventional anti-*S. aureus* medicine were used in combination therapy, and according to fractional inhibitory concentration (FIC), there is an antagonistic effect between the two drugs. While the current study contributes to the identification of novel MMV compounds with promising anti-*S. aureus* efficacy, the inhibitory effects of these hits might be amplified when combined with one another. For additional clinical applications, future research must examine the two-drug interactions of MMV hits when taken in combination. The results obtained suggest that the Malaria Box holds abundant potential in terms of anti-*S. aureus* candidates.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/dairy4040049/s1, Figure S1. *S. aureus* isolates on Tryptose Soya Agar; Figure S2. *S. aureus* under the microscope; Figure S3. A heatmap for the molecular weight correlation between MMV665941, MMV666021, and MMV001038. Compound CID: 2767330 = MMV666021; 2226126 = MMV001038; 103714763 = MMV665941; 23665637 = Augmentin. **Author Contributions:** Conceptualization, R.E., A.A. and M.A.R.; methodology, A.M. and R.E.; validation, R.E., A.A. and M.A.R.; formal analysis, M.A.R.; investigation, R.E., A.A. and M.A.R.; resources, A.M., R.E., A.A. and M.A.R.; data curation, R.E., A.A. and M.A.R.; writing original draft preparation, A.M., R.E., A.A. and M.A.R.; writing review and editing, R.E., A.A. and M.A.R.; visualization, R.E., A.A. and M.A.R.; supervision, R.E., A.A. and M.A.R.; project administration, R.E., A.A. and M.A.R.; hunding acquisition, A.M., R.E., A.A. and M.A.R. All authors have read and agreed to the published version of the manuscript.

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