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Gut Bacteria of *Rattus rattus* (Rat) Produce Broad-Spectrum Antibacterial Lipopeptides

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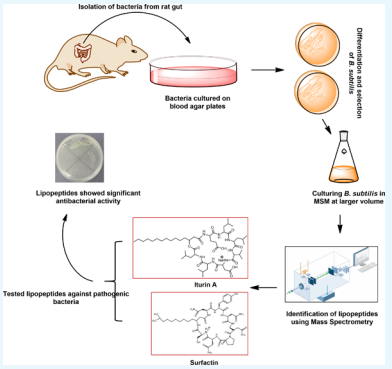
ABSTRACT: Among several animals, *Rattus rattus* (rat) lives in polluted environments and feeds on organic waste/small invertebrates, suggesting the presence of inherent mechanisms to thwart infections. In this study, we isolated gut bacteria of rats for their antibacterial activities. Using antibacterial assays, the findings showed that the conditioned media from selected bacteria exhibited bactericidal activities against Gram-negative (*Escherichia coli* K1, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Salmonella enterica*) and Gram-positive (*Bacillus cereus*, methicillin-resistant *Staphylococcus aureus*, and *Streptococcus pyogenes*) pathogenic bacteria. The conditioned media retained their antibacterial properties upon heat treatment at boiling temperature for 10 min. Using MTT assays, the conditioned media showed minimal cytotoxic effects against human keratinocyte cells. Active conditioned media were subjected to tandem mass spectrometry, and the results showed that conditioned media from *Bacillus subtilis* produced a large repertoire of surfactin and iturin A (lipopeptides) molecules. To our knowledge, this is the first report of isolation of lipopeptides from bacteria isolated from the rat gut. In short, these findings are important and provide a platform to develop effective antibacterial drugs.



INTRODUCTION

Increasing multidrug resistance in pathogenic bacteria highlights an urgent need to discover alternative antibacterials.[1−3] Although bacteria isolated from soil have been a tremendous source of antibiotics, bacteria inhabiting unique niches such as the gastrointestinal tract of animals (rats) living in polluted environments may also prove to be a source of potentially novel antimicrobials. In addition to conventional antibiotics, antimicrobial peptides are one of the most promising options that can reduce the risk of antibiotic-resistant bacteria.[4−6] In recent years, antimicrobial biosurfactants (lipopeptides) have been extensively studied as a novel class of antimicrobial drugs. These peptides are shown to possess both narrow- and broad-spectrum antibacterial activities against Gram-negative and Gram-positive bacteria and fungi.[1,6] Lipopeptides are mostly produced by Gram-positive bacteria including *Bacillus* species, while a few Gram-negative bacteria especially *Pseudomonas* have been reported to produce these antimicrobial peptides.[1,7] In addition, *Bacillus* sp. produce several metabolites/molecules including antibacterial polyketides, bacillaenes, isobutanols, polyhydroxyalkanoates, difficidins, and dozens of structurally distinct antibacterial molecules. *B. subtilis* has key genes in its genome involved in the production of several antibacterial molecules.[7] The most valuable and active among them are the cyclic lipopeptides such as surfactin, iturin A, bacillibactin, and fengycin.[7−9] Lipopeptides produced by Gram-positive bacteria have been classified into different types based on the amino acids and the length of fatty acid chains.[10] The presence of a lipid moiety in the lipopeptides increases their biological activity.[1] For example, the chain length of carbon atoms (C_{10}−C_{12}) possesses bactericidal activity, while lipopeptides with 14 or 16 carbons in their lipid chain show higher antifungal activity in addition to antibacterial activity.[1,5] These peptides have unique properties such as being easily biodegradable, eco-friendly, highly stable, and nonpolluting biomolecules and nontoxic in nature.[5,11] Due to these properties, lipopeptides are gaining importance in several applications such as pharmaceuticals, bioremediation, and food preservation.[5,12,13]

We have hypothesized previously that gut bacteria of animals/pests are a potential source of novel antibacterials.[3] *Rattus rattus* (rat) inhabits polluted environments. They are omnivores eating a wide range of foods including organic wastes, seeds, fruit, stems, leaves, fungi, and several invertebrates and vertebrates.[14,15] Rats are among the world's most invasive species living in close contact with humans and share ecological niches.[16] Rats act as a reservoir for many

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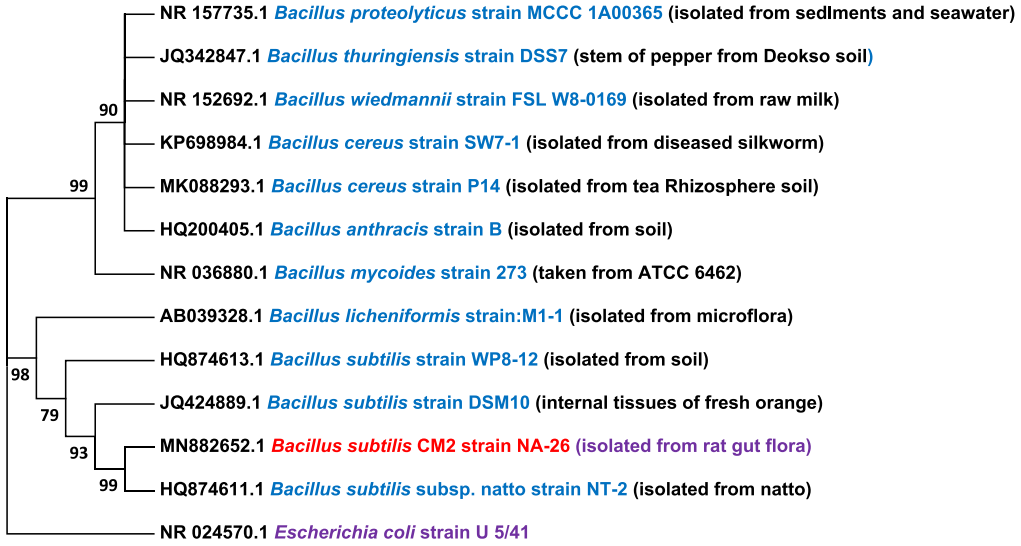

Figure 1. Phylogenetic tree representing 15 *Bacillus* strains with an *E. coli* strain U 5/41 (purple bold) as the outgroup and the *B. subtilis* (CM2) strain used in this study (red bold) based on the phylogenetic analysis of 16S rRNA genes. The phylogenetic tree was reconstructed using the maximum likelihood (ML) method (MEGA 7.) based on a GTR+G (8) model with concatenated 16S rRNA sequences. Percentage bootstrap values that were higher than 50% of 1000 replicates are indicated at branching nodes.

pathogenic species that are transmitted to mammals including humans.[17,18] Although rats are known to play a key role in the transmission of several human and domestic animal diseases, it is not clear how they counter infections. A possible explanation is that their gut bacteria produce antibacterials to thwart bacterial infections. Thus, the aim of this study was to mine gut bacteria of a rat for potential antibacterials. We have isolated 30 different bacteria, and among these bacteria, three *Bacillus* strains showed significant antibacterial properties. Finally, *B. subtilis* (CM2) exhibited the highest bactericidal activities with minimal cytotoxicity; hence, CM2 was subjected to detailed characterization by tandem mass spectrometry.

RESULTS

Bacterial Species Isolated from the Gut of *Rattus rattus* (Rat). Several bacterial species were isolated from the gut of *Rattus rattus* (rat). Bacteria isolated were screened against Gram-positive and Gram-negative pathogenic bacteria. Bacteria with profound antibacterial activities were selected, and their identification was done using 16S rRNA gene amplification and sequencing. The results revealed *B. velezensis* (accession no. MN882653), *B. cereus* (accession no. MN882654), and *B. subtilis* (accession no. MN882652) (Figure 1). *B. subtilis* showed 99 bootstrap values with *Bacillus subtilis* subsp. natto strain NT-2 sharing the same clade. Next, conditioned media of *Bacillus* species isolated were prepared as discussed in the materials and methods (Table 1), and codes were given to all CM as shown in Table 2. The CM were then tested against a range of selected Gram-positive and Gram-negative pathogenic bacteria (Table 1).

Conditioned Media of *Rattus rattus* Gut Bacteria Exhibited Notable Antibacterial Activities against Gram-Negative and Gram-Positive Bacteria. Conditioned media were tested for their bactericidal effects against pathogenic bacteria, and the results revealed that all CM except CM4 showed significant antibacterial activities against *S. enterica* and *P. aeruginosa* ($P < 0.05$ using Student's *t*-test, two-tailed distribution) (Figure 2a,b and Table 3). When tested against *K. pneumoniae*, only CM1 showed promising bactericidal properties ($P < 0.05$) (Figure 2c). CM2 and CM3 presented important antibacterial effects when tested against *E. coli* K1 ($P < 0.05$) (Figure 2d), while in the case of *S. marcescens*, only CM3 exhibited significant bactericidal activities ($P < 0.05$) (Figure 2e).

Similarly, when CM were evaluated against Gram-positive bacteria, all CM except CM4 possessed antibacterial properties against *B. cereus* ($P < 0.05$) (Figure 3a and Table 3). CM2 showed the highest activity among all CM tested (Figure 3a). When CM were tested against *S. pyogenes*, CM2 and CM3 revealed notable bactericidal activities ($P < 0.05$) (Figure 3b), whereas only CM2 showed remarkable bacterial killing properties against MRSA ($P < 0.05$) (Figure 3c).

Table 1. Bacteria Used in This Study

bacteria	strain
Bacillus cereus	MTCC 131621 (clinical isolate)
methicillin-resistant *Staphylococcus aureus*	MTCC 381123 (clinical isolate)
Streptococcus pyogenes	ATCC 49399 (clinical isolate)
Escherichia coli K1	MTCC 710859 (clinical isolate)
Escherichia coli K-12	MTCC 817356 (nonclinical isolate)
Klebsiella pneumoniae	ATCC 13883 (clinical isolate)
Pseudomonas aeruginosa	ATCC 10145 (clinical isolate)
Salmonella enterica	ATTC 14028 (clinical isolate)
Serratia marcescens	MTTC 13880 (clinical isolate)

Table 2. Bacterial Species Isolated from a Rat's Gut and Their Conditioned Media

conditioned media	bacterial source
CM1	*Bacillus cereus*
CM2	*Bacillus subtilis*
CM3	*Bacillus velezensis*
CM4	*E. coli* K-12

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Conditioned media	Bacterial source
CM1	*Bacillus cereus*
CM2	*Bacillus subtilis*
CM3	*Bacillus velezensis*
CM4	*E. coli* K-12

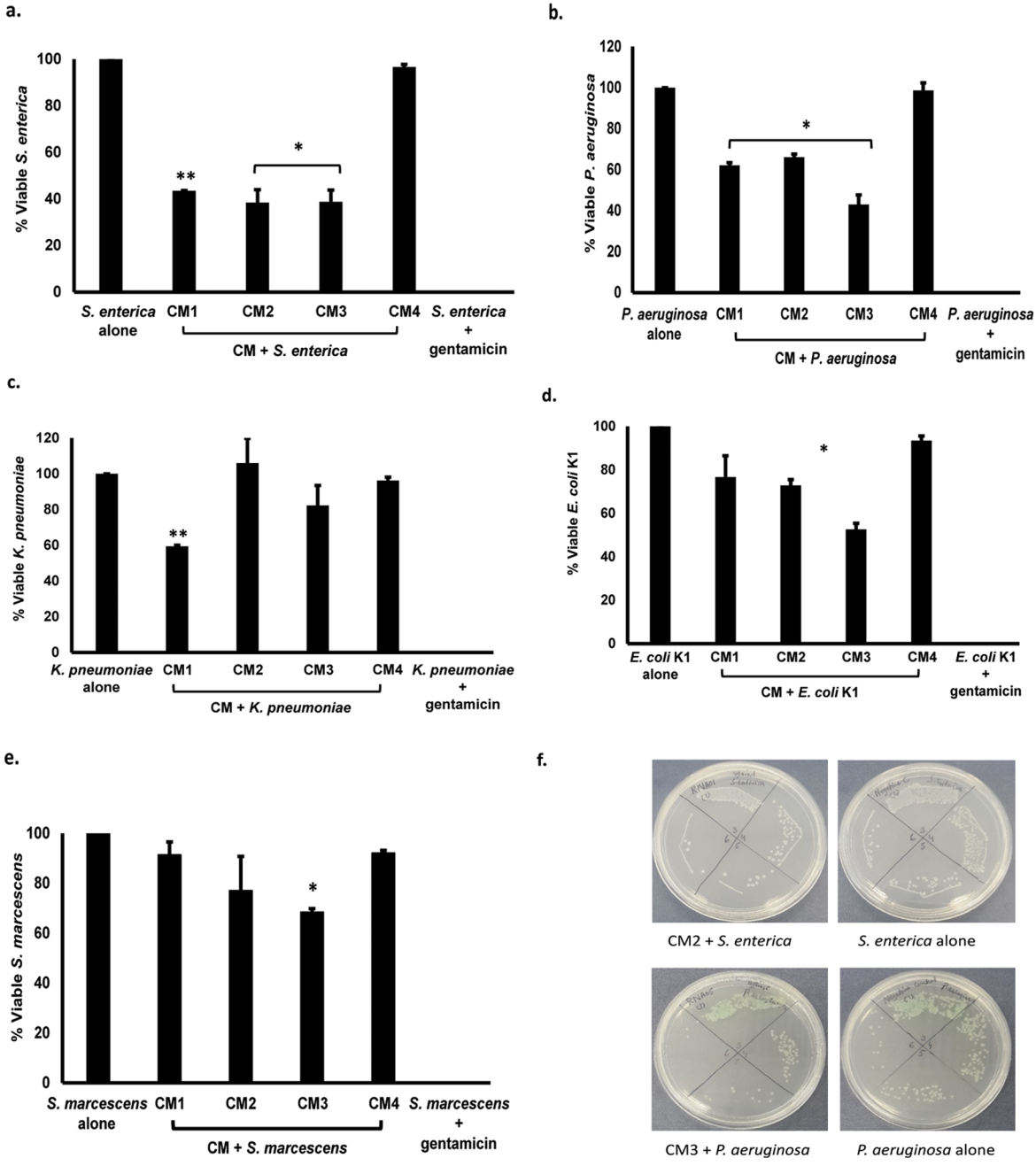

Figure 2. Conditioned media of rat gut bacteria possessed significant antibacterial activities against Gram-negative bacteria. CM were incubated with 1 million bacteria for 2 h at 37 °C. Next, cultures were serially diluted and plated onto nutrient agar plates, and plates were incubated overnight at 37 °C. Viable bacterial cfu were calculated, and results were recorded. (a) CM tested against *S. enterica*, (b) against *P. aeruginosa*, (c) against *K. pneumoniae*, (d) against *E. coli* K1, and (e) against *S. marcescens* and (f) representative effects of CM against *S. enterica* and *P. aeruginosa*. Data are expressed as the mean ± standard error of several independent experiments performed in duplicate. *P* values were determined using Student's *t*-test. An asterisk (*) denotes $P \leq 0.05$.

In some experiments, CM were incubated at 95 °C for 10 min, and then, their antibacterial properties were tested.

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Table 3. Overall Representation of Antibacterial Activities of Conditioned Media against Gram-Positive and Gram-Negative Bacteria

	antibacterial activities against Gram-positive bacteria			antibacterial activities against Gram-negative bacteria				
conditioned media	MRSA	*B. cereus*	*S. pyogenes*	*E. coli* K1	*P. aeruginosa*	*S. enterica*	*S. marcescens*	*K. pneumoniae*
CM1	−	+	−	−	+	+	−	+
CM2	+	+	+	+	+	+	−	−
CM3	−	+	+	+	+	+	+	−
CM4	−	−	−	−	−	−	−	−

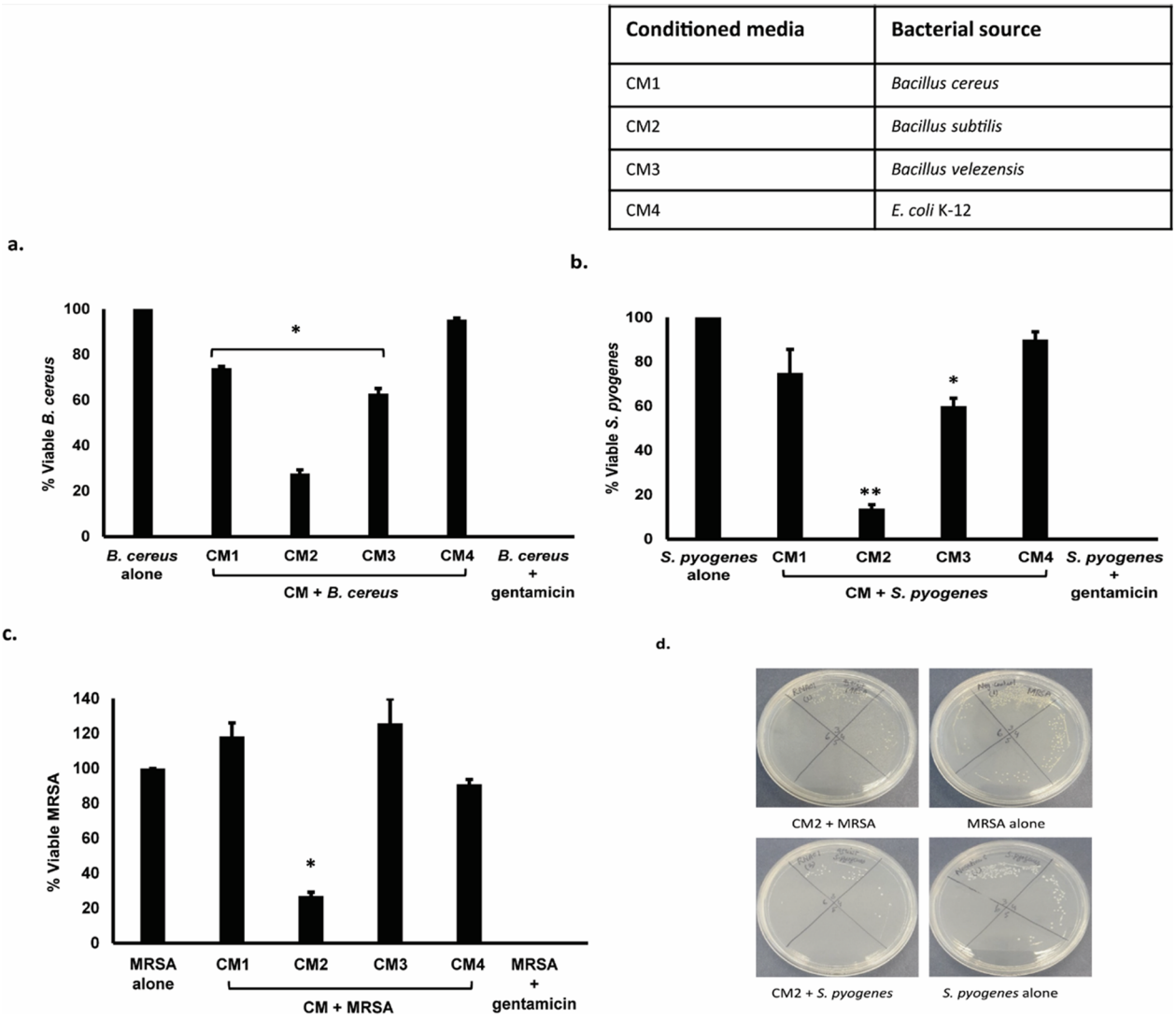

Figure 3. Conditioned media exhibited important bactericidal effects against Gram-positive bacteria. Approximately 1×10^6 bacterial cells were exposed to CM from rat gut bacteria and incubated at 37 °C for 2 h. After this incubation, cultures were serially diluted, plated on nutrient agar, and incubated at 37 °C for 24 h. Bacterial colonies were enumerated on the following day. Data are expressed as the mean ± standard error of several independent experiments performed in duplicate. *P* values were determined using two sample *t*-tests. An asterisk (*) denotes $P \leq 0.05$ (a) when CM were tested against *B. cereus*, (b) against *S. pyogenes*, and (c) against MRSA and (d) demonstrative effects of CM against MRSA and *S. pyogenes*.

Results showed that heat treatment did not abolish CM2 and CM3 and exhibited significant bactericidal effects against *P. aeruginosa* and *S. pyogenes* ($P < 0.05$) (Figure 4).

Conditioned Media Showed Limited Cytotoxicity. To determine the cytotoxic effects of CM, lactate dehydrogenase assays were performed against HaCaT cell lines. Results from cytotoxicity assays revealed that CM from *Rattus rattus* gut bacteria showed minimal effects against human cells except CM1 (*B. cereus*) with 55% cytotoxicity (Figure 5a,b).

For MTT assays, CM were tested against human cell lines in a graduated concentration as discussed in the materials and methods. Results showed parallel results with LDH assays where CM1 showed moderate to higher cytotoxic effects, while other CM (CM2−CM4) showed negligible cytotoxic effects against human cell lines (Figure 6). Values for CC_{50}

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Conditioned media	Bacterial source
CM1	*Bacillus cereus*
CM2	*Bacillus subtilis*
CM3	*Bacillus velezensis*
CM4	*E. coli* K-12

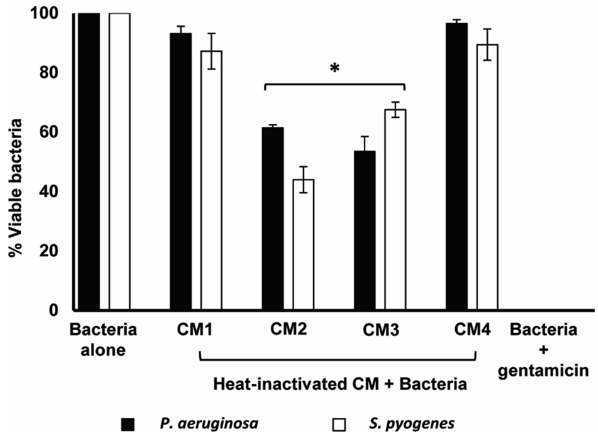
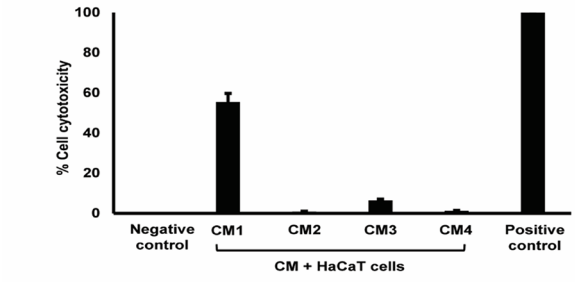


Figure 4. Heat-treated CM-mediated antibacterial properties against MRSA and *S. pyogenes*. Heat-treated CM were incubated with MRSA and *S. pyogenes* for 2 h at 37 °C. Cultures were serially diluted and subsequently plated on nutrient agar plates, and plates were incubated at 37 °C overnight. The next day, bacterial cfu were enumerated, and results were recorded. Data are expressed as the mean ± standard error of three independent experiments performed in duplicate.

Conditioned media	Bacterial source
CM1	*Bacillus cereus*
CM2	*Bacillus subtilis*
CM3	*Bacillus velezensis*
CM4	*E. coli* K-12

a.



b.

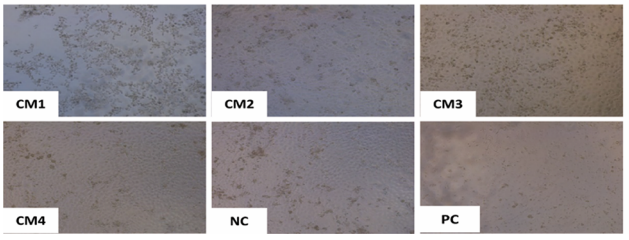


Figure 5. Conditioned media showed limited cytotoxicity properties against HaCaT cell lines. Briefly, HaCaT cells were challenged with CM from the rat gut bacteria for 24 h at 37 °C in the presence of 95% humidity and 5% CO_2. The next day, LDH released from HaCaT cells was measured as described in the materials and methods. (a) CM tested were nontoxic against HaCaT cells except CM1 (55%) and (b) representation of CM cytotoxic effects incubated with a human cell monolayer.

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Conditioned media	Bacterial source
CM1	*Bacillus cereus*
CM2	*Bacillus subtilis*
CM3	*Bacillus velezensis*
CM4	*E. coli* K-12

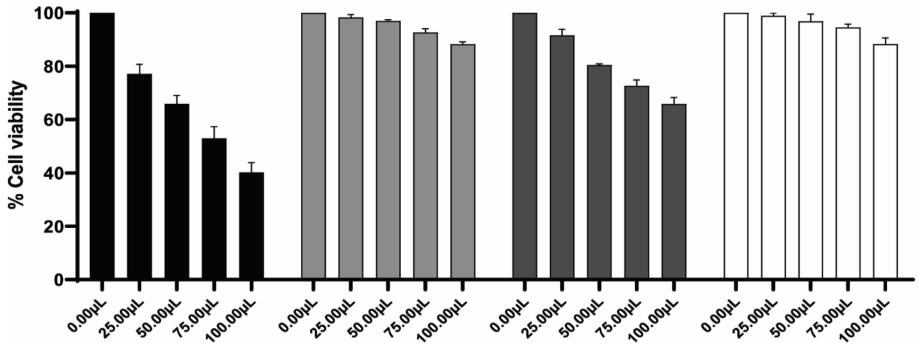

Figure 6. Conditioned media of rat gut bacteria produced negligible cytotoxicity against human cell lines. CM from all three *Bacillus* species were tested against human cells at their graduated volume/concentration overnight at 37 °C with 5% CO_2 and 95% humidity. Only CM1 (*B. cereus*) showed moderate to high cytotoxic effects, and other CM showed limited effects. Data are presented as the mean ± SE of three independent experiments performed in duplicate.

and $MNTD_{90}$ highlighting CM2 (*B. subtilis*) as a safe antibacterial drug candidate used for its medicinal therapeutic use are shown in Table 4.

Table 4. Cytotoxic Concentration 50 (CC_{50}) and Maximum Nontoxic Dose ($MNTD_{90}$) Values of CM of Rat Gut Bacteria[a]

sample/conditioned media	CC_{50}	$MNTD_{90}$
CM1	78.51	12.54
CM2	295.3	90.76
CM3	171.5	27.04
CM4	281.9	97.51

[a]CC_{50} = cytotoxic concentration. $MNTD_{90}$ = maximum nontoxic dose. CC_{50} and $MNTD_{90}$ are the concentrations at which 50 and 90% cells survive.

Mass Spectrometry Revealed Lipopeptides (Surfactin and Iturin). Among all CM tested, CM2 (*B. subtilis* with accession no. MN882652) showed the highest bactericidal effects, and it was further subjected to electrospray ionization (ESI)-MS/MS analysis. The direct syringe pump method was employed, and CM were injected. Tandem mass spectrometry was performed at positive total ion full scan mode. Results showed that CM2 (*B. subtilis*) revealed numerous lipopeptide molecules (Figure 7 and Table 5).

In this study, we identified and characterized lipopeptide (surfactin and Iturin A) molecules ranging from *m/z* 1000 to 1100 (Figure 7a,b). Lipopeptides produced by *B. subtilis* isolated from the rat gut were first analyzed through ESI-MS in positive full scan mode. The initial set of peaks observed belonged to the surfactin family with strong signals at *m/z* 1030.67, 1044.92, and 1058.75 $[M+Na]^+$ corresponding to sodiated peaks; moderate signals of 994.75, 1008.83, 1022.92, 1036.92 $[M+H]^+$; and finally 1046.92, 1060.67, 1074.83 and 1096.83 corresponding to $[M+K]^+$ (Figure 7a, Supplementary Figure S1a–e). Surfactins are believed to be a potent alternative to antibiotic compounds.[19] The presence of these metabolites was confirmed by tandem mass spectrometry as well as through comparing the values with literature data.

Among surfactin molecular peaks, sodiated adduct at *m/z* 1030.67 $[M+Na]^+$ was further analyzed by tandem mass spectrometry MS/MS analysis due to its higher ion intensity, and the fragmentation pattern revealed two different series of daughter ion peaks with the first series ranging from *m/z* 590.50 to 1012.75 (Figure 8).

Fragmentation of these surfactin molecules resulted in two different series. Series 1 represents loss of amino acid residues (Figure 8), while Series 2 shows loss of fatty acid chain length (CH_2) (Figure 8). Upon CID@20, product ions of *m/z* 1030.67 yielded both patterns (*m/z* 707.58–1012.75, Series 1, and *m/z* 382.25 to 707.58, Series 2). In detail, the peak at *m/z* 1030.67 $[M+Na]^+$ (**1**) loses two consecutive leucine (Δ = 113) molecules with one mole of H_2O (Δ = 18) leaving 917.67 (**2**) and 804.58 (**3**) and 786.58 (**4**). Further dissociation yielded 689.58 (**5**) (−asparagine), 590.50 (**6**) (−valine), 481.33 (**9b**), 463.33 (**9a**), and 382.25 (**10**) with loss of leucine, H_2O, and finally valine (Figure 8). On the other hand, when CID was applied, molecule (**1**) generated 707.58 (**7**) that further dissociated into (**8**), (**9a**), (**9b**), and (**10**) as shown in Figure 8. Correspondingly, another sodiated peak at *m/z* 1058.75 $[M+Na]^+$ (**1**) resulted in a similar fragmentation pattern and produced its fingerprint daughter ion peaks such as 707.50 (**5**), 594.50 (**6**), 481.33 (**7a**), 463.33 (**7b**), and 382.33 (**8**) (Figure 9). In both cases, both variants (Leu and Val) are present. For example, in the case of *m/z* at 1030.67 (Figure 8), the difference between 707.58 (**7**) and 594.42 (**8**) highlighted the loss of leucine molecules (−113), while at the same time, the difference between 689.58 (**5**) and 590.50 (**6**) signifies the loss of a valine molecule (−99). A similar pattern has been observed for *m/z* at 1058.75 where due to the loss of leucine (−113) from 707.50 (**5**) resulted in 594.50 (**6**), and the alternate loss of valine (−99) from 717.50 (**4**) produced 618.58 (not labeled). All the other surfactin homologues showed almost

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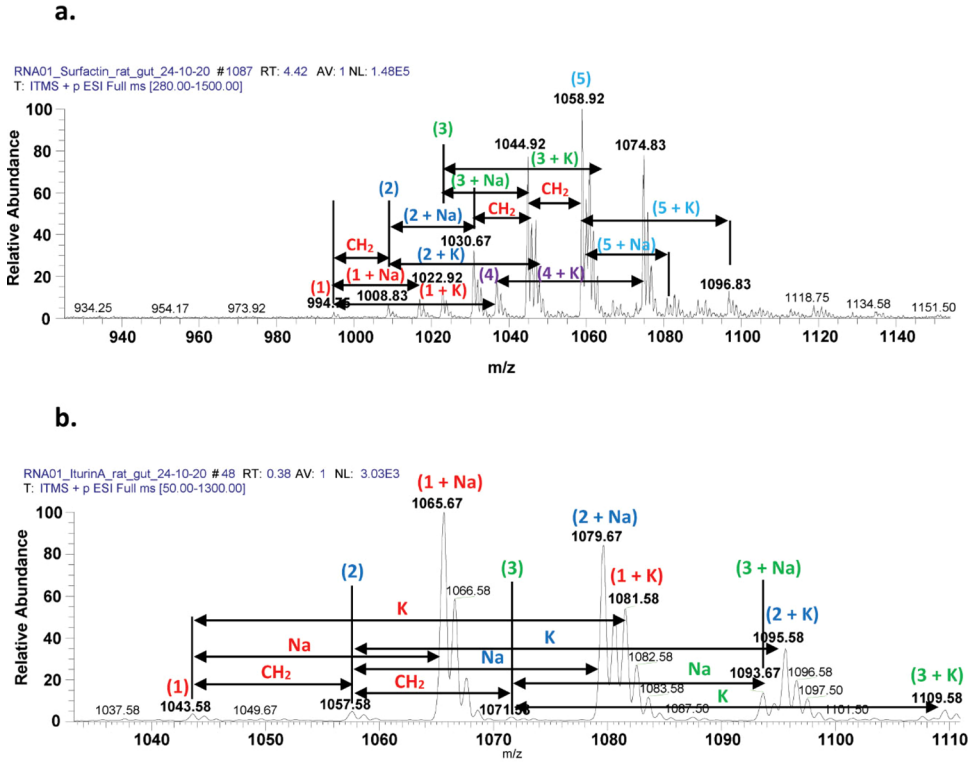

Figure 7. LCMS spectrum of *B. subtilis* isolated from the rat gut revealed the presence of several homologues of lipopeptides labeled as (a) surfactin molecules and (b) spectrum illustrating iturin A molecular ion species at positive ion mode.

Table 5. Identification of Lipopeptides Using ESI MS/MS Analysis Produced by *B. subtilis* Isolated from the Rat Gut

metabolites/ homologues	M	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	references
C_{12} surfactin	993.92	994.75	1016.83	1033.75	28
C_{13} surfactin	1007.75	1008.83	1030.92	1047.83	7, 28
C_{14} surfactin	1021.83	1022.92	1044.92	1061.75	7, 27, 28
C_{15} surfactin	1035.50	1036.83	1058.75	1074.75	7, 27, 28
C_{16} surfactin	1049.83	1050.92	1072.83	1089.83	7, 27
C_{14} iturin A	1042.67	1043.58	1065.67	1081.58	7, 27
C_{15} iturin A	1056.50	1057.58	1079.67	1095.58	7, 27
C_{16} iturin A	1070.67	1071.58	1093.67	1109.58	29

the same pattern of fragmentation upon tandem mass spectrometry analysis (Supplementary Figure S1a−e).

The second class of lipopeptides produced by *B. subtilis* corresponds to prominent peaks of iturin A molecules (Figure 7b, Supplementary Figure S2a−c). Among them, when 1043.83 $[M+H]^+$ was subjected to tandem mass spectrometry analysis (MS^2), upon CID @10, the iturin A precursor ion produced 915.17 after losing one glutamine molecule (−128) (Figure 10). Further fragmentation (MS^3 and MS^n) upon CID produced several daughter ion peaks, i.e., 801.67 (loss of asparagine −114), 638.42 (loss of tyrosine −163), 524.58 (loss of asparagine, −144), 437.25 (loss of serine, −87) and 322.92 (loss of asparagine, −114), leaving the proline (−98) moiety with a fatty acid chain (Figure 10). Next, the sodiated peak 1065.42 $[M+Na]^+$ was characterized by MS/MS analysis (Figure 11). The fragmentation pattern revealed its daughter ion peaks that confirmed its identity as an iturin A molecule as previously characterized.[20,21] When CID @11.0 was applied, the sodiated peak yielded 937.50 after losing one mole of glutamine residues (−128). Likewise, further fragmentation generated 823.42 (shed off asparagine, −114), 726.17 (loss of proline, −97), 639.33 (loss of serine, −87), 525.25 (loss of asparagine), and finally 362.08 (loss of tyrosine, −163) while leaving asparagine (114) with a sodiated fatty acid chain. A similar fragmentation pattern has been observed for the rest of iturin A homologues by ESI-MS/MS (Supplementary Figure S2a−c).

DISCUSSION AND CONCLUSIONS

Increasing antibiotic resistance and a decline in antibacterial discovery are of major concerns to human and animal health.[22,23] Therefore, there is an urgent need to search for safe and effective drugs to combat antibiotic-resistant bacteria. Natural products isolated from microbes are considered as the most promising source of future antibacterials.[24] For example, bacteria from panda feces were shown to produce bioactive molecules with strong antibacterial activities against enterotoxigenic *E. coli*, *Salmonella*, and *S. aureus*.[25] Similarly, gut bacteria of fish have been exploited for their antibacterial activities against multidrug-resistant pathogenic bacteria including *Aeromonas hydrophila*, *Salmonella enteritidis*, *Campylobacter jejuni*, *Vibrio cholera*, and *Enterobacter cloacae*.[26] Sponge-associated bacteria showed antimicrobial activities against *Candida albicans*, *S. aureus*, and *Vibrio parahaemolyticus*.[27] A recent study showed that actinomycetes isolated from insects produced a potent antimicrobial agent "cyphomycin". This antibacterial showed robust bioactivity against multidrug-resistant fungal species and Gram-negative bacteria.[28] A recent study reported lipopeptides from Gram-negative *P. aeruginosa* with potent antibacterial and antifungal activities.[29] Several other microbial species also produced structurally diverse lipopeptides

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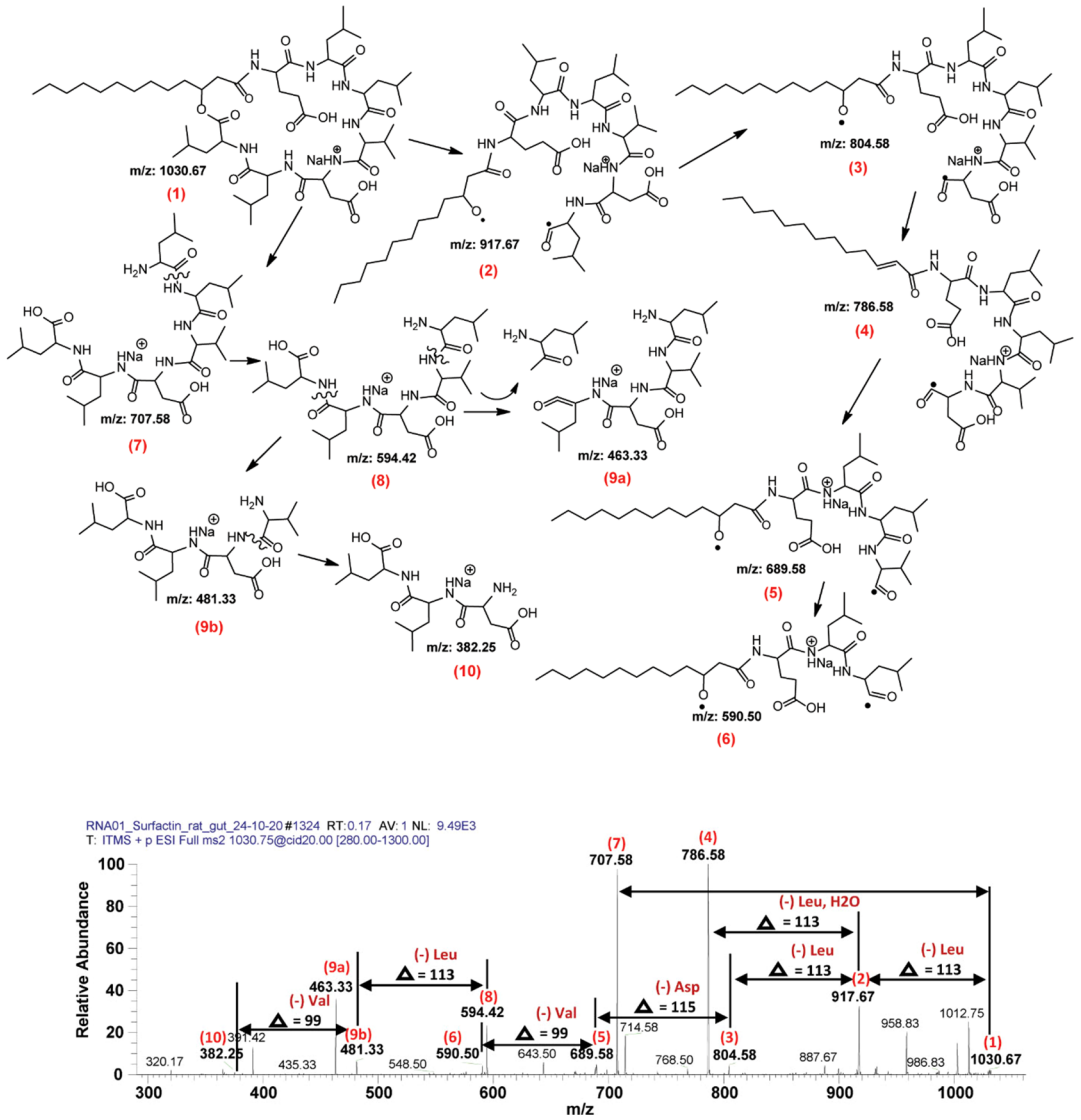

Figure 8. LCMS spectrum representing surfactin molecular ion species. The fragmentation pattern and product ion spectra of sodiated molecules of surfactin at m/z 1030.67 $[M+Na]^+$at positive ion mode.

having antagonistic effects against other pathogens.[30] Similarly, *B. subtilis* R1 significantly reduced surface tension and enhanced oil recovery.[31]

In this study, aerobic and culturable bacteria were isolated from the gut of *Rattus rattus*. Isolated bacteria were identified and cultured in RPMI (minimal medium) to prepare CM. CM were evaluated for their antibacterial activities before and after heat treatment at 95 °C for 10 min. CM exhibited significant antibacterial activities against selected pathogenic bacteria. CM2 and CM3 retained the activity post heat treatment and showed notable bactericidal effects against *P. aeruginosa* and *S. pyogenes*. CM were tested for their cytotoxicity against human cell lines, and only CM1 had 55% cytotoxic effects, while other CM showed no cytotoxicity against HaCaT cells. CM2 showed consistent and broad-spectrum activity and showed no human cell cytotoxicity. Furthermore, CM2 were subjected to liquid chromatography−mass spectrometry (LCMS) analysis. Tandem mass spectrometry analysis revealed that CM2 (*B. subtilis*) produced abundant isoforms of surfactant molecules during cultivation of potent antibacterial lipopeptides, i.e., "surfactin"[32,33] and pore-forming iturin A molecules.[34,35]

Molecular ion peaks with high intensity were subjected to MS/MS and MS^n analysis, and results showed that *B. subtilis* produced several lipopeptides and their derivatives. Previously, Mandal et al., (2013) isolated and identified lipopeptides from Enterobacter and Citrobacter species that showed significant antibacterial activities against *S. aureus* (MTCC1430) (1).[1] Likewise, in another study, lipopeptides were isolated and characterized for their antifungal activities against plant fungal pathogens.[36] Ali et al., (2014) isolated and identified *Bacillus* sp. from a plant rhizosphere. The bacterium was characterized for isolation of lipopeptides surfactin and iturin A. The lipopeptides exhibited broad-spectrum antifungal activities.[7] *Bacillus safensis* isolated from olive oil (contaminated with soil) produced several lipopeptide molecules with antibacterial activities against several bacterial pathogens and antibiofilm activity of *Staphylococcus epidermidis*.[37] A novel lipopeptide, paenibacterin, was identified from *Paenibacillus* sp. isolated from soil. The lipopeptide showed broad-spectrum antibacterial activities

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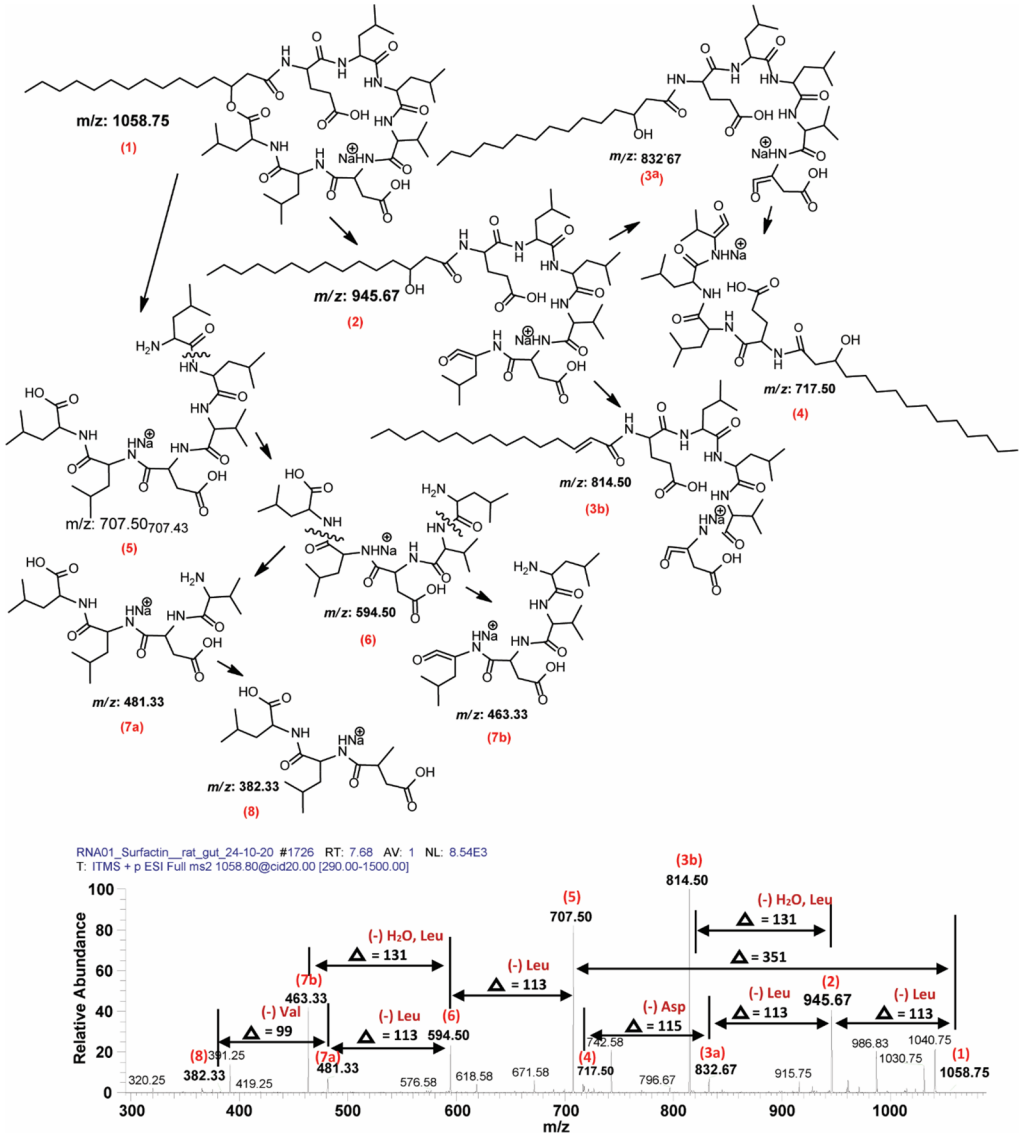

Figure 9. Profiling of fragmentation data obtained from tandem mass spectrometry of a surfactin homologue at m/z 1058.75 $[M+Na]^+$ at positive ion mode.

against Gram-positive and Gram-negative pathogenic bacteria.[38] Similarly, here we identified lipopeptides surfactin and Iturin A from bacteria isolated from the rat gut with broad-spectrum antibacterial activities against selected bacterial pathogens, and our results are in agreement with the abovementioned literature. Lipopetides (fengycin) were identified from *B. subtilis*, a Banyan endophyte with strong antifungal activities,[39] while surfactin molecules were identified from *B. subtilis*. Molecules showed significant antibacterial activities against both bacteriocin-sensitive and bacteriocin-resistant *Listeria monocytogenes* at lower concentrations. Antibacterial activity was retained after heat treatment for 10 min and 121 °C as well as proteolytic incubation.[40] In this study, rat gut bacteria produced several lipopeptides, and further characterization and functional studies of these molecules could be a basis for the development of novel antibacterial(s).

In summary, bacteria here were isolated from a unique source, i.e., rats, that inhibit polluted environments. The rat-derived gut bacteria showed broad-spectrum antibacterial activities against several Gram-positive and Gram-negative pathogenic bacteria. Mass spectrometry analysis revealed several lipopeptides from *B. subtilis* that could be the basis for rational development of antibacterial molecules.

EXPERIMENTAL SECTION

Isolation and Identification of Rat Gut Bacteria. The use of animals was approved by the Sunway University Research Ethics Committee, SUNREC 2017/042. We confirm that all experiments including dissection were done according to appropriate regulations and guidelines as described earlier.[3,41] Prior to dissection, all instruments were sterilized as well as surface sterilized using 70% alcohol throughout the dissection. The whole gut was removed aseptically. Following this, bacteria were isolated from the gut with sterile cotton swabs followed by plating on blood agar plates and incubated for 24 h at 37 °C. A number of bacterial species were isolated from the gut. These bacteria were differentiated from each other based on the appearance, color, shape, and texture on blood agar plates. Bacterial colonies selected were cultured on nutrient agar plates at 37

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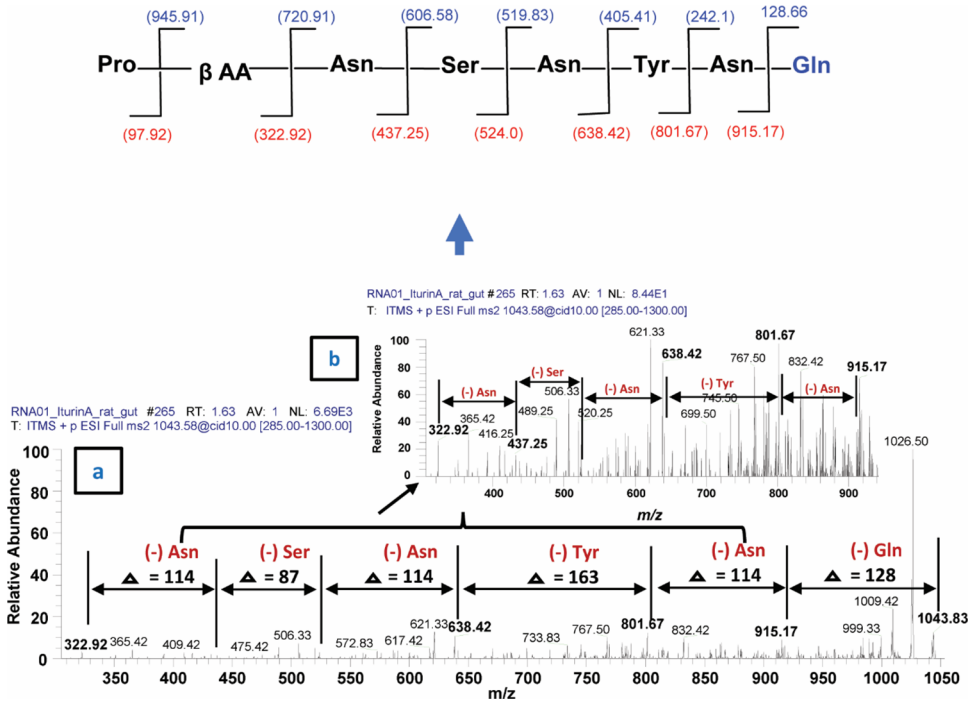

Figure 10. Fragmentation pattern and product ion spectra of sodiated molecules of the iturin A homologue having m/z at 1043.83 $[M+H]^+$.

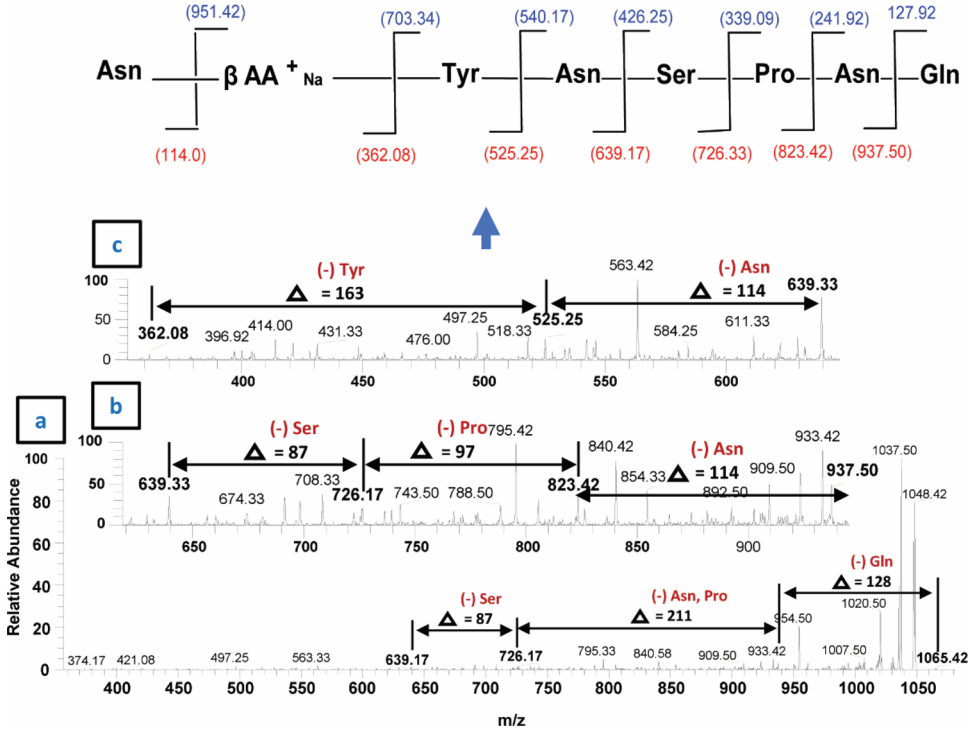

Figure 11. ESI−MS/MS spectrum of the iturin A precursor ion $[M+Na]^+$ at m/z 1065.42 and their daughter ion peaks upon CID.

°C overnight. Subsequently, bacterial identification was performed using molecular identification by 16S rRNA amplification and sequencing.[42] Single pure bacterial colonies were cultured in RPMI (minimal medium) at 37 °C for 24 h aerobically to prepare their conditioned media (CM). Next, overnight cultures were centrifuged at 10,000g at 4 °C for 1 h. Finally, culture supernatants were collected, filter-sterilized using a 0.22 μm pore size filter, and CM were stored at −80 °C until further use.

Bacterial Cultures. Several bacteria used in this study including Gram-positive (*B. cereus*, methicillin-resistant *S. aureus* (MRSA), and *S. pyogenes*) and Gram-negative neuropathogenic *E. coli* K1, *E. coli* K-12, *K. pneumoniae*, *P. aeruginosa*, *S. enterica*, and *S. marcescens*) (Table 1). MRSA was isolated from the blood culture of a patient with sepsis, while *E. coli* K1 (018: K1:H7), strain E44, was originally isolated from the cerebrospinal fluid (CSF) of a meningitis patient (obtained from the Luton & Dunstable NHS

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Foundation Trust, Luton, England, UK). All other bacteria were isolated from clinical samples including *B. cereus*, *K. pneumoniae*, *P. aeruginosa*, *S. enterica*, *S. marcescens*, and *S. pyogenes* (Table 1). Bacteria were grown in nutrient broth at 37 °C overnight aerobically prior to experiments as previously described.[3,43−45]

Evaluation of Bacterial Supernatants for Antibacterial Assays. To determine bactericidal properties of CM, antibacterial assays were performed as previously described.[3,44,46] Briefly, 1×10^6 bacteria were challenged with 100 μL of CM at 37 °C for 2 h. Following this, bacterial cultures were 10-fold serially diluted, plated on nutrient agar plates, and incubated overnight at 37 °C. Bacterial colonies were counted the following day. Bacteria incubated in PBS and *E. coli* K-12 CM were used as the negative control, while bacteria incubated with gentamicin (100 μg/mL) were used as the positive control. Additionally, in some experiments, CM were heat-treated at 95 °C for 10 min and then tested for their antibacterial activities.[3,47]

In Vitro Cytotoxicity Assays. To determine host cell cytotoxic effects of CM, cytotoxicity assays were performed using the human cell line (HaCaT).[48,49] Briefly, HaCaT cells (P14) were obtained from a cell tissue culture laboratory (Department of Biological Sciences, Sunway University). HaCaT monolayers were exposed to CM (100 μL) at 37 °C with 5% CO_2 and 95% humidity for 24 h. Next, cell supernatants were collected, and cytotoxicity was evaluated using the lactate dehydrogenase (LDH) assay kit (cytotoxicity detection kit). To estimate LDH released by human cells, percent cytotoxicity was determined as follows

cytotoxicity (%) = (sample value − negative control value)/(positive control value − negative control value) × 100.

For the negative control, cells were grown in RPMI alone and incubated with *E. coli* K-12 CM, while cells incubated with Triton X-100 (0.1%) were taken as the positive control. In addition (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT assay was performed by growing a HaCaT cell up to 80−90% confluency in a 96-well plate for 24 h at 37 °C in the presence of 95% humidity and 5% CO_2.[44] Subsequently, cells were exposed to CM at graduated concentrations, i.e., 25, 50, 75, and 100 μL and incubated at 37 °C for 24 h with 5% CO_2 and humidified conditions. After this, freshly prepared MTT dye solution (10 μL) was added and incubated for 3−4 h. DMSO (100 μL) was added to each well to dissolve formazan crystals formed by live cells. HaCaT monolayer cells incubated with DMSO alone were taken as the negative control, and absorbance was measured at 540 nm, and percent viability was determined using the given formula, % viability = mean OD of the test sample/mean OD of the negative control × 100.

Calculations for 50% cytotoxic concentration (CC_{50}) and maximum nontoxic dose ($MNTD_{90}$) were performed using GraphPad Prism 8.0.2 software.

Extraction and Purification of Lipopeptides. *B. subtilis* was grown in 1 L of the minimal salt medium (MSM) at 30 ± 2 °C for 24 h as described earlier.[7] After this incubation, the culture was centrifuged at 10000*g* for 45 min at 4 °C, and the culture supernatant was extracted using 1:3 of *n*-butanol in three rounds, and bacterial extracts were precipitated by pH adjustment of the medium to 2.0 with 6 N hydrochloric acid (HCL). The mixture was incubated at 4 °C for 2 h. Next, the final precipitate was achieved through centrifugation at 4 °C for 30 min, and resultant residues were dissolved in 2 mL of LCMS grade methanol (MeOH) and type 1 water at 2:1. It was further filtered through a 0.22 μm syringe filter to avoid contamination. Finally, the extract was evaporated under reduced pressure by rotary evaporation and resuspended in 2 mL of LCMS grade MeOH and stored at −20 °C until further use.

Metabolic Profiling of *Bacillus subtilis* (CM2) Supernatant Extracts Using Mass Spectrometric Analysis. For characterization, the extract (lipopeptides) was subjected to tandem mass spectrometry analysis using a mass spectrometer (LTQ XL Linear Ion Trap mass spectrophotometer, Thermo Scientific, USA) equipped with an ESI source as described previously.[44] Samples were filter-sterilized first and then injected through a direct syringe pump with a flow rate of 5 μL min^{-1}. Samples were scanned at positive total ion full scan mode (mass scan range m/z 50−2000) with a source voltage and a capillary voltage of 4.8 kV and 23 V, respectively. Sheath gas flow (N_2) and capillary temperature were 30 arbitrary units and 350 °C, respectively, in both scan modes. Selected analytes were further fragmented at positive ion modes by employing a collision-induced dissociation (CID) energy of 35 (percentage of 5 V). Mass spectra for molecule(s) present in CM2 were compared against the NIST Mass Spectral Search Program for identification of their analogues. Compounds were identified after correlation with already published data.

Statistical Analysis. Data analysis was done using Student's *t*-test to determine statistical significance. Data are presented as the mean ± standard error of several independent experiments performed in duplicate. P values ≤ 0.05 were considered statistically significant. Cytotoxic concentration (CC_{50}) and maximum nontoxic dose (MNTD), i.e., CC_{90} values, were determined with GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA) software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01137.

ESI−MS spectrum of the *B. subtilis* CM2 extract demonstrating the presence of surfactin analogues analyzed at positive ion mode (Figure S1); putative structures of the fragment ion generated through CID of m/z at 1057.58, 1079.58, and 1081.50 $[M+H]^+$ (Figure S2); ESI−MS spectrum of the surfactin standard (Figure S3); and ESI−MS spectrum of the iturin A standard (Figure S4) (PDF)

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Author Contributions

K.S. and N.A. sourced the animals and carried out dissections. N.A. performed all the experiments under the supervision of R.S., M.I., K.S.K., and N.A.K. and wrote the first draft of the manuscript. N.A. carried out LC/MS data analyses under the supervision of M.I. F.H. helped N.A. in LCMS sample analysis. R.S. and N.A.K. conceived the idea, obtained funding, supervised all work, and corrected the manuscript.

Notes

The authors declare no competing financial interest.

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