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In silico prediction and *in vitro* analysis of bacteriocin and probiotic properties of *Weissella cibaria* NM1 isolated from Asian sea bass

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ABSTRACT

Aims: This study aims to predict the presence of bacteriocin- and probiotic-associated genes in the genome of *Weissella cibaria* NM1 isolated from Asian sea bass using a machine learning-based NeuBI prediction approach, followed by the investigation of the crude bacteriocin antimicrobial and probiotic properties via *in vitro* analysis.
Methodology and results: This study utilized the machine learning-based NeuBI prediction approach with a homology search of highly conserved bacteriocin-associated genes present in the genome of *W. cibaria* NM1. This approach discovered a putative bacteriocin gene (WC_2064) and bacteriocin operon with complete immunity, transporter, regulator and modifier genes. Furthermore, the genome of *W. cibaria* NM1 was found to harboured specific probiotic-associated genes that would contribute to acid and bile tolerance, adhesion on the host cell and exhibited cholesterol-reducing ability. On top of that, the genome also shows the absence of virulence and antibiotic resistance genes, which signifies the safety of *W. cibaria* NM1 as a potential probiotic candidate. *In vitro* study has confirmed the antipseudomonal activity of crude bacteriocin NM1 with MIC of 62.5 mg/mL. *Weissella cibaria* NM1 can tolerate 0.3% (v/v) of bile salt condition and the transit through the simulated gastric (pH 3 and 4) and small intestinal (pH 8) tract.
Conclusion, significance and impact of study: Current findings suggested *in silico* approach can speed up the search for putative bacteriocin and probiotic-associated genes from the genome of *W. cibaria* NM1. Nevertheless, further verification through experimental works will be deemed essential.

Keywords: Weissella cibaria, bacteriocin, probiotic, *in silico* prediction, machine learning

INTRODUCTION

The publicly available genomic data opens the possibility of investigating new bacteriocins through different *in silico* approaches. The approaches that have been used include finding highly conserved bacteriocin regulatory gene (driver gene) that is often linked to a bacteriocin structural gene (Begley *et al*., 2009), extensive search on one bacterial genome of bacteriocin structural gene and genes that are associated to bacteriocin production. The latter approach is the working principle of the BAGEL4 tool (Walsh *et al.*, 2015). BAGEL4 is a database and a homology-based search tool that includes many experimentally verified annotated bacteriocin sequences (van Heel *et al*., 2013). The development of the BAGEL4 tool web server enables researchers to detect the putative bacteriocin gene clusters and secondary metabolites such as RiPPs (ribosomally synthesised and posttranslational modified peptides) in the bacterial genome (van Heel *et al*., 2018). Walsh *et al*. (2015) has successfully identified the gene clusters for key bacteriocins such as bacteriolysins, lantibiotics and sactipeptides from 59 unique members of Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria and Synergistetes using this BAGEL approach.

Another recent *in silico* approach is by using a machine learning prediction tool, the Neural Bacteriocin Identifier (NeuBI). NeuBI is a recurrent neural network-based software to predict bacteriocins from protein sequences. It was developed to overcome the limitation of homology-based prediction used by BAGEL4 (Hamid and Friedberg, 2019). Through NeuBI, the hunt of bacteriocin from novel isolates can be improved to discover new putative bacteriocins. The study from Vezina *et al*. (2020) has discovered a putative circular bacteriocin through mining the NCBI database against experimentally verified circular bacteriocin sequence. This study highlights the swiftness of the bacteriocin prediction approach in finding

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new bacteriocin compared to the conventional method, which requires many isolates to be tested against indicator strains. The bioinformatics approach serves as an initial screening process that speeds up the discovering of new bacteriocin producers. However, the presence of bacteriocin genes does not necessitate the strain to exhibits antimicrobials in some cases, where the bacteriocin producing trait of the strain is inactive due to missing essential genes linked to bacteriocin production (Colins *et al*., 2018). Hence, the validation of experimental data to support bioinformatic prediction is of utmost importance (Oliveira *et al.*, 2017).

In this work, we have isolated a least explored Gram-positive bacterium, *Weissella cibaria* NM1, from Asian sea bass. The genus *Weissella* is a member of the lactic acid bacteria group that is found in many environments such as humans, fermented vegetables and animal gut. Much research attention has been focused on the probiotic characteristics exhibited in *Weissella* species, but only limited reports were found on the production of bacteriocins. To the author's knowledge, only one bacteriocin (Weissellicin 110) produced by *W. cibaria* 110 has been reported by Srionnual *et al.* (2007). Other Weissellicins -Y, -M and -L being reported are from closely related species, *W. hellenica* (Masuda *et al.*, 2000; Leong *et al.*, 2013). Hence, this study aims to adopt *in silico* approaches to screen for the presence of bacteriocin and probiotic-associated genes in the genome of *W. cibaria* NM1, followed by the investigation of its antimicrobial and probiotic activity using *in vitro* assays.

MATERIALS AND METHODS

Bacterial culture and maintenance

Weissella cibaria NM1 was isolated from the gut of Asian sea bass by Dr Ivan Chiew and deposited at the Microbial Culture Collection Unit (UNICC), Institute of Biosciences, University Putra Malaysia under the accession number of UPMC1203. The bacterium was subculture in MRS broth (Oxoid, UK) and incubated at 37 °C for 24 h prior to use. The frozen stock culture was stored in MRS broth containing glycerol (20% v/v) at –20 and –80 °C freezers.

Genomic DNA extraction

A 24 h-old *W. cibaria* NM1 culture was used for genomic DNA extraction. The lytic enzyme solution was prepared by adding 50 mg of lysozyme (Sigma L6876) into 5 mL of 50 mM EDTA (pH 8.0). The solution was then mixed thoroughly prior to use. The DNA extraction was done according to the protocol of the Promega Wizard® Genomic DNA Purification kit (Promega Corporation, 2021). The purified DNA was stored at 4 °C until further use. The DNA quantity and purity were measured using a NanoDrop Spectrophotometer (Thermofisher, USA). The quality of DNA was further validated using 1.5% agarose gel electrophoresis stained by SYBR® safe DNA gel stain (Thermo Fisher Scientific, USA). The DNA band was viewed using the Chemidoc XRS+ system (Biorad, USA).

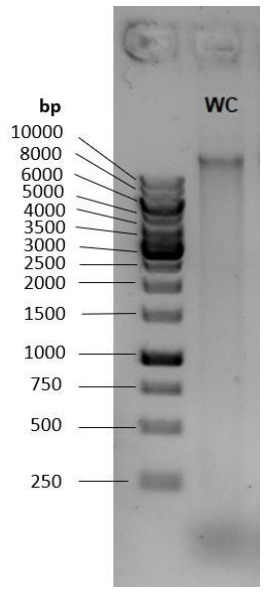

Figure 1: Result from gel electrophoresis of genomic DNA for *W. cibaria* NM1. Lane 1: 1 kb DNA Ladder; Lane 2: *W. cibaria* NM1.

The agarose gel with DNA band was presented in Figure 1.

Genome sequencing

The DNA of *W. cibaria* NM1 was sent to BioEasy Sdn. Bhd. (Selangor, Malaysia) for genome sequencing service. The sequencing of *W. cibaria* NM1 was performed on the Illumina MiSeq® platform using 2 × 251 paired-end method (Malaysia Genome Institute, Malaysia). The resulted DNA sequence reads were adapted and quality trimmed. The contaminant and read length filtered with a threshold of 30 using BBDuk (Bushnell *et al*., 2017). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JABXJP000000000. The version described in this paper is version JABXJP010000000.

Genome assembly and annotation

Raw sequences were assembled using St. Petersburg genome assembler (SPAdes) (Bankevich *et al*., 2012) and annotated using Prokaryotic dynamic programming gene-finding algorithm (Prodigal) for protein-coding gene prediction (Hyatt *et al*., 2010), ARAGORN (Laslett and Canback, 2004) for transfer RNA gene prediction, RNAmmer for ribosomal RNA gene prediction (Lagesen *et al*., 2007) and HMMER for protein domain search (https://www.ebi.ac.uk/Tools/hmmer/) (Potter *et al.*, 2018). The 16S rDNA sequence of *W. cibaria* NM1 was searched against NCBI's 16S rRNA database, and the phylogenetic tree was constructed using 16S rDNA from the BLAST hits through the MEGA-X tool and using the Neighbour-joining statistical method (Kumar *et al*., 2008).

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BAGEL4 and NeuBI bacteriocin gene prediction tools

The *W. cibaria* 110 nucleotide sequences obtained from the NCBI repository were used as reference strain in this study. This is due to the bacteriocin (Weissellicin 110) sequences of *W. cibaria* 110 have been validated experimentally (Srionnual *et al.*, 2007; Li *et al.*, 2017) and suitable for use as positive control.

In this study, the nucleotide sequences for targeted strain, *W. cibaria* NM1 and control, *W. cibaria* 110 were used for BAGEL4 prediction through BAGEL4 dedicated web server (http://BAGEL4.molgenrug.nl/index.php) (van Heel *et al*., 2018). While the protein sequences for both strains were used for NeuBI prediction, following the instruction provided in the Github repository (https://github.com/nafizh/NeuBI) (Hamid and Friedberg, 2019). The protein sequences with a score higher than 0.9 were shortlisted. Accession ID of the context genes (genes associated with bacteriocin production, transport, regulator, immunity, modifier) was collected from the NeuBI's Github and then were fetched from NCBI through the Entrez tool (Maglott *et al.*, 2011).

A custom BLAST database was created using makeblastdb from the BLAST standalone tool suite (Camacho *et al.*, 2009). Protein sequences of *W. cibaria* NM1 and *W. cibaria* 110 were searched against the custom database with the cut-off value of e-value greater than -5. Positive hits from the BLAST search were combined with NeuBI's bacteriocin prediction hits and were arranged in a coordinated manner according to its location in the genome. Bacteriocin gene that is surrounded with all types of context gene (modifier, immunity, regulator and transport) in the vicinity of 50 kb were selected as potential bacteriocin gene. The gene operon is visualised using DNA_feature_viewer python script (https://github.com/Edinburgh-Genome-Foundry/DnaFeaturesViewer) (Zulkower and Rosser, 2020). Potential bacteriocin gene was searched against NCBI non-redundant (NR) database. InterPro analysis was also done to provide functional analysis of the proteins and BACTIBASE database for the characterization of bacterial antimicrobial peptides (Blum *et al.*, 2021).

In silico screening of probiotic-associated genes

Screening of probiotic-associated genes of *W. cibaria* NM1 was done through BLASTP search (Camacho *et al*., 2009) based on the probiotic-associated genes reported in the work of Kumari *et al*. (2020). The protein sequences of *W. cibaria* NM1 were searching for antimicrobial resistance genes using ResFinder3.0 server (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari *et al.*, 2012) and through Resistome data (RGI) from the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca) (Alcock *et al.*, 2020). Virulence genes were detected using VirulenceFinder (Joensen *et al.*, 2014).

In vitro assessment of antimicrobial activity

Production of crude bacteriocin

In this assay, 1% (v/v) of 24 h-old *W. cibaria* NM1 (8 Log_{10} CFU/mL) was grown in 10 mL MRS broth for 24 h at 37 °C. After incubation, the culture was centrifuged at 9600 rpm for 20 min. The cell-free supernatant (CFS) was withdrawn, neutralised to pH 6.5 using 1 M NaOH to eliminate the inhibitory effect due to acid production, and then filter sterilised with 0.45 µm syringe filter (Minisart, Sigma Aldrich, United States). The neutralised and filtrated CFS was then stored in a –80 °C freezer for 24 h and then subjected to freeze-drying using a Freeze-Dryer (Christ Alpha 1-2 Ldplus, Denmark) for 72 h. The freeze-dried CFS powder was designated as "crude bacteriocin". A stock solution of 1 g of crude bacteriocin dissolved in 1 mL of sterile water was prepared freshly prior to quantification of bacteriocin inhibitory activity.

Minimum inhibitory concentration (MIC) assay

The MIC microdilution assay was carried out to quantify the antimicrobial activity of crude bacteriocin NM1 according to the protocol of CLSI guidelines (Subramaniam and Nandan, 2011). The Mueller Hinton (MH) broth was used as the negative control and the antibiotic Ciprofloxacin was used as the positive control in this MIC assay. MIC was defined as the concentration of antimicrobials (crude bacteriocin or antibiotic) producing complete inhibition on the visible growth of the indicator *P. aeruginosa* ATCC 10145.

In vitro assessment of probiotic properties

Bile tolerance assay

The bile tolerance assay was carried out to determine the ability of *W. cibaria* NM1 to grow in the presence of bile based on the method of Vinderola and Reinheimer (2003). A 2% (v/v) of 24 h-old *W. cibaria* NM1 (8 Log_{10} CFU/mL) was inoculated into 10 mL MRS broth with concentrations of bile salt ranging from 0 to 1.0% (w/v). The treated cultures were then incubated at 37 °C for 24 h and followed by determining total viable plate count using the Mile Misra method (Miles *et al*., 1938).

In vitro simulated gastrointestinal transit tolerance assay

The method of Charteris *et al*. (1998) and Huang and Adams (2004) were used to determine the tolerance of *W. cibaria* NM1 in the simulated gastrointestinal transit. In simulated gastric transit assay, a 0.3% (w/v) pepsin (Sigma, USA) was mixed in sterile 0.5% (w/v) NaCl. Then, the mixture was adjusted to pH 2, 3 and 4, respectively, with 5 M of HCl or NaOH to produce fresh simulated gastric juice. The washed cell suspension of *W. cibaria* NM1 (8 Log_{10} CFU/mL) with a volume of 200 µL was inoculated into 0.3 mL of NaCl (0.5% w/v) in a sterile microcentrifuge tube. Then, 1.0 mL of simulated gastric

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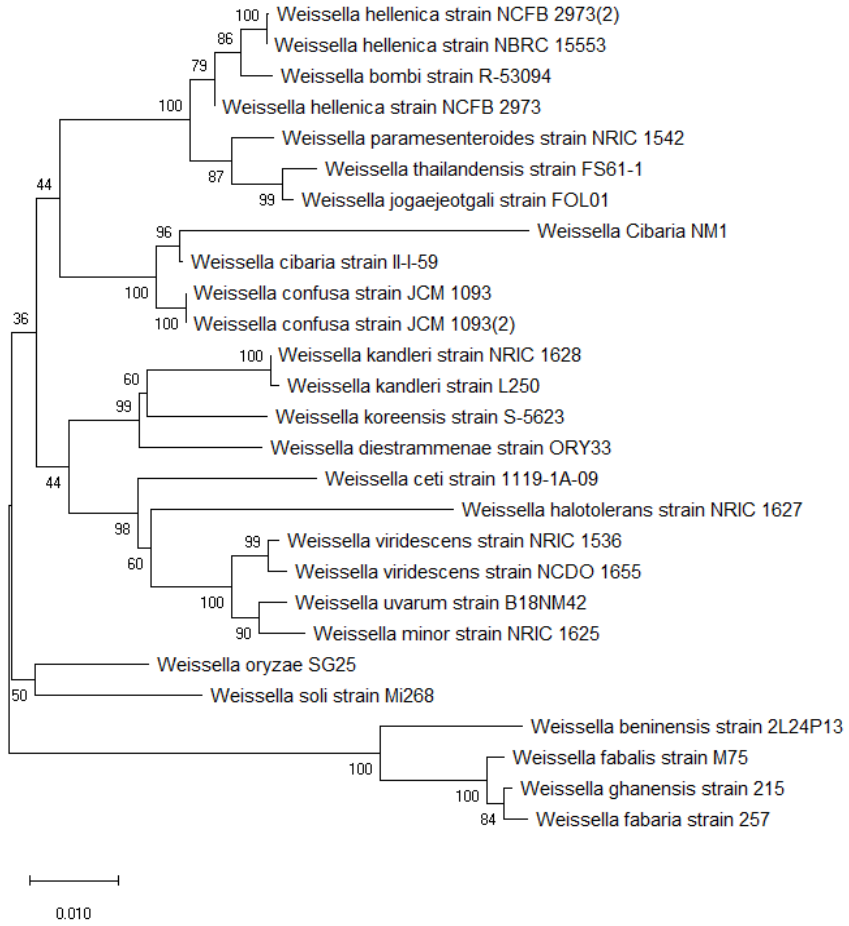

Figure 2: Phylogenetic tree of *W. cibaria* NM1.

juice with different pH (pH 2, 3 and 4) were added into the mixture, respectively. Next, the mixtures were vortexed for 10 s and incubated in a 37 °C water bath for 180 min. At an interval of 0, 60, 120 and 180 min of incubation, 100 µL of the aliquot was withdrawn to determine the total viable plate count.

In small intestinal transit assay, the pancreatin USP (0.1% w/v) (Sigma, USA) was suspended in sterile 0.5% (w/v) NaCl. Then, the pH of the simulated small intestinal juice was adjusted to pH 8 by using 5 M of NaOH. The washed cell suspension of *W. cibaria* NM1 (8 Log_{10} CFU/mL) with a volume of 200 µL was inoculated into 0.3 mL of NaCl (0.5% w/v) in a sterile microcentrifuge tube. Then, 1.0 mL of simulated small intestine juices (pH 8) (Sigma, USA) was added into the mixtures. The mixture was then vortexed for 10 s and incubated in a water bath at 37 °C for 240 min. At an interval of 0, 60, 120, 180 and 240 min of incubation, 100 µL of the aliquot was withdrawn to determine the total viable plate count.

Statistical analysis

Data were analysed using IBM SPSS Statistic version 24.0 (SPSS Inc, Chicago, IL, USA). All experiment was done in three independent replicates. All data obtained are the average of three replicates and are represented as the mean ± standard deviation. Paired t-test was used to compare bacteriocin activity of crude bacteriocin and CFS. All assays were conducted in three independent replicates. One-way ANOVA was used to compare data between different treatments. $P<0.05$ was considered as being statistically significant.

RESULTS

Assembly and analysis of the *W. cibaria* NM1 genome

The whole-genome sequencing for *W. cibaria* NM1 generated a draft genome of 2,421,541 bp, with a GC content of 44.86%. The genome was assembled into 36 contigs with N50 of 151,860. The estimated assembly coverage was 42.32. Prodigal annotation predicted 2,248 coding sequences out of 2,320 genes (96% coding intensity). There are 72 RNA genes consisted of 67 tRNA and 5 rRNA. The sequences of the rDNA gene of *W. cibaria* NM1 showed 98.16% similarity to *W. cibaria* II-59, resulting in the same cluster under the phylogenetic tree (Figure 2).

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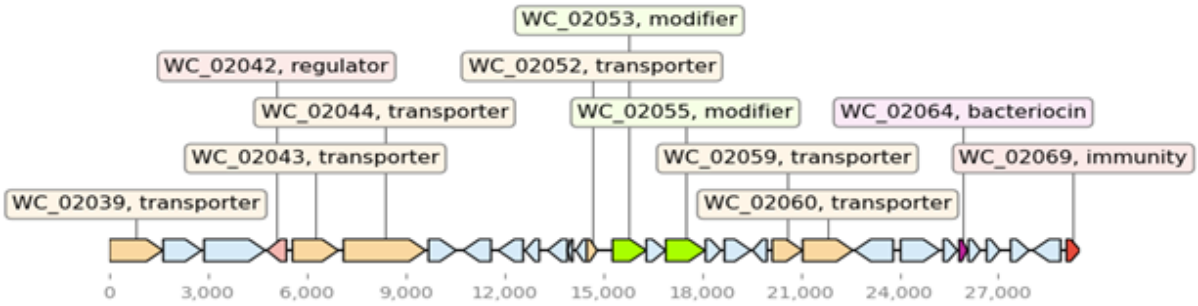

Figure 3: Illustration of predicted bacteriocin gene operon of *W. cibaria* NM1. The unlabelled gene boxes are non-related genes.

A

WC_02064

VSLIAVPQFVVLMILFLLAGVIYPLTTPAMIIDFKAVGG TLMLATGFRMIKVRMFPTADMIPAMLFIMPISWAWTTWVLPLLAK

B

```
   V  S  L  I  A  V  P  Q  F  V  V  L  M  I  L  F  L  L  A  G
 1 GTGTCATTGATTGCCGTACCGCAGTTTGTTGTGTTGATGATTCTGTTTTTGTTAGCCGGG 60

   V  I  Y  P  L  T  T  P  A  M  I  I  D  F  K  A  V  G  G  T
61 GTGATCTATCCACTCACGACGCCAGCCATGATAATTGACTTTAAGGCGGTTGGTGGTACC 120

   L  M  L  A  T  G  F  R  M  I  K  V  R  M  F  P  T  A  D  M
121 CTTATGTTAGCAACGGGGTTCCGAATGATTAAGGTGCGCATGTTCCCGACGGCTGACATG 180

   I  P  A  M  L  F  I  M  P  I  S  W  A  W  T  T  W  V  L  P
181 ATTCCAGCGATGCTGTTTATTATGCCAATTAGTTGGGCGTGGACGACCTGGGTCTTGCCG 240

   L  L  A  K  *
241 TTGCTAGCAAAGTAA 255
```

Figure 4: (A) Protein sequence of WC_2064. The double glycine motif in the sequence is shown by the arrow. (B) Alignment of the protein sequence and nucleotide sequence of WC_2064.

In silico prediction of bacteriocin genes using BAGEL4 and NeuBI

In silico prediction of bacteriocin genes for the targeted strain, *W. cibaria* NM1 and the reference strain, *W. cibaria* 110 were initially performed using BAGEL4. However, a negative prediction result was obtained with no bacteriocin was found using BAGEL4 for both tested strains. The BAGEL4 prediction was then extended for all the *W. cibaria* genomes deposited in the NCBI database, the similar negative outcome was obtained.

Due to the limitation of homology-based prediction used by BAGEL4, the NeuBI tool was used to predict bacteriocins from the protein sequences of both *W. cibaria* NM1 and *W. cibaria* 110. NeuBI successfully predicted the presence of Weissellicin 110 from the protein sequences of *W. cibaria* 110, with the probability of 0.96. NeuBI predicted the presence of 21 protein sequences that have scored probability higher than 0.9 in *W. cibaria* NM1. However, only one bacteriocin gene candidate, WC_2064, with a prediction score of 0.97, and has all four types of context genes associated with bacteriocin production (namely the transporter, regulator, immunity and modifier genes) in the 25 kb vicinity (Figure 3). Figure 3 shows the genetic organisation of bacteriocin operon for *W. cibaria* NM1 generated through the custom BLAST analysis. The immunity gene was found upstream of the bacteriocin gene. Whereas several transporter genes, modifier genes, and regulator genes were found downstream of the bacteriocin gene. Table 1 also shows the complete set of context genes and predicted gene function involved in bacteriocin biosynthesis from the GenBank database.

Figure 4 shows the complete gene sequence of WC_2064 based on the gene annotation output from Prodigal gene prediction software. The sequence of WC_2064 contains a double glycine leader at the N-terminal, which indicates the sequence has a precursor peptide that could be cleaved off by the N-terminal domain of the transporter (Dirix *et al.*, 2004). However,

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Table 1: Predicted gene function involved in bacteriocin biosynthesis.

Gene ID	Function	Gene	E-value	Similarity (%)	GenBank ID
WC_02039	Transporter	ABC Transporter	1.00e-75	30.43	EFM66193.1
WC_02042	Regulator	TetR Family Transcriptional Regulator	2.00e-08	25.82	KKD14008.1
WC_02043	Transporter	MFS Family Major Facilitator Transporter	1.00e-39	28.6	AEA57849.1
WC_02044	Transporter	ABC Transporter ATP-Binding Protein	1.00e-09	31.25	COL50160.1
WC_02052	Transporter	Multidrug Transporter	8.00e-08	21.57	KQB72399.1
WC_02053	Modifier	Riboflavin Biosynthesis	9.00e-56	36.86	AIG26540.1
WC_02055	Modifier	Riboflavin Biosynthesis	5.00e-124	49.23	AIG26542.1
WC_02059	Transporter	Glycine Betaine Carnitine Choline ABC Transporter ATP-Binding Protein	3.00e-94	52.20	KRL50892.1
WC_02060	Transporter	Glycine Betaine Carnitine Choline ABC Transporter ATP-Binding Protein	5.00e-164	52.82	AGE38097.1
WC_02064	Bacteriocin	Putative membrane protein YdfK	8.00e-50	100	KIU20424.1
WC_02069	Immunity	Putative Leucocin B Immunity Protein	3.00e-06	28.16	YP 009090140.1

Table 2: BLAST hits of WC_2064 against BACTIBASE database of bacteriocin genes.

Hits	Score (bits)	Query cover	E-value	Identity
Carocin D	19.5947	57%	0.478	15%
lactococcin-G β	17.6254	10%	0.937	5%
Enterocin EJ97	17.6254	23%	1.098	7%
Gassericin T (gassericin K7 B)	15.6562	13%	6.459	8%
Lacticin Z	15.6562	19%	6.480	5%
Lacticin Q	15.3748	7%	7.163	3%

through the BLASTP search in the NR nucleotide database, WC_2064 was annotated as a hypothetical gene by Prodigal gene prediction software. As a result, WC_2064 was found to be a putative membrane protein YdfK with unknown function (DUF554) (Table 1). A similar DUF554 outcome was obtained from the InterPro functional and structural analysis of protein sequences. The WC_2064 was further search through the BACTIBASE database for bacterial antimicrobial peptides and resulted in low homology hits (15% similarity) with a bacteriocin known as Carocin D (Table 2).

Genome screening of probiotic-associated genes

The genome screening of probiotic-associated genes of *W. cibaria* NM1 was done using BLASTP search and based on the closely related *W. cibaria* CH2 reported by Kumari *et al.* (2020). Table 3 shows the list of probiotic-associated genes present in the *W. cibaria* NM1 genome. These probiotic-associated genes are responsible for functions such as acid tolerance (e.g. ATP synthase and esterase), bile tolerance (e.g. ABC transporter and ATP dependent proteases subunit), adhesion to the epithelial lining (e.g. mucus binding protein) and functional traits such as cholesterol reduction (catabolite control protein A). In addition, no virulence gene and antibiotic resistance gene were found encoded in *W. cibaria* NM1 after mining through the ResFinder3.0 server (Zankari *et al.*, 2012), Resistome data (RGI) from the Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al.*, 2020) and VirulenceFinder (Joensen *et al.*, 2014).

***In vitro* determination of the antimicrobial activity of crude bacteriocin NM1**

In this study, the crude bacteriocin NM1 was neutralised to pH 6.5 to eliminate the inhibitory effect due to acid production, a key metabolite produced by a member of the lactic acid bacteria group. After that, the crude bacteriocin was filter sterilised with a 0.45 µm syringe filter to reduce other contaminants. Bacteriocins are antimicrobial peptides with sizes ranging from less than 3 kDa to more than 30 kDa (Yang *et al.*, 2014), and hence these peptides will be retained in the filtrate. The current study only focused on testing the antimicrobial activity of the crude bacteriocin. Once this antimicrobial activity is determined, the future studies can be conducted to purify the bacteriocin and re-examine its antimicrobial activity. The antimicrobial activity of the crude bacteriocin NM1 was quantified using a microbroth dilution assay. Table 4 shows the MIC of crude bacteriocin NM1 and controls against *P. aeruginosa* ATCC10145. The crude bacteriocin NM1 exhibited a MIC of 62.5 mg/mL against *P. aeruginosa* ATCC10145. The antibiotic ciprofloxacin (positive control) showed stronger inhibitory activity than crude bacteriocin NM1.

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Table 3: List of probiotic related genes present in *W. cibaria* NM1 genome.

Function	Protein	Gene hits	Identity (%)	E-value
Acid tolerance	Amino acid permease	WC_02193	100	9.31e-157
	ATP synthase F0F1 subunit C	WC_00515	100	1.97e-44
	F0F1 ATP synthase subunit A	WC_00516	99.583	4.10e-173
	ATP synthase F0F1 subunit B	WC_00514	100	9.72e-121
	ATP synthase F0F1 subunit delta	WC_00513	99.444	1.22e-129
	ATP synthase F0F1 subunit gamma	WC_00511	100	0
	Glucose-6-phosphate isomerase	WC_00687	99.776	0
	GTP pyrophosphokinase	WC_01382	99.731	0
	L-Lactate dehydrogenase	WC_00329	100	0
	Putative esterase	WC_00481	99.377	0
	Pyruvate kinase	WC_00797	99.577	0
Adhesion	Sortase	WC_01443	75.934	9.07e-136
	Fibronectin-binding protein	WC_01897	79.9	0
	Mucus binding protein	WC_01688	95.038	0
	Competence protein ComGC	WC_00823	100	9e-75
	Elongation factor Tu	WC_01897	79.9	0
	Enolase	WC_00733	100	0
	Triosephosphate isomerase	WC_00047	100	1e-171
Acid and bile tolerance	Arginine-ornithine antiporter	WC_00584	100	0
	ATP-dependent Clp protease proteolytic subunit	WC_00358	100	8.94e-148
	Cyclopropane-fatty-acyl-phospholipid synthase	WC_01335	98.992	0
	ATP-dependent Clp protease ATP-binding subunit ClpC	WC_00500	99.88	0
	ATP-dependent Clp protease ATP-binding subunit ClpE	WC_01650	99.711	0
	ATP-dependent Clp protease ATP-binding subunit ClpX	WC_01894	64.216	0
	D-Alanyl-lipoteichoic acid biosynthesis protein DltB	WC_01553	100	0
	Multidrug resistance protein	WC_01775	47.951	9.40e-146
Cholesterol reduction	Catabolite control protein A	WC_00896	99.7	0

Table 4: Minimum inhibitory concentration of crude bacteriocin NM1 against *P. aeruginosa* ATCC10145.

Antimicrobial agents	Minimum inhibitory concentration (MIC)
Crude bacteriocin NM1	62.5 ± 0.0 mg/mL
Ciprofloxacin (Positive control)	1.0 ± 0.0 µg/mL
Mueller Hinton broth (Negative control)	0.0 ± 0.0 µg/mL

Table 5: Simulated gastric transit tolerance of *W. cibaria* NM1.

Incubation time (h)	Viable cell count of *Weissella cibaria* NM1 (Log_{10} CFU/mL)		
	pH 2	pH 3	pH 4
0	7.61 ± 0.03^{c}	8.19 ± 0.15^{a}	7.59 ± 0.42^{a}
1	4.32 ± 0.35^{b}	8.10 ± 0.20^{a}	7.59 ± 0.23^{a}
2	4.24 ± 0.45^{b}	8.08 ± 0.14^{a}	7.56 ± 0.12^{a}
3	3.77 ± 0.05^{a}	7.95 ± 0.18^{a}	7.45 ± 0.17^{a}

Notes: Values are mean ± standard deviation; abc within a column, values with different superscripts are significantly different at $P<0.05$.

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In vitro determination of probiotic properties of W. cibaria NM1

The ability to tolerate a harsh gastrointestinal environment is the key feature of probiotics. In the bile tolerance assay, *W. cibaria* NM1 was able to maintain a viable cell count of 1.54 Log_{10} CFU/mL when treated with 0.3% (v/v) bile salt but failed to remain viable when treated with 0.5 and 1.0% (v/v) bile salt. *W. cibaria* NM1 was treated with simulated gastric juice of pH 2, 3 and 4 (Table 5). *Weissella cibaria* NM1 exhibited high tolerance at pH 3 and 4, with no significant reduction in cell viability during the 3 h of incubation. However, the growth of *W. cibaria* NM1 was significantly reduced by 43% after 1 h of incubation at pH 2. *Weissella cibaria* NM1 was treated with simulated small intestinal juice adjusted to pH 8 and exhibited strong growth (7.90 Log_{10} CFU/mL) in the presence of pancreatin and with no significant changes in viable cell count after 4 h incubation.

DISCUSSION

Wet-lab screening is one of the commonly used approaches to identify new bacteriocin producers and probiotics. However, this method is time-consuming, filled with trials and errors. Hence, the recent advent of *in silico* screening process was adopted to discover new bacteriocin producers and probiotic candidates.

In silico prediction of bacteriocin using BAGEL4

BAGEL4 is a homology-based search tool that includes many experimentally verified annotated bacteriocin sequences. However, in this study, the prediction of bacteriocin genes with BAGEL4 for the targeted strain, *W. cibaria* NM1 and the reference strain, *W. cibaria* 110 was unsuccessful. This BAGEL4 prediction was also extended to all *W. cibaria* strains deposited in NCBI and similar outcome was observed.

Abdul Karim *et al.* (2020) reported that using BAGEL 4 to predict bacteriocin gene on newly isolated *W. cibaria* D1 also failed to detect any bacteriocin gene found from the genome. This outcome could be due to the bacteriocin associated genes potentially harbouring in the genome have low similarity with those found on the database of BAGEL4. Walsh *et al.* (2015) also reported a similar observation on identifying bacteriocin gene clusters in the gastrointestinal tract using Human Microbiome Genome database where the author stated that *in silico* prediction of BAGEL is dependent on the similarity to previously described bacteriocin associated genes and the arrangement of the context genes which are not in close vicinity with one another. This arrangement also dampens the possibility of bacteriocin genetic organisation being detected (Hols *et al*., 2019).

In silico prediction of bacteriocin using NeuBI

The NeuBI, a machine-based prediction tool was employed to unlock the cryptic and novel gene that are undetected through BAGEL4 homology search. NeuBI was developed with the notion to differentiate between bacteriocin and non-bacteriocin sequences using a deep recurrent Neural Network (RNN). Thus, NeuBI allows for a potential bacteriocin with low similarity with other bacteriocins to be detected, and the prediction will be further verified with the presence of highly conserved context genes that contribute to the bacteriocin biosynthesis (Hamid and Friedberg, 2019).

Out of the 21 protein sequences of *W. cibaria* NM1 resulted from NeuBI, only one bacteriocin gene candidate, WC_2064, was found with bacteriocin conserved context genes in the 25 kb vicinity of WC_2064 (Figure 3). The presence of context genes such as ABC transporter and immunity genes around WC_2064 also resemble those bacteriocin context genes found in Weissellicin 110 (Li *et al*., 2017). However, this ABC transporter gene alone is not substantial to classify the WC_2064 as a putative bacteriocin gene. This is because the ABC transporter gene does not contain any C39 peptidase domain that could process bacteriocin that is with a double glycine secretion signal (Dirix *et al*., 2004). Nevertheless, the report of Kjos *et al.* (2010) discovered the putative bacteriocin through screening of a bacteriocin immunity gene, called Abi protein. The author listed several criteria such as length, containing N terminal secretion signal (double glycine motif), cationic protein sequence and related transport and immunity gene, that are required for a sequence to be classified as putative bacteriocin. However, no specific requirement for the ABC transporter was listed.

An extended search through the BACTIBASE database for bacterial antimicrobial peptides was conducted to investigate the identity of WC_2064. This search has resulted in a low homology similarity with bacteriocin Carnocin D. Current findings suggested that the sequence of WC_2064 might not be deposited in the respective databases for bacteriocins and antimicrobial peptides, hence resulted in no high homology similarity with known or experimental validated bacteriocin genes.

Interestingly, Kjos *et al.* (2010) also conducted a heterologous expression study that was able to assess the antimicrobial activity of the predicted bacteriocin genes. Hence, a future study on the heterologous expression of the antimicrobial activity of WC_2064 is recommended to provide further elucidation on the relationship of the conserved context genes and the bacteriocin gene.

Probiotic assessment through genome screening

Weissella cibaria has been studied for its probiotic potentials (Lee *et al*., 2012; Kang *et al.*, 2019; Kumari *et al.*, 2020). Probiotic is defined by Food and Agriculture Organization as a live microorganism which, when administered in sufficient amounts, convene a health value to the host (FAO/WHO, 2001). Probiotics have health-promoting effects by influencing gut microbiota and improve the intestinal immune response (Hemarajata and Versalovic, 2013). However, probiotic effects are strain-

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specific, and each new probiotic candidate will require an experimental assessment to ensure it does not incur detrimental health effects on the host.

Thus, current work utilises a genome-centric strategy to assess the probiotic potential of *W. cibaria* NM1. The genome of *W. cibaria* NM1 was searched for genetic determinants based on three important criteria: (i) survivability in the gut, (ii) not harbouring any virulence and antibiotic resistance genes and (iii) producing beneficial effects. Our finding shows that *W. cibaria* NM1 harbours genes that contribute to tolerance toward acid and bile, such as the various subunits of FoF1 ATP synthase, which are one of the contributing factors for acid tolerance ability (Cotter and Hill, 2003). The Clp proteases found in the genome of *W. cibaria* NM1 contribute to bile tolerance ability. In the study reported by Whitehead *et al.* (2008), the mutants without the Clp protease are unable to tolerate bile stress. Acid and bile tolerance are the criteria required for any probiotic to survive and adapt during the transit through the gastrointestinal tract. The acid and bile tolerance genes listed in Table 2 are in accordance with the report by Lee *et al.* (2012) that studied on the probiotic traits of multiple *Weissella* strains such as *W. confusa* and *W. cibaria.* All the genes related to adhesion ability found in *W. cibaria* CH2 were also found harboured in the genome of *W. cibaria* NM1, such as mucus binding domain protein and fibronectin-binding protein. Adhesion characteristic of the probiotic is crucial for the increased gut residence time, which help in colonisation of the intestine and competitive exclusion of the pathogen (Colins *et al.*, 1998).

In terms of exhibiting beneficial effects, *W. cibaria* NM1 was found to harbour a Catabolite control protein A gene that contributes to cholesterol reduction, as reported by Lee *et al*. (2010). The study by Lee *et al*. (2010) was done through a random mutagenesis approach and further verified through *in vivo* approaches and confirmed the reduction of rat's total serum cholesterol by 20%. Recent work of Lakra *et al.* (2020) demonstrated both *W. cibaria* MD1 and MD2 extracted from fermented batter exhibited the cholesterol-reducing ability of 67.11% and 78%, respectively. In this study, no virulence and antibiotic resistance genes were found in the genome of *W. cibaria* NM1 through the RGI analysis. A similar finding on safety assessment was also reported for *W. cibaria* CH2 through genome screening of antibiotic resistance (Kumari *et al.*, 2020).

In vitro determination of antimicrobial and probiotic potential

Although *in silico* prediction approach allowed swiftness in discovering new bacteriocin producers and probiotic candidates, the efficacy of this approach relies strongly on laboratory works for verification. Hence *in vitro* experiments were conducted to verify the bacteriocin and probiotic properties of *W. cibaria* NM1. Crude bacteriocin produced by *W. cibaria* NM1 exhibited inhibitory activity against *P. aeruginosa* ATCC 10145 with MIC of 62.5 mg/mL. To the author's knowledge, the published work related to the antipseudomonal effect of bacteriocins is scarce. Only one report from Lin and Pan (2019) showed that the CFS sample from *Lactobacillus plantarum* NTU 102 exhibited a MIC of >3.35 mg/mL against *P. aeruginosa.* Nevertheless, there are a few published reports on the antimicrobial activity of the bacteriocins produced by *W. cibaria*, such as Weissellicin 110 produced by *W. cibaria* 110 have a narrow inhibitory activity against strains from the *Lactococcus* genus and related species such as *W. kandleri*, *W. halotolerans*, *W paramesenteroides* and *Leuconostoc mesenteroides* (Srionnual *et al*., 2007).

In silico study discovered that *W. cibaria* NM1 harbours a gene that contributes to tolerance toward acid and bile, and this is also proven in the *in vitro* simulated gastrointestinal assay. *Weissella cibaria* NM1 was able to tolerate bile salt, harsh gastric and small intestinal environment. Bile tolerance is an essential characteristic for probiotics in order to survive and cultivate in the proximal segment of the small intestine (De Smet *et al.*, 1995). According to Gilliland *et al.* (1984), in the selection of bile tolerant strain, 0.3% (w/v) of bile salt concentration is classified as a critical concentration that can be used. By raising the bile salt concentration, the growth of *W. cibaria* NM1 declined. In contrast, Elavarasi *et al.* (2014) reported that *W. cibaria* was able to survive more than 80% viability in 0.3, 0.5 and 1.0% (w/v) of bile salt treatment after 4 h of incubation.

Holzapfel *et al.* (1998) described the low gastric pH value and antimicrobial effects of pepsin as an active protector against bacteria entrance into the bowel tract. Similar to the finding for *W. cibaria* NM1, several studies reported that *Weissella* strain and most probiotics such as *Lactobacillus* strains are able to grow better in pH 3 compared to pH 2 (Anandharaj *et al*., 2015; Davati *et al.*, 2015). Lee *et al*. (2012) have isolated eight *Weissella* strains, including *W. cibaria* from human faeces and found that the strains showed good tolerance in pH 3 but very poor growth in pH 2 treatments with 2 hours incubation period. According to Liong and Shah (2005), tolerance towards pH 3 has become the benchmark for acidic condition tolerance of probiotics.

After successfully survived the acid condition in the stomach, the bile salt and pancreatin in the small intestine is another harmful environment that the probiotic bacteria need to tolerate in order to adhere and colonise the intestine and exhibit health benefits (Huang and Adams, 2004). *W. cibaria* NM1 show good tolerance to 0.3% bile salt and the simulated small intestinal environment. The current finding is similar to a study by Tokatlı *et al.* (2015), where the author reported that the reduction of probiotic growth in the simulated small intestine juice contained 1 mg/mL pancreatin and 0.3% (w/v) bile salt. The positive outcomes of these in vitro assays have validated the bacteriocin and probiotic potential of *W. cibaria* NM1.

CONCLUSION

This work provided an insight into the efficacy of *in silico* prediction approach in discovering new bacteriocin

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producers and probiotic candidates. *In silico* bacteriocin prediction of *W. cibaria* NM1 via NeuBI suggested that the genome harbouring a bacteriocin operon consists of potential conserved context genes with transportation, immunity, modification and regulatory function. The *W. cibaria* NM1 also exhibited probiotic-associated genes and did not harbour any virulence and antibiotic resistance gene that will cause detrimental effects to the host. Although the bacteriocin function of the WC_2064 gene required further experimental validation, this NeuBI prediction approach allowed swiftness in discovering new bacteriocin producers. We also noted the limitations of this study and the efficacy of *in silico* approach relies strongly on the genome databases available, and the laboratory works through data reconciliation or verification will be deemed essential.

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