JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF BACTERIOCINOGENIC LACTIC ACID BACTERIAL STRAINS FOR POSSIBLE BENEFICIAL, VIRULENCE, AND ANTIBIOTIC RESISTANCE TRAITS

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https://doi.org/10.55251/jmbfs.4990

ARTICLE INFO

Received 5. 7. 2021
Revised 10. 11. 2021
Accepted 12. 11. 2021
Published 1. 4. 2022

Regular article

OPEN ACCESS

ABSTRACT

Lactic acid bacteria (LAB) are a diverse group of microorganisms that can be found in natural habitats and are used in a wide range of industries, especially in the food sector. They are regarded "Generally Recognized as Safe" and are commonly used as a starter culture, probiotics, and biopreservative. However, some LAB genera are excluded from the "Qualified Presumption of Safety" (QPS) list due to the possibility that they have virulence and antibiotic resistance genetic determinants. The objective of this study was to investigate the beneficial, virulence, and antibiotic resistance-related genes in 10 well-characterized bacteriocinogenic LAB strains. The auto-aggregation, co-aggregation, cell surface hydrophobicity, and bile salt tolerance were also evaluated to examine their potential as bacteriocinogenic probiotics and/or starter culture. Results showed that all tested strains showed abilities for auto-aggregation at 4ºC and 37ºC, co-aggregation with *S. aureus* JCM8704, *S. typhimurium* BIOTECH1826, and *E. coli* DH5α, and significant cell-surface hydrophobicity. However, only a few strains were able to withstand the media treated with 0.3% bile salt. Among the tested bacteriocinogenic LAB strains, *L. lactis* IO-1 and *C. divergens* V41 had the maximum values for auto-aggregation at 4ºC and 37ºC, respectively. *C. divergens* V41 also exhibited the highest percentage for cell surface hydrophobicity. *E. faecium* NKR-5-3 showed the highest co-aggregation percentages with all indicator strains. Our findings also showed that the tested isolates presented distinct combinations of virulence-related genes. Only two of ten bacteriocinogenic LAB strains exhibited presence of multiple virulence genes. *Lactococcus* sp. QU12 was found to have a high frequency of beneficial and virulence genes, with 2 out of 7 genes present encoding beneficial factor and 11 out of 13 genes encoding virulence factor. *Lactococcus* sp. QU12 and *L. lactis* IO-1 were also positive for tetracycline resistance gene *tetM* and aminoglycoside resistance gene *aphA-2*, respectively, and transposon genes. Moreover, only a few LAB isolates tested positive for 2 out of 8 antibiotic resistance classes. Although few, the substantial danger of these genes being transferred and acquired cannot be overlooked as this could potentially cause serious health concerns in the future. These results suggest that despite the promising properties of bacteriocinogenic LAB, careful safety evaluation of these strains should be a prerequisite before using these in food systems.

Keywords: lactic acid bacteria, bacteriocins, antibiotic resistance, virulence genes, beneficial genes

INTRODUCTION

For centuries, LAB and its by-products have been extensively used in the food industry, often as starter culture, probiotics, and as biopreservative. These microorganisms perform an essential role in the preparation, preservation, and production of food products and are considered as "Generally Recognized as Safe". Many LAB strains have been used as starter cultures in food fermentations such as cheese, yoghurt, rice wine, pickled fruits & vegetables and many others. (**Vázquez-Velázquez, *et al.*, 2018, De Pasquale *et al.*, 2019, Aforijiku *et al.*, 2020, Hassen *et al.*, 2018**). LAB produces organic acids, primarily lactic acid, which promote its fast acidification. Their ability to produce acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and some enzymes contribute in the extension of shelf life, improved microbiological safety, and enhance the sensory features of the final product (**Madi & Boushaba, 2017, Sathe *et al.*, 2007, Sedaghat *et al.*, 2016, Tuntisuwanno *et al.*, 2014**).

The utility of LAB as probiotic has also gained increasing attention in recent years due to the upward trend of health-conscious population. Numerous studies have shown the positive effects of many probiotic LAB strains on the immunological response, morpho-physiological, productive, and health indices of the host. The health-promoting effects of probiotics are conferred through the balanced host microbiota, improvement of the barrier function of the gut mucosa, modulation of the host's immune response, and production of beneficial metabolites such as antimicrobial factors and enzymes among others (**Gareau *et al.*, 2010, Butel, 2014**).

Production of antimicrobial compounds, such as bacteriocins, in LAB is a highly desirable trait as it enhances its utility in different applications. Bacteriocins are ribosomally synthesized small proteins that have been shown to prevent food spoilage and inhibit growth of pathogenic microorganism (**Sukrama *et al.*, 2020**). For instance, **Retnaningrum *et al.* (2020)** showed that all crude bacteriocin-producing LAB isolates from a traditional Indonesian fermented product revealed a broad antibacterial spectrum against different Gram-positive and Gram-negative indicator bacterial strains. Isolates from the study by **Yang *et al.* (2012)** also revealed the bacteriocinogenic ability of LAB present in cheeses and yogurts which may have the potential to be used as biopreservatives in food. The use of bacteriocin-producing starter cultures could be an effective control to prevent pathogens from contaminating fermented food products upon consuming it. In a study by **Chang & Chang (2011)**, the number of *S. aureus*, *S. typhimurium*, and *E. coli* significantly reduced when the starter culture capable of producing bacteriocin was used for kimchi fermentation. Bacteriocin-producing probiotic strain *L. salivarus* DPC6005 was able to dominate over co-administered strains both in the ileum digesta and in mucosa of the host (**Walsh *et al.*, 2008**).

However, some genera of LAB, such as genera *Streptococcus* and *Enterococcus* and others containing opportunistic pathogens, are not included in the "Qualified Presumption of Safety" (QPS) list due to their susceptibility to acquiring virulence and antibiotic resistance genes and their opportunistic properties (**Mattila-Sandholm *et al.*, 1999, Dapkevicius *et al.*, 2021**). This resulted in a major issue of concern in the safety of the utilization of these microorganisms for consumption.

Consequently, using isolated LAB strains requires caution due to their potential for virulence. *Enterococci*, for example, is being used in traditional dairy fermentation such as in cheese technology but are also potentially dangerous to humans (**Hammad *et al.*, 2015, Gaglio *et al.*, 2016, Câmara *et al.*, 2020**). The virulence genes that are potentially carried by this genus can be expressed in food products, representing serious public health hazards for consumers (**Hammad *et al.*, 2015**). LAB has also been speculated to act as reservoirs of intrinsic or extrinsic antibiotic resistance genetic determinants (**Ouwehand *et al.*, 2016, Xu *et al.*, 2020, Lee *et al.*, 2021**). The main threat associated with LAB is their potential to transfer these genes to pathogenic bacteria which can result in multidrug-resistant pathogens (**Perin *et al.*, 2014**). Thus, antimicrobials for these pathogens will become ineffective and these microorganisms will be able to survive and transfer these genes to other microorganisms (**Álvarez-Cisneros & Ponce-Alquicira, 2018**).
Despite the interest of the food sector in using LAB, it is still necessary to assess its safety. Within this context, the purpose of the present study was to investigate 10 well-characterized bacteriocinogenic LAB strains for beneficial, virulence, and antibiotic resistance traits to ensure the safety and know the limitations of their use as potential probiotics and/or starter culture in food fermentations. These LAB strains were also tested for auto-aggregation, co-aggregation, cell surface hydrophobicity, and bile salt tolerance to examine their potential as bacteriocinogenic probiotics and/or starter culture.

MATERIALS AND METHODS

Bacterial strains and reagents

The strains used in this study are summarized in Table 1. All LAB strains were cultivated in MRS medium (Oxoid, UK) and incubated at 30ºC for 24h. Non-LAB strains used for co-aggregation assay were cultivated in Tryptic Soy Broth (Titan Media Ltd., India), supplemented with 0.6% Yeast Extract (Titan Media Ltd., India) (TSBYE), and incubated at 30ºC for 24h. Stock cultures of all bacterial strains were prepared in their actively growing state in MRS or TSBYE medium supplemented with 30% glycerol and stored at −80°C. The strains were reactivated by cultivating them twice prior to every use.

Table 1 Bacterial strains used in this study

Strain or plasmid	Description	Reference or source[a,b,c]
Strains		
Enterococcus faecium NKR-5-3	enterocin NKR-5-3B producer strain	**(Ishibashi *et al.*, 2012)**
Lactococcus lactis QU5	lacticin Q producer strain	**(Fujita *et al.*, 2007)**
L. lactis QU 7	lactococcin Z producer strain	**(Ishibashi *et al.*, 2015)**
L. lactis QU 14	lacticin Z producer strain	**(Iwatani *et al.*, 2007)**
Lactococcus sp. QU12	lactocyclicin Q producer strain	**(Sawa *et al.*, 2009)**
L. lactis IO-1	nisin Z producer strain	**(Matsusaki *et al.*, 1998)**
L. lactis NCDO 497	Nisin A producer strain	**NCDO[a]**
Leuconostoc mesenteroides TK41401	leucocyclicin Q producer strain	(**Masuda *et al.*, 2011)**
Carnobacterium divergens V41	divercin V41 producer strain	**(Métivier *et al.*, 1998)**
Enterococcus sp. M4-3	enterocin W producer strain	**(Sawa *et al.*, 2012)**
Staphylococcus aureus JCM8704	Indicator strain for co-aggregation assay	**JCM[b]**
Salmonella typhimurium BIOTECH1826	Indicator strain for co-aggregation assay	**PNCM[c]**
Escherichia coli DH5α	Indicator strain for co-aggregation assay	**Novagen**

[a] NCDO, National Collection of Food Bacteria, Reading, UK.
[b] JCM, Japan Collection of Microorganisms, RIKEN, Tsukuba, Japan.
[c] PNCM, Philippine National Collection of Microorganisms, UPLB, Philippines.

Aggregation Assay

The aggregation abilities of bacteriocinogenic LAB strains were evaluated according to the modified method reported by **Todorov *et al.* (2011)**. The strains were grown at 37°C for 18h in MRS broth. The cultures were harvested by centrifugation at 6000 × *g* for 10 min, washed, and resuspended using 2 mL phosphate buffer saline (PBS) to an OD_{600nm} of 0.3 to 0.5. Then, samples were incubated at 4°C, to resemble the refrigerated probiotic temperature condition, and 37°C, to simulate the normal human body temperature, for 60 min. The cell suspension was harvested by centrifugation (300 × *g* for 2 min) and OD_{600nm} of supernatant was determined. Auto-aggregation was calculated as follows: % auto-aggregation = $[(OD_0\text{-}OD_{60})/ OD_0\text{-}] \times 100$, wherein OD_0 represents the initial optical density and OD_{60} represents the absorbance after 60 min.
The bacterial suspensions for co-aggregation were prepared in the same way as auto-aggregation. The LAB strains were cultivated in MRS broth and the indicator strains *S. aureus* JCM8704, *S. typhimurium* BIOTECH1826, and *E.coli* DH5α were cultivated in TSBYE at 37°C for 18h. The cultures were harvested by centrifugation at 6000 × *g* for 10 min, washed, and resuspended in phosphate buffer saline (PBS) to obtain an optical density of 0.3 to 0.5. The degree of co-aggregation ability was determined by reading the absorbance values of paired 500 µL LAB cells and 500 µL co-aggregation partner cells in a sterile plastic cuvette. The cell suspension of each LAB strain and indicator strain was incubated at 37°C for 60 min. Cells were harvested by centrifugation (300 × *g* for 2 min) and the OD_{600nm} of supernatant was determined. The co-aggregation rates were calculated as follows: % co-aggregation = $[(OD_0\text{-}OD_{60})/ OD_0\text{-}] \times 100$, wherein OD_0 represents the initial optical density and OD_{60} represents the absorbance after 60 min.

Cell Surface Hydrophobicity Assay

The cell surface hydrophobicity tests were performed according to **Todorov and Dicks (2008)** with few modifications. The strains were cultivated in MRS broth at 37°C for 18h. The cultures were harvested by centrifugation at 6000 × *g* for 10 min, washed, and resuspended in phosphate buffer saline (PBS) to an OD_{600nm} of 0.3 to 0.5. Cell suspension (1.5 mL) was added to 0.5 mL of 99.9% hexadecane (Sigma Aldrich, USA) and vortexed for 2 min. The mixture was left at room temperature for 30 min to allow phase stabilization. Then, an aliquot of 1.5 mL of the aqueous phase was taken to measure the OD_{600nm}. The cell surface hydrophobicity was calculated as follows: % hydrophobicity = $[(OD_0\text{-}OD_{30})/ OD_0\text{-}] \times 100$, wherein OD_0 represents the initial optical density and OD_{30} represents the absorbance after 30 min.

Bile Salt Tolerance Assay

The bile tolerance of bacteriocinogenic LAB strains were tested using the modified methods described by **Nami *et al.* (2019)**.The strains were grown in MRS broth with and without 0.3% bile salt at 37°C for 8h. The optical density at 600 nm was recorded every 2h (2h, 4h, 6h, and 8h). The bile salt tolerance was calculated as follows: % bile salt $\text{tolerance}_t = (OD_{\text{bile salt}}/OD_{\text{control}}) \times 100$, wherein $OD_{\text{bile salt}}$ represents the optical density of samples with 0.3% bile salt and OD_{control} represents the absorbance of the control, at t=2h, 4h, 6h, and 8h.

Extraction of Bacterial Genomic DNA

The genomic DNA of the LAB strains was extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's protocol. The genomic DNA concentration was measured using NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA) and standardized to 10 ng/µL using sterile ultrapure water before it was stored at -20°C.

Genetic Profiling of Bacterial Strains

PCR amplification was performed in a 20 µL reaction mixture using EmeraldAmp MAX HS PCR Master Mix (TaKaRa Biotechnology, Japan), added with 0.5 ng of DNA, and 0.2 µM of each primer. Amplifications were carried out using MultiGene Gradient Thermal Cycler (Labnet International, USA). Thermocycler amplification reactions were set as follows: an initial cycle of 98°C for 2 min, 30 cycles consisting of denaturation of 98°C for 10 seconds, annealing at an appropriate temperature for 30 seconds (Table 2 & 3), and elongation of 72°C for 2 min, then a final extension step at 72°C for 2 min. The annealing temperatures used were based on the Tm values of each primer set. The amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels in 0.5× TAE buffer with ViSafe Red Gel Stain (Vivantis Technologies, Malaysia). The primers used in this study are summarized in Tables 2 and 3.

Table 2 PCR Primers used in this study to detect beneficial and virulence genetic determinants from the bacteriocinogenic LAB strains

Category	Primer Name	Primer Sequence (5' → 3')	Amplicon Size (bp)	Annealing Temp (°C)	References
Beneficial	*mub*	F-GTAGTTACTCAGTGACGATCAATG R-TAATTGTAAAGGTATAATCGGAGG	150	50	**Ramiah *et al*., 2007**
	map	F-TAATTGTAAAGGTATAATCGGAGG R-TGGATTCTGCTTGAGGTAAG	156	52	**Ramiah *et al*., 2007**
	EFTu	F-GACTAGTAATAACGCGACCG R-TTCTGGTCGTATCGATCGTG	161	55	**Ramiah *et al*., 2007**
	prgB	F-CCACGTAATAACGCACCAAC R-GCCGTCGACTCGAGGAGAATGATACATGAAT	3917	64	**Kao *et al*., 1991**
	EF2662-cbp	F-CCTGCGGCCGCGTCCTTCTTTTCGTCTTCAA R-GGCGTCGACCACTTAAACTGATAGAGAGGAAT	1121	59	**Fortina *et al*., 2008**
	EF1249-fbp	F-GCGGTCGACAAACGAGGGATTTATTATG R-CTGGCGGCCGCGTTTAATACAATTAGGAAGCAGA	1712	61	**Fortina *et al*., 2008**
	EF2380-maz	F-GCGGTCGACGACATCTATGAAAACAAT R-TCCGCGCCGCCTTAAACTTTCTCCTT	1268	60	**Fortina *et al*., 2008**
Virulence	*gelE*	F-TATGACAATGCTTTTTGGGAT R-AGATGCACCCGAAATAATATA	213	49	**Todorov *et al*., 2017**
	hyl	F-ACAGAAGAGCTGCAGGAAATG R-GACTGACGTCCAAGTTTCCAA	276	56	**Todorov *et al*., 2017**
	asa1	F-GCACGCTATTACGAACTATGA R-TAAGAAAGAACATCACCACGA	375	52	**Todorov *et al*., 2017**
	esp	F-AGATTTCATCTTTGATTCTTG R-AATTGATTCTTTAGCATCTGG	510	46	**Todorov *et al*., 2017**
	cylA	F-ACTCGGGGATTGATAGGC R-GCTGCTAAAGCTGCGCTT	688	55	**Todorov *et al*., 2017**
	efaA	F-GCCAATTGGGACAGACCCTC R-CGCCTTCTGTTCCTTCTTTGGC	688	60	**Todorov *et al*., 2017**
	ace	F-GAATTGAGCAAAAGTTCAATCG R-GTCTGTCTTTTCACTTGTTTC	1008	50	**Todorov *et al*., 2017**
	ccf	F-GGGAATTGAGTAGTGAAGAAG R-AGCCGCTAAAATCGGTAAAAT	543	51	**Todorov *et al*., 2017**
	cob	F-AACATTCAGCAAACAAAGC R-TTGTCATAAAGAGTGGTCAT	1405	49	**Todorov *et al*., 2017**
	cpd	F-TGGTGGGTTATTTTTCAATTC R-TACGGCTCTGGCTTACTA	782	49	**Todorov *et al*., 2017**
	int	F-GCGTGATTGTATCTCACT R-GACGCTCCTGTTGCTTCT	1028	49	**Todorov *et al*., 2017**
	int-Tn	F-TGACACTCTGCCAGCTTTAC R-CCATAGGAACTTGACGTTCG	579	54	**De Barbeyrac *et al*., 1996**
	sprE	F-TTGAGCTCCGTTCCTGCCGAAAGTCATTC R-TTGGTACCGATTGGGGAACCAGATTGACC	591	65	**Todorov *et al*., 2017**

Table 3 PCR primers used in this study to detect antimicrobial resistance genetic determinants from the bacteriocinogenic LAB strains

Antimicrobial Drug Class	Primer Name	Primer Sequence (5' → 3')	Amplicon Size (bp)	Annealing Temp (°C)	References
Aminoglycosides	*aphA-1*	F-ATGGGCTCGCGATAATGTC R-CTCACCGAGGCAGTTCCAT	600	56	**Sáenz *et al*., 2004**
	aphA-2	F-GAACAAGATGGATTGCACGC R-GCTCTTCAGCAATATCACGG	680	54	**Sáenz *et al*., 2004**
	aphA-3	F-GGGGTACCTTTAAATACTGTAG R-TCTGGATCCTAAAACAATTCATCC	848	50	**Poyart *et al*., 2003**
	aadA1/2	F-GCAGCGCAATGACATTCTTG R-ATCCTTCGGCGCGATTTTG	282	56	**Sáenz *et al*., 2004**
	aad6	F-AGAAGATGTAATAATATAG R-CTGTAATCACTGTTCCCGCCT	978	36	**Poyart *et al*., 2003**
β-Lactams	*blaIMP*	F-CTACCGCAGCAGAGTCTTTG R-AACCAGTTTTGCCTTACCAT	587	53	**Sundsfjord *et al*., 2004**
	blaSHV	F-ATGCGTTATATTCGCCTGTG R-TTAGCGTTGCCAGTGCTCGA	860	54	**Sundsfjord *et al*., 2004**
	blaSPM-1	F-CCTACAATCTAACGGCGACC R-TCGCCGTGTCCAGGTATAAC	649	56	**Sundsfjord *et al*., 2004**
	blaTEM	F-ATGAGTATTCAACATTTTCGTG R-TTACCAATGCTTAATCAGTGAG	860	49	**Sundsfjord *et al*., 2004**
	blaVIM	F-ATTCCGGTCGGAGAGGTCCG R-GAGCAAGTCTAGACCGCCCG	633	62	**Sundsfjord *et al*., 2004**
	mecA	F-TGGCTATCGTGTCACAATCG R-CTGGAACTTGTTGAGCAGAG	310	54	**Sundsfjord *et al*., 2004**
Macrolides	*ermA/TR*	F-TCAGGAAAAGGACATTTTACC R-ATACTTTTTGTAGTCCTTCTT	432	46	**Sutcliffe *et al*., 1996**
	ermB	F-GAAAAGGTACTCAACCAAATA R-AGTAACGGTACTTAAATTGTTTAC	639	48	**Sutcliffe *et al*., 1996**
	ermC	F-TCAAAACATAATATAGATAAA R-GCTAATATTGTTTAAATCGTCAA	642	38	**Sutcliffe *et al*., 1996**
	mdfA/mefE	F-AGTATCATTAATCACTAGTGC R-TTCTTCTGGTACTAAAAGTGG	348	47	**Sutcliffe *et al*., 1996**
Quinolones	*gyrA*	F-TTCTCCGATTTCCTCATG R-AGAAGGGTACGAATGTGG	458	49	**de Toro *et al*., 2010**

	parC	F-TGGGTTGAAGCCGGTTCA R-CAAGACCGTTGGTTCTTTC	361	52	**de Toro *et al*., 2010**
Streptomycin	*rpsL*	F-GGCCGACAAACAGAACGT R-GTTCACCAACTGGGTGAC	501	54	**Sreevatsan *et al*., 1996**
	rrs	F-GAGAGTTTGATCCTGGCTCAG R-TGCACACAGGCCACAAGGGA	1042	56	**Sreevatsan *et al*., 1996**
Sulfonamides	*sul1*	F-TGGTGACGGTGTTCGGCATTC R-GCGAGGGTTTCCGAGAAGGTG	789	62	**Sáenz *et al*., 2004**
	sul2	F-CGGCATCGTCAACATAACC R-GTGTGCGGATGAAGTCAG	722	54	**Sáenz *et al*., 2004**
	sul3	F-CATTCTAGAAAACAGTCGTAGTTCG R-CATCTGCAGCTAACCTAGGGCTTTGGA	990	54	**Sáenz *et al*., 2004**
Tetracyclines	*tetA*	F-GTAATTCTGAGCACTGTCGC R-CTGCCTGGACAACATTGCTT	937	54	**Sáenz *et al*., 2004**
	tetB	F-CTCAGTATTCCAAGCCTTTG R-CTAAGCACTTGTCTCCTGTT	416	51	**Sáenz *et al*., 2004**
	tetC	F-TCTAACAATGCGCTCATCGT R-GGTTGAAGGCTCTCAAGGGC	570	55	**Sáenz *et al*., 2004**
	tetD	F-ATTACACTGCTGGACGCGAT R-CTGATCAGCAGACAGATTGC	1104	54	**Sáenz *et al*., 2004**
	tetE	F-GTGATGATGGCACTGGTCAT R-CTCTGCTGTACATCGCTCTT	1179	55	**Sáenz *et al*., 2004**
	tetK	F-GTAGGATCTGCTGCATTCCC R-CACTATTACCTATTGTCGC	552	48	**Poyart *et al*., 2003**
	tetL	F-GGATCGATAGTAGCCATGGG R-GTATCCCACCAATGTAGCCG	516	56	**Poyart *et al*., 2003**
	tetM	F-GTGGAGTACTACATTTACGAG R-GAAGCGGATCACTATCTGAG	359	50	**Poyart *et al*., 2003**
	tetO	F-GCGGAACATTGCATTTGAGGG R-CTCTATGGACAACCCGACAGAAG	538	58	**Poyart *et al*., 2003**
Lincosamides	*linB*	F-CCTACCTATTGTTTGTGGAA R-ATAACGTTACTCTCCTATTC	-	46	**Bozdogan *et al*., 1999**

RESULTS AND DISCUSSION

Beneficial and Virulence Genotyping

The incidence of beneficial and virulence factors among tested bacteriocinogenic LAB isolates is shown in Table 4. Results from PCR amplification revealed that several isolates carried beneficial genes coded for choline-binding protein (*EF2662-cbp*, 1 out of 10), fibrinogen binding protein (*EF1249-fbp*, 1/10), and virulence genes coded for production of gelatinase (*gelE*, 1/10), aggregation substance (*asa1*, 1/10), enterococcal surface protein (*esp*, 1/10), cytolisin (*cylA*, 1/10), endocarditis antigen (*efaA*, 2/10), adhesion of collagen (*ace*, 1/10), sex pheromone (*ccf*, 2/10 and *cob*, 2/10), transposon (*int*, 2/10 and *int-Tn*, 1/10) and serine protease (*sprE*, 2/10). Only one strain (*Lactococcus* sp. QU12) from the tested LAB was positive for beneficial genes (*EF2662-cbp* and *EF1249-fbp*) and only four strains (*L. lactis* IO-1, *Lactococcus* sp. QU12, *Enterococcus* spp. M4-3, *L. lactis* QU 14) presented virulence genes. *C. divergens* V41, *L. mesenteroides* TK41401, *L. lactis* NCDO 497, *L. lactis* QU5, *E. faecium* NKR-5-3, and *L. lactis* QU 7 were negative for both beneficial and virulence genetic determinants. A high frequency of incidence of beneficial and virulence genes was observed for *Lactococcus* sp. QU12, which was positive for 2 out of 7 genes encoding beneficial factor and 11 out of 13 genes encoding virulence factor.

The genes encoding for endocarditis antigen *efaA*, sex pheromone *ccf*, and serine protease *sprE* were present in both *Lactococcus* sp. QU12 and *Enterococcus* spp. M4-3. *efaA* plays a critical role in the adhesion of bacteria to surfaces and biofilm formation of *Enterococci* spp, one of the main causes of nosocomial infections (**Kafil *et al.*, 2016, Narenji *et al.*, 2020**). Results from research conducted by **Domingos-Lopes *et al.* (2017)** showed that 99% of their *Enterococci* isolates and all *Lactococcus* isolates were positive for *efaA*. Sex pheromone peptides are also very common in *Enterococci* (**Eaton & Gasson, 2001, Choho *et al.*, 2008, Valenzuela *et al.*, 2009**). These are thought to play a role in inducing an inflammatory response as well. Sex pheromones have a chemotactic effect on human leukocytes and facilitate conjugation. Strains with sex pheromone genes have a chance of acquiring the corresponding sex pheromone plasmids and, as a result, the related virulence determinants can also be acquired (**Eaton & Gasson, 2001**). The *sprE* together with *gelE*, especially in strains of *E. faecalis*, plays a crucial role in inciting diseases in humans and animals as well (**Shin *et al.*, 2007, Wu *et al.*, 2007, Chávez de Paz *et al.*, 2015**). The presence of *sprE* in LAB has also been reported in a study by **Arbulu *et al.* (2016)**.

Screening for beneficial genes can be considered as a preliminary step in evaluating possible strains for probiotics. The detection of virulence genes can be used as well to further assess the strains for their future application. Strains that presented virulence-related factors may be eliminated from the screening to avoid the use of potentially hazardous strains. Here, the tested bacteriocinogenic LAB strains presented virulence genes. However, the presence of specific genes does not imply that they will be expressed and that the strain holding this genetic material would guarantee its virulence (**de Castilho *et al.*, 2019**). According to **Perin *et al.* (2014)**, different virulence factors can be found in *Lactococcus* spp., however, their presence in the genome is not a proper indicator of their pathogenicity. Distinct combinations of virulence-related genes were present in their examined isolates, but not necessarily their expression. According to **Lopes *et al.* (2006)**, the presence of the *gelE* gene is not sufficient for gelatinase activity, given the complete *fsr* operon appears to be essential for the expression of its virulence. The *fsr* operon, on the other hand, appears to be easily damaged, lost, or subjected to deletions, mainly during the freezing of cells in the laboratory. Thus, *gelE* expression is significantly influenced by culture conditions, and laboratory manipulations of strains can lead to the loss of structural genes, which can also explain how gelatinase activity was lost during in vitro tests (**Eaton & Gasson, 2001**). Based on these considerations, it is always necessary to address and evaluate the presence of these beneficial and virulence genetic determinants in a more comprehensive manner, taking into account the future applications of these strains for specific use.

Antibiotic Resistance Genotyping

The prevalence of antibiotic resistance genes among 10 bacteriocinogenic LAB strains is shown in Table 5. Results showed that few LAB strains were positive to 2 out of 8 classes of antibiotic resistance. *Lactococcus* sp. QU12 indicated the presence of *tetM*, coding for tetracycline resistance. Antimicrobials such as tetracycline are commonly used, putting pressure on LAB to acquire antimicrobial resistance determinants (**Rao Thumu & Halami, 2012**). Tetracycline is an antibiotic that often inhibits protein synthesis (**Todorov *et al.*, 2017**). The presence of tetracycline resistance genes in *Lactococcus* was also reported in previous researches (**Flórez *et al.*, 2008, Zycka-Krzesinska *et al.*, 2015**). Moreover, three out of 10 strains (*L. lactis* IO-1, *L. lactis* NCDO 497, and *L. lactis* QU 7) were positive for *aphA-2*, which encodes for aminoglycoside resistance. Aminoglycosides such as neomycin, kanamycin, streptomycin, gentamicin, often interfere with the synthesis of protein (**Francis *et al.*, 2013, Pacifici & Marchini, 2017, Sharma *et al.*, 2016**). This inhibition mechanism was achieved by binding to the A-site on the 30S ribosome's 16S ribosomal RNA with high affinity (**Krause *et al.*, 2016)**. *Lactococci* isolates have also been reported to be resistant to aminoglycosides based on previous studies (**Rodríguez-Alonso *et al.*, 2009, Aditi *et al.*, 2017**).

In the present study, transposon genes *int* and *int-Tn* and tetracycline resistance gene *tetM* were detected in *Lactococcus* sp. QU12. *L. lactis* IO-1 also presented *int* and aminoglycoside resistance gene *aphA-2*. **Boguslawska *et al.* (2009)** investigated the capacity of tetraclycline-resistant *L. lactis* strains to transfer its antibiotic resistance genetic determinants. Results showed that several strains were able to transfer resistance genes in vitro to *L. lactis* Bu2-60 and *E. faecalis* JH2-2. The *tetM* gene was shown to be identical to the *tetM* gene found on the conjugative transposon Tn*916* after molecular study of *in vivo* transconjugants carrying the gene.

Table 4 Presence of genes related to beneficial and virulence factors in bacteriocinogenic lactic acid bacterial strains and its gene description

Category	Gene	Gene Description	Strains									
			V41	IO-1	TK41401	NCDO 497	QU5	NKR-5-3	QU7	QU12	M4-3	QU14
Beneficial	*mub*	Adhesion	-	-	-	-	-	-	-	-	-	-
	map	Adhesion	-	-	-	-	-	-	-	-	-	-
	EFTu	Adhesion	-	-	-	-	-	-	-	-	-	-
	prgB	Surface Protein	-	-	-	-	-	-	-	-	-	-
	EF2662-cbp	Choline-binding protein	-	-	-	-	-	-	-	+	-	-
	EF1249-fbp	Fibrinogen-binding protein	-	-	-	-	-	-	-	+	-	-
	EF2380-maz	Membrane-associated zinc metalloprotease	-	-	-	-	-	-	-	-	-	-
	gelE	Gelatinase	-	-	-	-	-	-	-	+	-	-
	hyl	Hyaluronidase	-	-	-	-	-	-	-	-	-	-
	asa1	Aggregation substance	-	-	-	-	-	-	-	+	-	-
	esp	Enterococcal surface protein	-	-	-	-	-	-	-	+	-	-
	cylA	Cytolisin	-	-	-	-	-	-	-	+	-	-
	efaA	Endocarditis antigen	-	-	-	-	-	-	-	+	+	-
	ace	Adhesion of collagen	-	-	-	-	-	-	-	+	-	-
Virulence	*ccf*	Related to sex pheromones	-	-	-	-	-	-	-	+	+	-
	cob	Related to sex pheromones	-	-	-	-	-	-	-	+	-	+
	cpd	Related to sex pheromones	-	-	-	-	-	-	-	-	-	-
	int	Transposon	-	+	-	-	-	-	-	+	-	-
	int-Tn	Transposon	-	-	-	-	-	-	-	+	-	-
	sprE	Serine protease	-	-	-	-	-	-	-	+	+	-

Transposons are a group of mobile genetic elements. They are known as jumping genes because they can jump into different parts of the genome. Bacterial transposons are members of the DNA transposons and Tn family, which commonly carry antibiotic resistance genes. Transposons can move from one plasmid to another or from a DNA chromosome to a plasmid and vice versa, causing the spread of genes coding for antibiotic resistance in bacteria (**Babakhani & Oloomi, 2018**). As a result, it will be more difficult to treat bacterial infectious diseases since antibiotics for these microorganisms will become ineffective and these pathogens will be able to live and transfer these genes to other pathogenic microorganisms (**Álvarez-Cisneros & Ponce-Alquicira, 2018**). Thus, contamination of combined transposons and other virulence and antibiotic resistance genes limits the utility of several bacteriocinogenic LAB strains, such as *L. lactis* IO-1 and *Lactococcus* sp. QU12. This suggests that the investigated LAB strains cannot be directly used in the food industry.

Table 5 Presence of genes related to antibiotic resistance factors in bacteriocinogenic lactic acid bacterial strains

Class	Gene	Strains									
		V41	IO-1	TK4101	NCDO 497	QU5	NKR-5-3	QU7	QU12	M4-3	QU14
Aminoglycosides	*aphA-1*	-	-	-	-	-	-	-	-	-	-
	aphA-2	-	+	-	+	-	-	+	-	-	-
	aphA-3	-	-	-	-	-	-	-	-	-	-
	aadA1/2	-	-	-	-	-	-	-	-	-	-
	aad6	-	-	-	-	-	-	-	-	-	-
B-Lactams	*blaIMP*	-	-	-	-	-	-	-	-	-	-
	blaSHV	-	-	-	-	-	-	-	-	-	-
	blaSPM-1	-	-	-	-	-	-	-	-	-	-
	blaTEM										
	blaVIM	-	-	-	-	-	-	-	-	-	-
	mecA	-	-	-	-	-	-	-	-	-	-
Macrolides	*ermA/TR*	-	-	-	-	-	-	-	-	-	-
	ermB	-	-	-	-	-	-	-	-	-	-
	ermC	-	-	-	-	-	-	-	-	-	-
	mdfA/mefE	-	-	-	-	-	-	-	-	-	-
Quinolones	*gyrA*	-	-	-	-	-	-	-	-	-	-
	parC	-	-	-	-	-	-	-	-	-	-
Streptomycin	*rpsL*	-	-	-	-	-	-	-	-	-	-
	rrs	-	-	-	-	-	-	-	-	-	-
Sulfonamides	*sul1*	-	-	-	-	-	-	-	-	-	-
	sul2	-	-	-	-	-	-	-	-	-	-
	sul3	-	-	-	-	-	-	-	-	-	-
Tetracyclines	*tetA*	-	-	-	-	-	-	-	-	-	-
	tetB	-	-	-	-	-	-	-	-	-	-
	tetC	-	-	-	-	-	-	-	-	-	-
	tetD	-	-	-	-	-	-	-	-	-	-
	tetE	-	-	-	-	-	-	-	-	-	-
	tetK	-	-	-	-	-	-	-	-	-	-
	tetL	-	-	-	-	-	-	-	-	-	-
	tetM	-	-	-	-	-	-	-	+	-	-
	tetO	-	-	-	-	-	-	-	-	-	-
Lincosamides	*linB*	-	-	-	-	-	-	-	-	-	-

Safety assessment of using these strains especially in food products should be exercised due to the risk that they may serve as a reservoir of antibiotic resistance genetic determinants in the human microbiome. These LAB strains may also have the potential to spread and transfer these genes to other LAB strains and pathogens, hence causing serious health hazards, particularly for consumers.

Although *L. lactis* IO-1 and *Lactococcus* sp. QU12 strains carry combined potential virulence traits, such as transposon genes, and antibiotic resistance genes, these strains can be subjected to genomic editing (i.e. CRISPR-Cas9 technology) to manipulate these potentially dangerous genes present. CRISPR (clustered regular interspaced short palindromic repeats) - Cas (CRISPR-associated) systems provide powerful tools to perform genetic manipulations such as gene deletion, insertion, and point mutation. The CRISPR/Cas system is an important part of the adaptive immunity system that protects bacteria and archaea from mobile genetic elements (**Mei *et al.*, 2016, Peters *et al.*, 2017**). To date, CRISPR-Cas9 is creating a technological breakthrough in gene editing of numerous LAB species (**Oh & Van Pijkeren, 2014, Huang *et al.*, 2019**). **Song *et al.*** (**2020**) recently developed a RecE/T-assisted CRISPR genome editing toolbox for *Lactobacillus plantarum* WCFS1 and *Lactobacillus brevis* ATCC367. This technology, as demonstrated by **Song *et al.*** (**2020**), is an effective genome editing tool that can be easily deployed in *Lactobacilli*.

Aggregation Properties

The aggregation ability of LAB is one of its desirable properties. Auto-aggregation is important for probiotic LAB strains since it is correlated with the adherence capability to epithelial cells (**Somashekaraiah *et al.*, 2019, Kong *et al.*, 2020, Reuben *et al.*, 2020**). LAB competes with pathogens by preventing them to adhere to the binding sites of intestinal epithelial cells, thus reducing its colonization (**Campana *et al.*, 2017, Srisesharam *et al.*, 2018**). In our study, the auto-aggregation and co-aggregation capacities of these 10 bacteriocinogenic LAB strains were measured (Figures 1 & 2). The results indicated that each strain can auto-aggregate and the OD increased after 60 min incubation at 4°C and 37°C. Among these LAB strains, *L. lactis* IO-1 showed the highest auto-aggregation percentage of 53.8 ± 9.54% at 4°C after 60 min. The results in our are in agreement with that of **Kazancıgil *et al.*** (**2019**), which showed that compared to other strains tested, *L. lactis* NTH10 and *L. lactis* NTH7 had higher auto-aggregation abilities. **Kong *et al.*** (**2020**) also confirmed that *L. lactis* L19 exhibits good auto-aggregation property compared with other isolates. Although there were no reported data on the auto-aggregation *C. divergens* V41, results showed that among other strains, it had the highest auto-aggregation percentage of 37.9 ± 2.81% at 37°C after 60 min. Another mechanism of surviving the adverse conditions of the intestinal tract is through co-aggregation or the accumulation of bacteria of different species. This prevents intestinal surface colonization of pathogenic microorganisms (**Campana *et al.*, 2017**). The beneficial LAB strain produces antimicrobial substances, thus destroying or eliminating the pathogens (**Srisesharam *et al.*, 2018**). Co-aggregation of these 10 bacteriocinogenic LAB strains was tested using three different indicator strains after 60 min. All the tested LAB strains showed capacities for aggregation with the tested pathogenic bacteria (Figures 2). Compared to the tested strains, *E. faecium* NKR-5-3 showed the maximum co-aggregation percentages with *S. aureus* JCM8704 (72.9 ± 0.13%), *S. typhimurium* BIOTECH1826 (72.4 ± 4.93%), and *E.coli* DH5α (75.9 ± 0.17%). It is a positive feature of *E. faecium* NKR-5-3 since its co-aggregation to indicator strains may aid in the elimination of these pathogenic microorganisms from the intestinal tract (**de Souza *et al.*, 2019**). The results obtained in this study are in agreement with those of **Arellano-Ayala *et al.*** (**2020**), who reported high co-aggregation of *E. faecium* Col1-1C against *S. typhimurium* and *E. coli*. **dos Santos *et al.*** (**2015**) also observed high levels of co-aggregation with *E. coli* INCQS 00033 of *E. faecium* EM485 (78 ± 2%) and *E. faecium* EM925 (74 ± 4%).

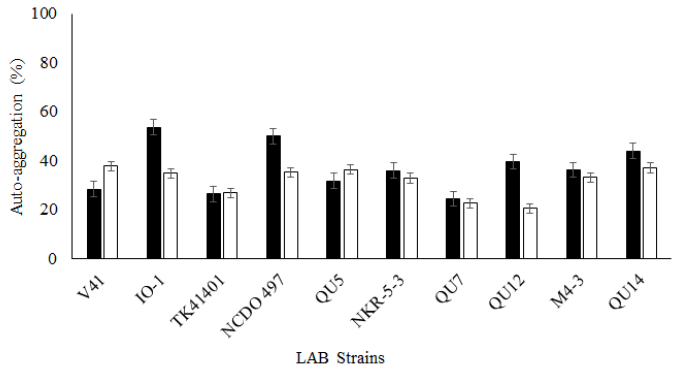

Figure 1 Percentage auto-aggregation of bacteriocinogenic lactic acid bacterial isolates at 4°C (black) and at 37°C (white). Data represented as means from 2 replicates ± standard deviations.

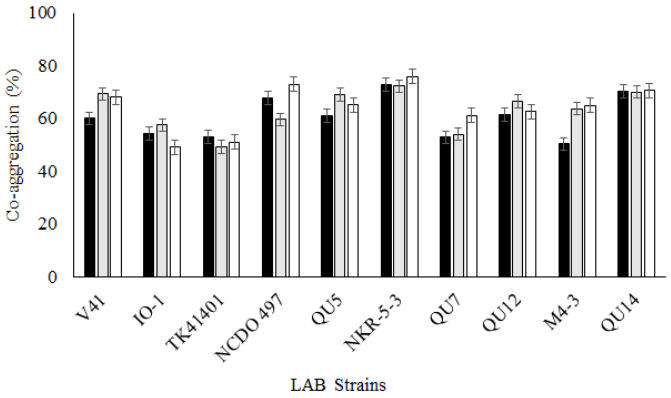

Figure 2 Percentage co-aggregation of bacteriocinogenic lactic acid bacterial isolates with *S. aureus* JCM8704 (black), *S. typhimurium* BIOTECH1826 (gray) and *E. coli* DH5α (white). Data represented as means from replicates ± standard deviations.

Cell Surface Hydrophobicity

One of the physicochemical features that can enable the first contact between microorganisms and the intestinal wall cells of the host is the cell surface hydrophobicity. The hydrophobicity abilities of a bacteria determine its capacity to adhere to intestinal mucosa cells (**de Souza *et al.*, 2019**). To colonize the gastrointestinal tract, probiotic strains must attach to the intestinal epithelium. This feature is usually linked to cell adhesion, which can influence the aggregation and adhesion of bacteria to different surfaces (**Dlamini *et al.*, 2019**).

Here, the cell surface hydrophobicity of these 10 bacteriocinogenic LAB strains was measured (Figure 3). Among these strains, *C. divergens* V41 showed the highest cell surface hydrophobicity percentage of 43.5 ± 2.53% and *L. mesenteroides* TK41401 showed the lowest percentage of 4.98 ± 9.92%. This is in contrast with the results of the study conducted by **Xu *et al.*** (**2009**), which showed that *L. mesenteroides* is one of the most hydrophobic strains among others. High levels of hydrophobicity in *L. mesenteroides* subsp. *mesenteroides* SJRP58 was also observed in a study by **Jeronymo-Ceneviva *et al.*** (**2014**).

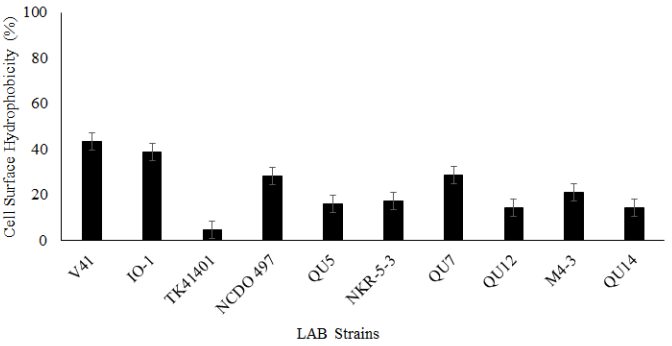

Figure 3 Percentage cell surface hydrophobicity of bacteriocinogenic lactic acid bacterial isolates. Data represented as means from 2 replicates ± standard deviations.

Bile Salt Tolerance

Another essential factor in niche adaptation is the ability to withstand stressful environments, such as those found in the human gut (**Fontana *et al.*, 2019**). Bile salt tolerance is one of the most important characteristics of LAB to be able to survive and colonize in the human intestine. This feature of LAB measures the survival and performance of probiotic strains in exerting their beneficial functions in the host (**Fortina *et al.*, 2008**). Probiotics must be able to survive passage through the stomach and small intestine because they are usually taken orally (**Shehata *et al.*, 2016**).

The bile salt tolerance of 10 bacteriocinogenic LAB strains was investigated (Figure 4). The results indicated that the tolerance to bile salt of these LAB strains decreases over time. Different strains had the highest bile salt tolerance percentage every 2h. *E.faecium* NKR-5-3, *L. mesenteroides* TK41401, *C. divergens* V41, and *L. lactis* QU14 had the maximum bile salt tolerance of 89.4 ± 12.5% at 2h, 45.9 ± 4.09% at 4h, 5.62 ± 2.41% at 6h, and 3.64 ± 1.19% at 8h respectively. Only a few LAB strains were able to survive in the media with 0.3% bile salt. To further enhance the bile salt tolerance, **Gou *et al.*** (**2021**) studied the effects of soybean lecithin and whey protein concentrate (WPC) 80 on the tolerance to bile salt of *Lacticaseibacillus paracasei* L9. Results showed that the optimized method improved the survival rate of *L. paracasei* treated with 0.3% bile salt. This method

can be applied to other LAB strains, such as these 10 bacteriocinogenic LAB strains used in the present study, to improve the tolerance of LAB to gastrointestinal stress.

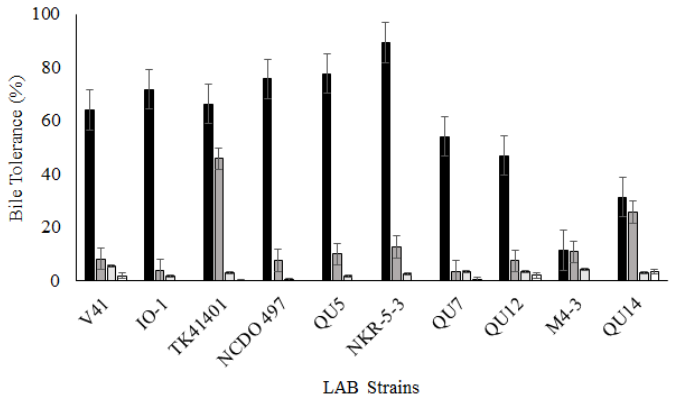

Figure 4 Percentage bile salt tolerance of bacteriocinogenic lactic acid bacterial isolates at 2h (black), 4h (dark gray), 6h (light gray), and 8h (white). Data represented as means from 2 replicates ± standard deviations.

CONCLUSION

In conclusion, we have identified the presence of beneficial, virulence, and antimicrobial resistance genetic determinants in 10 well-characterized bacteriocinogenic LAB strains. The aggregation, cell surface hydrophobicity, and bile salt tolerance abilities were evaluated as well to examine the potential of these strains as bacteriocinogenic probiotics and/or starter culture. The findings of this study suggest that the studied bacteriocinogenic LAB strains should be treated with caution since they may serve as a reservoir for virulence and antibiotic genes. The further application of these strains in food systems such as starter culture, probiotics, and biopreservatives should be carefully evaluated because they are potentially harmful to humans and could pose major public health risks to consumers. Identifying the strains with such traits is significant due to the possibility of transferring these genes to other bacteria that may express them. The presence of beneficial, virulence, and antibiotic resistance genes in LAB strains can be examined through *in vivo* tests to further confirm the findings of this study. Strains with no or lower incidence of such traits should be further studied for their potential utility in the food industry.

Acknowledgements: The work described here was financially supported by BIOTECH-Core Research Fund (Project Code: 16224) and the National Research Council of the Philippines, Department of Science and Technology (NRCP-DOST).

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