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Molecular Identification of Pathogen Antagonistic Gut-Lactic Acid Bacteria from *Periplaneta americana* using 16S rRNA Genes Sequencing

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ABSTRACT

Seven randomly chosen bacterial colonies were isolated from Cockroach *Periplaneta americana* gut and identify using 16S rRNA gene sequencing. NCBI Basic Local Alignment Search Tool revealed that isolate 1 had 93% similarity with *Enterococcus sp*. while isolate 2, isolate 3, isolate 4, isolate 5, isolate 6 are possibly different strains of *Enterococcus hirrea*. Isolate 7 had 98% similarity with *Lactobacillus fermentum.* Antibiotic sensitivity test using agar well method revealed that all seven isolates cell-free supernatant suppress the growth of *E. coli, B. subtilis*, and *S. aureus*. The isolates show different inhibition zones against these bacteria, in which isolates 4 was the most effective compared to isolate 1, 2 3, 5, 6, and 7. Results suggest that the lactic acid bacteria isolated from cockroach gut synthesized broad spectrum antibacterial substance that kills the three test strains.

Keywords: - Antibiotic sensitivity, *Enterococcus hirrea*, *Lactobacillus fermentum*.

INTRODUCTION

Lactic acid bacteria (LAB) are well known for diverse role in health, industry, and research [1]. In health, they were demonstrated to help for proper food digestion and participate in the regulation of metabolic activities such as carbohydrates [2]. LAB are being used in industry as probiotics for human consumption and as additives for feeds and many products [3]. Many types of research are still being conducted to elucidate the biotechnological application of Lactic Acid Bacteria.

One distinct characteristic of Lactic Acid Bacteria is to produce lactic acid as the by-product of fermentation of carbohydrate [4]. Apparently, this lactic acid can regulate the number of other microorganisms in the digestive tract of distinct species [5]. Disturbance of the normal population

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of probiotic bacteria in the human digestive tract was reported to cause constipation and digestive problems [6]. Consumption of Lactic acid bacteria fermented food products have been reported to reduce high cholesterol would improve human health by reducing the chance of heart problems [7].

Some industry shifted from making variants of old drugs to purchasing fundamentally new drugs with actively against resistant pathogens, using antimicrobial peptides. [8]. Lactic acid bacteria produce antibiotic peptides against pathogens called bacteriocins that are capable for microbial and pathogen inhibition [9]. The antimicrobial peptides produced by the lactic acid bacteria have enormous potential as both food preservatives and next generation antibiotics capable of targeting multiple drug resistant pathogens [10].

Cockroaches are considered as one of the dirtiest insects that carry different bacteria, viruses, and even fungi. They are found in any dirty environment like garbage, uncleaned house and some healthcare facilities [11]. Many microorganisms associated with cockroaches are pathogenic [12]. They are one of those organisms that can adapt well to changing environments. The potential of the gut microbiota of cockroaches has not been fully explored well. Thus, there is a need to exploit the microbiome for its potential pharmacological application.

This study presented some molecularly identified lactic acid bacteria(LAB) that are capable of inhibiting test organisms such as *E. coli, B. subtilis* and *S. aureus*.

METHODOLOGY

Research Design

To achieve the purpose of the study, the researcher made use of an experimental method of research. The methodology is designed to isolate, identify and screen the antimicrobial properties of the isolated lactic acid bacteria from the gut of cockroach. Pour plate method and serial dilution method was used for isolation followed by Antimicrobial spectrum analysis [13] using antibiotic sensitivity test. Molecular identification using 16S rRNA gene sequencing [14] was done to identify the LAB present in Cockroach Gut.

Dissecting out the cockroach

The five (5) cockroaches were collected from trash bins in Brgy. 50, Caloocan City, Metro Manila Philippines. The external body of the cockroach was cleaned using cotton and alcohol. The cockroach was medially dissected using scalpel and forceps. One (1g) gram of gut was transferred to sterile test tube supplied with 9ml peptone water.

Serial Dilution and Plating

From the gut and peptone mixture, serial dilutions were made taking 1ml aliquot which was diluted to another test tube containing 9ml peptone water.

Dilution from the 10 up to 10^6 was made to ensure acquisition of countable and isolatable colonies.

From each dilution 1 ml aliquot was taken and transferred to sterile petri dish separately. Pre-cooled MRS agar (40^{o}C) was poured into each petri dish with aliquot. The medium was allowed to solidify for 4 hours and then incubated at 37 ^{o}C for 24 hours.

Isolation and Purification of Lactic Acid Bacteria.

After incubation plate with least number of grown colonies was used for random selection. Seven colonies were pick-up from and streak on plated containing MRS agar. Isolates were initially coded as T1 for the first colony, T2 for the second, T3 third, T4 for the ; fourth, T5 Fifth, T6 for sixth and T7 for the seventh isolate.

From each plate, isolated colonies were re-picked and streak in test-tube slants using inoculating needle. All slants were incubated at the same temperature and time.

Bulk Culturing of Presumptive Lactic Acid Bacteria

Grown purified cultures were inoculated on a sterile flask with 500ml MRS Broth. The flask with inoculum was incubated at 35 ^{o}C for seven (7) days with shaking intervention daily.

Anti-Bacterial Spectrum Analyses by Cell-Free Supernatant *[15]*

Cell Free Supernatant

After incubation, 20 ml of the aliquot was centrifugated for 2000rpm for 5 minutes and filtered using 0.45 microfilter(Whatman). After filtration, the collected cell-free supernatant was subjected to an antibacterial sensitivity test. The 30 µl of the cell-free supernatant was used for antimicrobial- spectrum assay.

Antibacterial Spectrum Test

A hole on the plates containing solidified Muller Hinton Agar were made prior inoculation of the test strains. Ten (10) µl test bacterial strains (*E. coli, S. aureus and B. subtilis)* with a concentration of 10^8/ ml were inoculated to Muller Hinton Agar separately.

30µl of the cell-free supernatant were transferred onto the MHA well. The supernatants were allowed to diffuse for 4 hours. After drying the plate were incubated at 35 degree Celsius for 24 hours. Zones of inhibition were measured after incubation.

Molecular Identification of Presumptive Lactic Acid Bacteria

All isolated positive for inhibiting the test strain were submitted to Philippine Genome Centre(PGC) University of the Philippines Diliman for DNA extraction, amplification of 16S rRNA gene and sequencing.

Sequence editing and alignment

Bioedit was used for editing the obtain data sequence from PGC. The sequence was compared with the published sequences in GenBank using

Basic Local Alignment Search Tool-Nucleotide (BLAST-n) of the National Center for Biotechnology Information (NCBI to determine their molecular identity.

Treatment of Data

Data of zone of inhibition were subjected to One Way Analysis of Variance. The differences between the means were further analyzed using HSD test. Statistical analyses made use of the Graph Pad Prism software version 6.

RESULT AND DISCUSSION

Selection of Isolates

From the plates running from the dilution 10-1 up to 10-6 only the plate of 10-6 dilution factor obtains countable number colonies which are 52. Out of 52 colonies only 7 (top layer growing colonies) were chosen for purification and antimicrobial action.

Bacterial Antagonistic potential of cell-free supernatant of 8 Presumptive Lactic Acid Bacteria

Inhibitory potential cell-free supernatant of seven isolates against *E. coli*

Figure 1 shows the inhibition of supernatants of seven isolates against *E. coli*. Analysis of variance shows that there are significant differences among the treatments. T1, T2, T3, T4, T5, T6, T7, T+ (Positive control) inhibit *E. coli* with mean diameter 11.33mm, 12.66mm, 14.33mm, 11.33mm, 11.0mm, 12.16mm, 18.66mm. T- Negative did not inhibit *E. coli*. Comparison between the means revealed the T- (distilled water) is significantly different to the rest of the treatment. T1, T2 T3, T4, T5, T6, and T7 are comparable in terms of inhibiting *E. coli*, but significantly different T+ (positive control). All the seven isolates cell-free supernatant is capable of inhibiting *E. coli*.

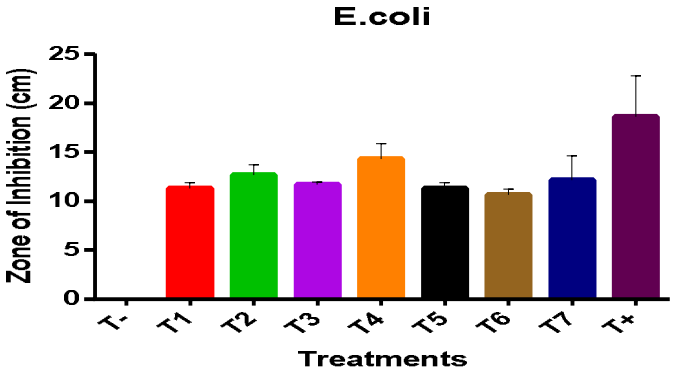


Figure1. Inhibitory potential cell-free supernatant of seven isolates against *E. coli*

Inhibitory potential cell-free supernatant of seven isolates against *B. subtilis*

Figure 2 shows the inhibition of seven isolates against *B. subtilis*. Analysis of variance shows (Table 3) that there is a significant difference among the treatments. T1, T2, T3, T4, T5, T6, T7, T+ (Positive control) inhibit *E. coli* with mean diameter 12mm, 10.33mm, 12.16mm, 14.66mm, 11.83mm, 11.33mm, 11.5mm, 27mm respectively. T- Negative did not inhibit *B. subtilis*. Comparison between the means revealed the T- (distilled water) is significantly different to the rest of the treatments. The positive control is not comparable to the inhibition given by T1, T2 T3, T4, T5, T6, and T7. Cell-free supernatant from T4 is significantly effective than T1, T2 T3, T5, T6 and T7, but less effective than the T+ (positive control). The cell-free supernatant of the seven isolates is capable of killing *B. subtilis*, suggesting a compound/ antimicrobial molecule is released by bacterial.

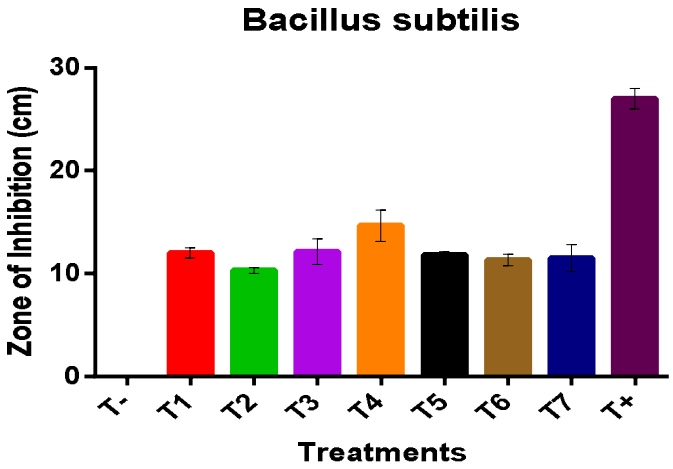


Figure 2. Inhibitory potential cell free supernatant of seven isolates against *B. subtilis*

Inhibitory potential cell free supernatant of seven isolates against *S. aureus*

Figure 3 shows the inhibition of seven isolates against *S. aureus*. Analysis of variance shows that there is a significant difference among the treatments. T1, T4, T5, T6, T7, T+ (Positive control) inhibit *S. aureus* with mean diameter 14.5mm, 18.5mm, 11.16mm, 10.5mm, 10.5mm, 23.66mm respectively. T-, T2, T3 did not inhibit *S. aureus*. T4 has a significantly higher zone of inhibition than T1, T5, T6, and T7. Furthermore, T4 is comparable with the T+ because its zone of inhibition is not significantly different from that of T+ (positive control). Five isolates had the

ability to inhibit the *S. aureus* during the cell-free supernatant method indicating that some kind of antibacterial substance might be release from these isolates

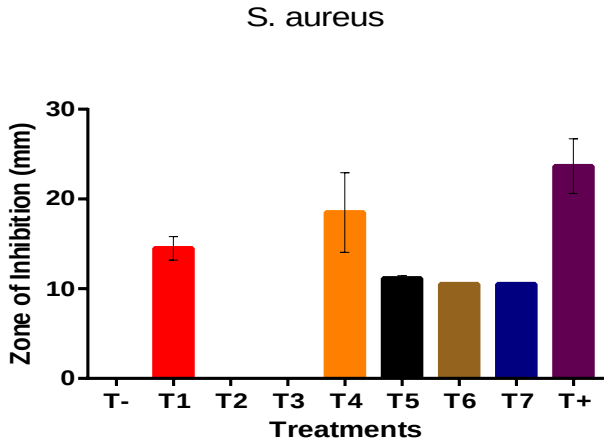


Figure 3. Inhibitory potential cell free supernatant of seven isolates against *S. aureus*

Molecular Identity of the Lactic Acid Bacteria

Base on 16S rRNA gene sequences, BLAST of NCBI reveals that T1 is 93% similarity With *Enterococcus sp*. T2, T3, T4, T5 and T6 isolate are *Enterococcus hirrea* with the percent identity of 99%, 99%, 99%, 98%, 100% respectively and T7 was to be *Lactobacillus fermentum* with the percent identity of 98%. The differential inhibitory potential of T2, T3, T4, T5 and T6 suggest a possible difference on their molecular identity at the strain level.

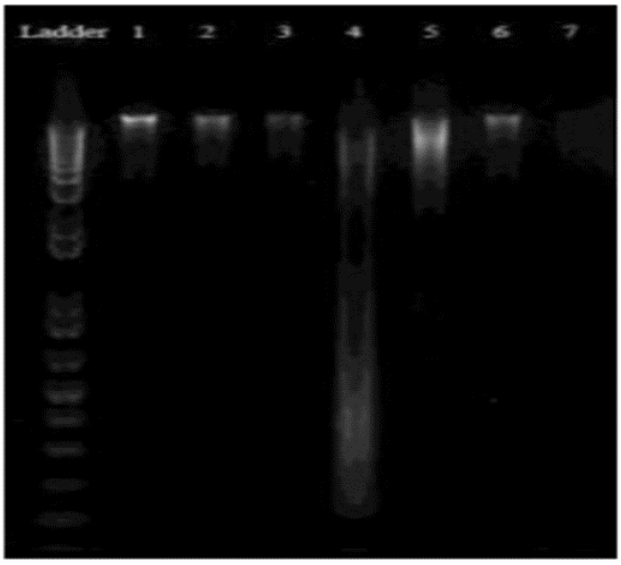
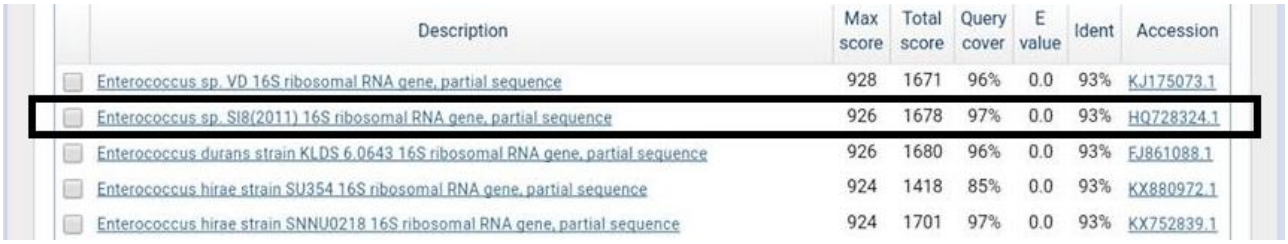
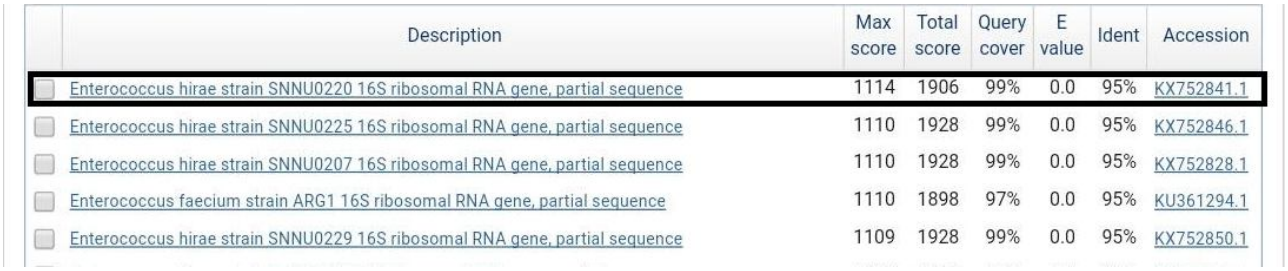


Figure 4. Amplicons of seven isolated lactic acid bacteria from the gut of *Periplaneta americana*



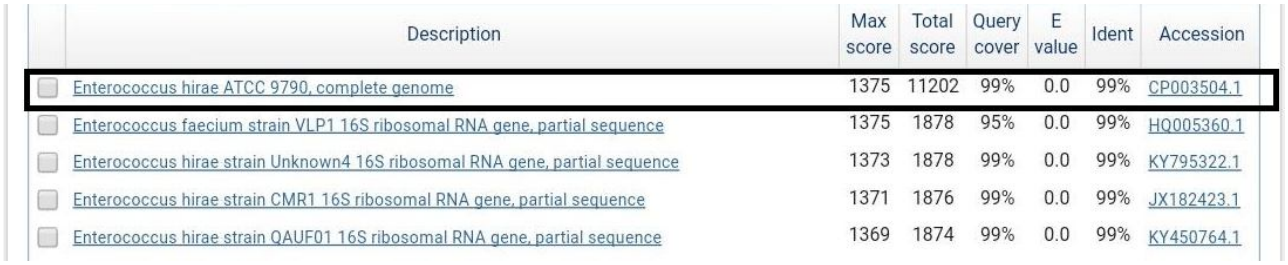
Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterococcus sp. VD 16S ribosomal RNA gene, partial sequence	928	1671	96%	0.0	93%	KJ175073.1
Enterococcus sp. SI8(2011) 16S ribosomal RNA gene, partial sequence	926	1678	97%	0.0	93%	HQ728324.1
Enterococcus durans strain KLDS 6.0643 16S ribosomal RNA gene, partial sequence	926	1680	96%	0.0	93%	FJ861088.1
Enterococcus hirae strain SU354 16S ribosomal RNA gene, partial sequence	924	1418	85%	0.0	93%	KX880972.1
Enterococcus hirae strain SNNU0218 16S ribosomal RNA gene, partial sequence	924	1701	97%	0.0	93%	KX752839.1

Figure 4. T1 Isolate Molecular Identity based on BLAST NCBI data



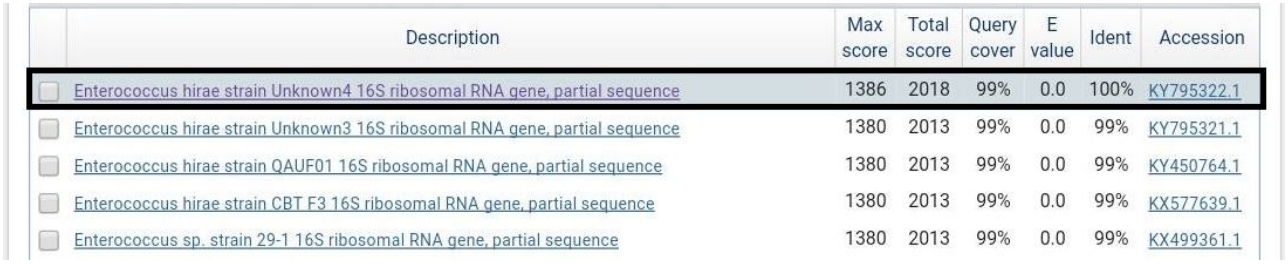
Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterococcus hirae strain SNNU0220 16S ribosomal RNA gene, partial sequence	1114	1906	99%	0.0	95%	KX752841.1
Enterococcus hirae strain SNNU0225 16S ribosomal RNA gene, partial sequence	1110	1928	99%	0.0	95%	KX752846.1
Enterococcus hirae strain SNNU0207 16S ribosomal RNA gene, partial sequence	1110	1928	99%	0.0	95%	KX752828.1
Enterococcus faecium strain ARG1 16S ribosomal RNA gene, partial sequence	1110	1898	97%	0.0	95%	KU361294.1
Enterococcus hirae strain SNNU0229 16S ribosomal RNA gene, partial sequence	1109	1928	99%	0.0	95%	KX752850.1

Figure 5. T2 Isolate Molecular Identity based on BLAST NCBI data



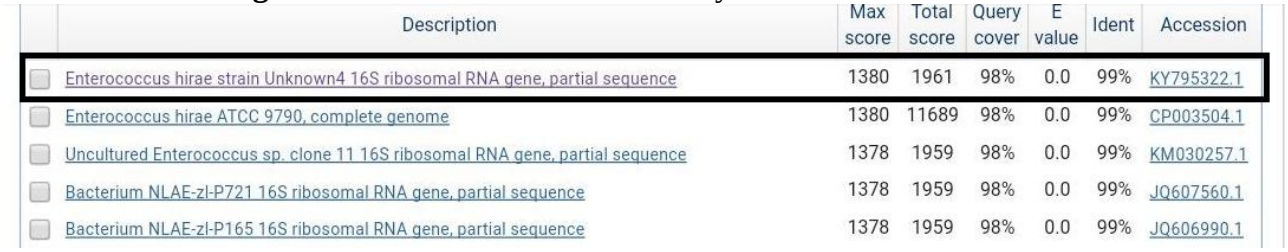
Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterococcus hirae ATCC 9790, complete genome	1375	11202	99%	0.0	99%	CP003504.1
Enterococcus faecium strain VLP1 16S ribosomal RNA gene, partial sequence	1375	1878	95%	0.0	99%	HQ005360.1
Enterococcus hirae strain Unknown4 16S ribosomal RNA gene, partial sequence	1373	1878	99%	0.0	99%	KY795322.1
Enterococcus hirae strain CMR1 16S ribosomal RNA gene, partial sequence	1371	1876	99%	0.0	99%	JX182423.1
Enterococcus hirae strain QAUF01 16S ribosomal RNA gene, partial sequence	1369	1874	99%	0.0	99%	KY450764.1

Figure 6. T3 Isolate Molecular Identity based on BLAST NCBI data



Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterococcus hirae strain Unknown4 16S ribosomal RNA gene, partial sequence	1386	2018	99%	0.0	100%	KY795322.1
Enterococcus hirae strain Unknown3 16S ribosomal RNA gene, partial sequence	1380	2013	99%	0.0	99%	KY795321.1
Enterococcus hirae strain QAUF01 16S ribosomal RNA gene, partial sequence	1380	2013	99%	0.0	99%	KY450764.1
Enterococcus hirae strain CBT F3 16S ribosomal RNA gene, partial sequence	1380	2013	99%	0.0	99%	KX577639.1
Enterococcus sp. strain 29-1 16S ribosomal RNA gene, partial sequence	1380	2013	99%	0.0	99%	KX499361.1

Figure 7. T4 Isolate Molecular Identity based on BLAST NCBI data



Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterococcus hirae strain Unknown4 16S ribosomal RNA gene, partial sequence	1380	1961	98%	0.0	99%	KY795322.1
Enterococcus hirae ATCC 9790, complete genome	1380	11689	98%	0.0	99%	CP003504.1
Uncultured Enterococcus sp. clone 11 16S ribosomal RNA gene, partial sequence	1378	1959	98%	0.0	99%	KM030257.1
Bacterium NLAE-zl-P721 16S ribosomal RNA gene, partial sequence	1378	1959	98%	0.0	99%	JQ607560.1
Bacterium NLAE-zl-P165 16S ribosomal RNA gene, partial sequence	1378	1959	98%	0.0	99%	JQ606990.1

Figure 8. T5 Isolate Molecular Identity based on BLAST NCBI data

Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterococcus hirae strain Unknown4 16S ribosomal RNA gene, partial sequence	1380	1961	98%	0.0	99%	KY795322.1
Enterococcus hirae ATCC 9790, complete genome	1380	11689	98%	0.0	99%	CP003504.1
Uncultured Enterococcus sp. clone 11 16S ribosomal RNA gene, partial sequence	1378	1959	98%	0.0	99%	KM030257.1
Bacterium NLAE-zl-P721 16S ribosomal RNA gene, partial sequence	1378	1959	98%	0.0	99%	JQ607560.1
Bacterium NLAE-zl-P165 16S ribosomal RNA gene, partial sequence	1378	1959	98%	0.0	99%	JQ606990.1

Figure 9. T6 Isolate Molecular Identity based on BLAST NCBI data

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactobacillus fermentum strain L39 16S ribosomal RNA gene, partial sequence	1386	1950	98%	0.0	99%	KP317708.1
Lactobacillus fermentum strain L7 16S ribosomal RNA gene, partial sequence	1386	1950	98%	0.0	99%	KP317680.1
Lactobacillus fermentum strain LBF 13A 16S ribosomal RNA gene, partial sequence	1386	1950	98%	0.0	99%	KY249642.1
Lactobacillus fermentum strain TW27-2 16S ribosomal RNA gene, partial sequence	1386	1950	98%	0.0	99%	KJ026616.1
Lactobacillus fermentum strain NS9 16S ribosomal RNA gene, partial sequence	1386	1950	98%	0.0	99%	JQ013298.1

Figure 10. T7 Isolate Molecular Identity based on BLAST NCBI data

DISCUSSION

Previous studies have shown that *Enterococcus* sp. has the ability to produce antimicrobial compounds against particular types of gram positive and negative bacteria. Some of the Enterococcus species showed the antimicrobial activity against the food pathogen *Listeria monocytogenes* ATCC35152 [16]. *Enterococcus hirae* from cow feces can inhibit the growth of *E. coli* bacteria. Further cell- free supernatant *Enterococcus hirae* showed largest inhibition zone against *E. coli* [17]. *Lactobacillus fermentum* has the capability to produce antimicrobial and antioxidative properties and can destroy the growth of *Salmonellas* causing reduction of liver spleen granulomas [18].

Results of the study demonstrated the capability of *Enterococcus sp. Enterococcus hirae* and *Lactococcus fermentum* isolated from cockroach gut to inhibit the growth of *E. coli. Bacillus subtilis and S. aureus.* The potent cell- free supernatant signify that antimicrobial peptide or agent is release by the bacterial isolates extracellularly.

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REFERENCES

1. Khalid K.(2011) An overview of lactic acid bacteria. 1(30); 1-13,

2. Lahtinen S. J. (2012). Probiotic viability – does it matter?Microbial Ecology in Health & Disease 2012. Co-action publishing. doi.org/10.3402/mehd.v23i0.18567
3. Maria Kechagia, Dimitrios Basoulis, Stavroula Konstantopoulou, et al., "Health Benefits of Probiotics: A Review," ISRN Nutrition, vol. 2013, Article ID 481651, 7 pages, 2013. doi:10.5402/2013/481651
4. Brüssow, Harald (2013). Nutrition, population growth and disease: a short history of lactose. in Environmental Microbiology. 15, 2154-2161.
5. Guarner, F., Sanders, M.E., Gibson, G. et al. (2011). Probiotic and prebiotic claims in Europe: seeking a clear roadmap. The British Journal of Nutr., 106, 1765-1767.
6. Gerritsen, J., Smidt, H., Rijkers, G. T., & de Vos, W. M. (2011). Intestinal microbiota in human health and disease: the impact of probiotics. Genes & Nutrition, 6(3), 209–240. http://doi.org/10.1007/s12263-011-0229-7
7. Ongol M.P. (2012), Lactic Acid Bacteria in Health and Disease, 1, (1) :39-50
8. Nathan C,& Otto C. .Antibiotic Resistance – Problems, Progress, and Prospects. November 6, 2014 N Eng J Med 2014; 371: 1761-1763
9. M.P. Zacharof, R.W. Lovitt (2012), Bacteriocins Produced by Lactic Acid Bacteria a Review Article, .2, 50–56
10. Perez, R. H., Zendo, T., & Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial Cell Factories*, *13*(Suppl 1), S3. http://doi.org/10.1186/1475-2859-13-S1-S3
11. Prado MA, Elucir G. IIi; PereiraI, M.S. Reis C, Fabiana C. P.I (2006), Profile of antimicrobial resistance of bacteria isolated from cockroaches (Periplaneta americana) in a Brazilian health care institution 10, 1
12. Al-bayati N.Y, Anfal S. Al-ubaidi, I. Al-Ubaidi K (2011), Risks Assosiated with cockroach Periplanata aamericana asa aTransmitter of Pathogen Agents. Diyala Journal of Medicine. 1, 91-96
13. Guevarra B.Q.(2005) A Guide Book on Plant Screening: Phytochemical and Biological. Research Center for Natural Sciences. UST Publishing House. Manila, Philippines.
14. Boye K. Hogdall E. and Borre M.(1999) Identification of Bacteria using two degenerate 16S rDNA sequencing primers. Microbiol Res. 154(1):23-6
15. Audisio M.C., Torres M.J., Sabaté D.C., Ibarguren C., Apella M.C. (2011), Properties of different lactic acid bacteria isolated from Apis mellifera L. bee-gut.Micro Res. 166(1):1–13, doi:10.1016/j.micres.2010.01.003
16. Comeriato CB, Julla Roberta Buboltz, Nalara Agular Santestevan, Amanda de Souzza da Motta and Ana Paula Guedes Frazzon (2016), Antimicrobial compounds produced by Enterococcus spp. isolates from fecal samples of wild south American fur seals. JMA. 8(3): 14-21 DOI: 10.5897/JMA2015.0360
17. Adeniyi BA, Adetoye A, Ayeni FA (2015). Antibacterial activities of lactic acid bacteria isolated from cow faeces against potential enteric pathogens. Afr. Health Sci. 15(3):888-895. http://dx.doi.org/10.4314/ahs.v15i3.24
18. Mikelsaar, M. & Zilmer, M. (2009). *Lactobacillus fermentum* ME-3 – an antimicrobial and antioxidative probiotic. *Microbial Ecology in Health and Disease*, *21*(1), 1–27. http://doi.org/10.1080/08910600902815561