

# Phylogenetic Analysis of Selected Lactic Acid Bacteria by Using Bioinformatic Tools

Priyanka Gautam, Anjuradha Vimal

**Abstract**— Bacteriocin is proteinaceous antimicrobial peptides secreted by bacteria that inhibit the growth of closely related microorganism. Bacteriocin produced by lactic acid bacteria have attained a great attention because of its huge potential to use in food product as preservative. In the present study we have done phylogenetic analysis of selected 25 lactic acid bacteria those are found in milk and ready to eat milk products and produces of bacteriocin. Phylogenetic analysis was done by using the distance based and character based methods. 16S rRNA gene sequences of selected 25 lactic acid bacteria retrieved from NCBI database and it was used to build phylogenetic tree using MEGA 5.2 software. In the tree topology obtained from all the 4 methods, likeness was observed in branches (48), nodes (22), polytomy (0), out group (0), the differences was observed only in monophyletic group, polyphyletic group, monotomy and dichotomy. On the comparison of all the prepared phylogenetic tree on the basis of morphological and biochemical characters, families and production of bacteriocin, superlative evolutionary tree of the selected lactic acid bacteria was obtained from UPGMA (Unweighted Paired Group with Arithmetic Mean) method. The further confirmation of the close relation on the basis of bacteriocin can be done to get alternative lactic acid bacteria to inhibit the growth of pathogenic microbes.

**Index Terms**— Lactic acid bacteria, phylogenetic analysis, bacteriocin, MEGA 5.2.

## I. INTRODUCTION

Phylogenetic analysis is the new concept for the research study, because it generates branching, treelike diagrams that represent an estimated pedigree of the inherited relationship among molecule (“gene tree”), organism or both. Phylogenetic tree shows how the families have been deriving during evolution. The branches of phylogenetic trees are sometime also called as cladistics because of the word “clade”, a set of descendants from a single ancestor, is derived from Greek word for branch. Phylogenetic tree can be prepared by using sequence data to visualize the evolutionary relationship between species [14]. The major assumptions of the phylogenetic tree are that the molecular sequences used in phylogenetic construction are homologous, meaning that they share a common origin and subsequently diverged through time. Phylogenetic divergence is assumed to be bifurcating meaning that a parent

branch splits in to two daughter branches at any given point. Another assumption in

phylogenetics is that each position in a sequence evolved independently. The variability among sequence is sufficiently informative for constructing unambiguous phylogenetic tree [24].

Lactic acid bacteria (LAB) are group of gram-positive, nonsporulating, rods and cocci with nonaerobic habitat but aerotolerant [21]. Most Lactic acid bacteria (LAB) are considered generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) [12, 1]. The lactic acid bacteria population has a profound effect on the long safe preservation of food quality and develops the aroma and flavor of the final products and also increases the nutritional quality of food. European Food Safety Agency (EFSA) (2007) reported 33 *Lactobacillus* species as Qualified Presumption of safety (QPS) status. In addition to *Lactobacillus* species, also other species have also been granted QPS-status. They include three *Leuconostoc* (*Ln. citreum*, *Ln. lactic*, *Ln. mesenteroides*), three *Pediococcus* (*P. acidilactici*, *P. dextrinicus* and *P. pentosaceus*), *Lc. Lactic* and *streptococcus theamophilus*. A recent work done by [11] with lactic acid bacteria isolated from traditional raw-milk, cheese, revealed several *Lactobacillus* having antimicrobial activity against pathogen such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella newport* and even *Escherichea coli*. Lactic acid bacteria have the ability to produce a number of antimicrobial substance, such as, organic acid (lactic acid and acetic acid), hydrogen peroxide, which have the capacity to inhibit growth of a variety of spoilage and pathogenic organism [9, 3]. Importance of LAB in milk productions such as they increase food safety through the release of lactic acid and bacteriocin.

Bacteriocins are proteinaceous substances and antimicrobial peptide secreted by bacteria that inhibit the growth of closely related microorganism [5, 20] and have feasible application to food such as meat and dairy product, fruits vegetable, cereals and beverage. They offer several desirable properties to which make them suitable for food preservation, are generally recognized as safe substance, bacteriocin are usually pH and heat-tolerant [11]. These compounds are produced by both gram-positive and gram-negative organism. Bacteriocin production has been observed among *Lactobacilli*, *Enterococci*, *Lactococci*, *Leuconostoc*, *Pediococci*, *Staphylococci* and *Carnobacteria*. Most bacteriocin inhibits food-born pathogen like *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus* and *Staphylococcus aureus*; hence they are considered as potential natural food preservative [4,15]. Several bacteriocin producing strain have been isolated from raw and fermented milk and milk product [18, 8]. Various other

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bacteriocin from LAB have been reported, e.g. nisin produced by *Lactococcus lactis* [6], Lactocin produced by *Lactobacillus acidophilus* [2], Pediocin produced by *Pediococcus acidilactici*, Mesentericin produced by *Leuconostoc mesenteroides* [7] and Enterocin produced by *Enterococcus faecium* [17, 13]. As lactic acid bacteria is a vast group and have the great food application due to the production of bacteriocin. So in the present study we have chosen the selected and most dominant lactic acid bacteria found in milk product they are able to produced bacteriocin, and have antimicrobial properties against pathogens and these are use for the preparation of Phylogenetic tree to show their evolutionary relationship.

II. MATERIALS AND METHODS:

Samples were collected by using various search engines as Google Chrome, PubMed, and Google Scholar. 16S rRNA sequences for all selected lactic acid bacteria were retrieving from the publically available databases NCBI, established in 1988.

16S rRNA:

There are conserved sequences and all are retrieved from the NCBI databases. In these sequences little changes occur during evolution.

Sequences alignment:

sequences alignment were done by two methods, one is the pair wise sequence alignment, BLAST (Basic Local Alignment Search Tool), and other one is the CLUSTAL W is the multiple sequence alignment and in the MEGA 5.2 software is automatically alignment the sequences by CLUSTAL W. MULTALIN is another multiple sequences alignment can also be used for the align the sequences.

Phylogenetic analysis

Phylogenetic analysis was prepared by two methods distance based and character based methods by using the MEGA 5.2 software [10, 16] and the tree were also statistically analyzed by bootstrap method.

III. RESULTS AND DISCUSSION:

After the literature survey 5 LAB were selected. All 25 selected lactic acid bacteria belong to phylum Firmicutes, class Bacillis and order Lactobacillales [23]. According to the morphology all selected 25 lactic acid bacterial species (*Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*, *Lactobacillus kefir*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Lactobacillus johnsonii*, *Lactobacillus acidophilus*, *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Leuconostoc citreum*, *Lactococcus raffinolactis*, *Lactococcus lactis subsp. lactis*, *Lactococcus garvieae*, *Streptococcus thermophilus*, *Streptococcus mutans*, *Streptococcus salivarius*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus mundtii*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*) are gram-positive, catalase negative and non-sporulating and all are found in milk and milk products such as butter milk, fermented milk, goat milk, dahi and cheese etc [19] (Table 1). The sequence used in phylogenetic tree analysis include the total 48 sites, out of which the conserved site are 8 and variable site are 32 and parsim-informative site are 20.

Table: 1 List of all selected lactic acid bacteria found in milk and milk products.

S. No	LAB	ASSOCIATION	BACTERIOCIN	CLASS
1.	<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	Yogurt, cheese	Lactocin	IIb
2.	<i>Lactobacillus brevis</i>	kefir	Brevicin	IIa
3.	<i>Lactobacillus casei</i>	Dairy product/probiotic milk	Lactocin	IIb
4.	<i>Lactobacillus fermentum</i>	Goat milk	Fermentacin	I
5.	<i>Lactobacillus helveticus</i>	Fermented milk, cheese	Helveticin	IIb
6.	<i>Lactobacillus hilgardii</i>	butter milk	Lactocin	IIb
7.	<i>Lactobacillus kefir</i>	Kefir	Lactacin	IIb
8.	<i>Lactobacillus plantarum</i>	Cheese	Plantaricin	IIb
9.	<i>Lactobacillus curvatus</i>	Butter milk	Curvacin	IIa
10.	<i>Lactobacillus johnsonii</i>	Fermented milk	Lactacin	IIb
11.	<i>Lactobacillus acidophilus</i>	Fermented, probiotic milk	Nisin	I
12.	<i>Leuconostoc mesenteroides</i>	Butter and buttermilk	Mesentericin	IIa
13.	<i>Leuconostoc lactis</i>	Fermented milk	Leucocin	IIa

14.	<i>Leuconostoc citreum</i>	Yourt and as probiotic	Leucocin	IIa
15.	<i>Lactococcus raffinolactis</i>	Butter milk and cheese	Lactacin	IIb
16.	<i>Lactococcus lactis subsp. lactis</i>	Dairy product	Lactacin	IIb
17.	<i>Lactococcus garvieae</i>	Raw milk	Garviecin	IIa
18.	<i>Streptococcus thermophilus</i>	Yogurt, cheese	Thermophilin	I
19.	<i>Streptococcus mutans</i>	Dahi and milk	Mutacin	I
20.	<i>Streptococcus salivarius</i>	Used as a probiotic	Salivaricin	I
21.	<i>Enterococcus faecium</i>	whey	Enterocin	IIa
22.	<i>Enterococcus faecalis</i>	Whey	Enterocin	I
23.	<i>Enterococcus mundtii</i>	Cheese	Mundtacin	IIa
24.	<i>Pediococcus acidilactici</i>	Butter and butter scotch	Pediocin	IIa
25.	<i>Pediococcus pentosaceus</i>	Butter milk	Pediocin	IIa

Results obtained all the four methods (Unpaired Group Method with Arithmetic mean (UPGMA), Neighbour Joining, Maximum Parsimony and Maximum Likelihood) each method shows the similarity between the species of 2

clade, in which one clade contains the species *Lactococcus garvieae*, *Streptococcus salivarius*, *Enterococcus faecalis* and *Lactococcus lactis subsp. lactis* and other clade contains the species *Streptococcus thermophilus*, *Lactobacillus curvatus* and *Leuconostoc lactis*.(Fig 1-4).

**Fig1. Phylogenetic tree of the lactic acid bacteria was prepared by maximum likelihood method (boot strap analysis)**

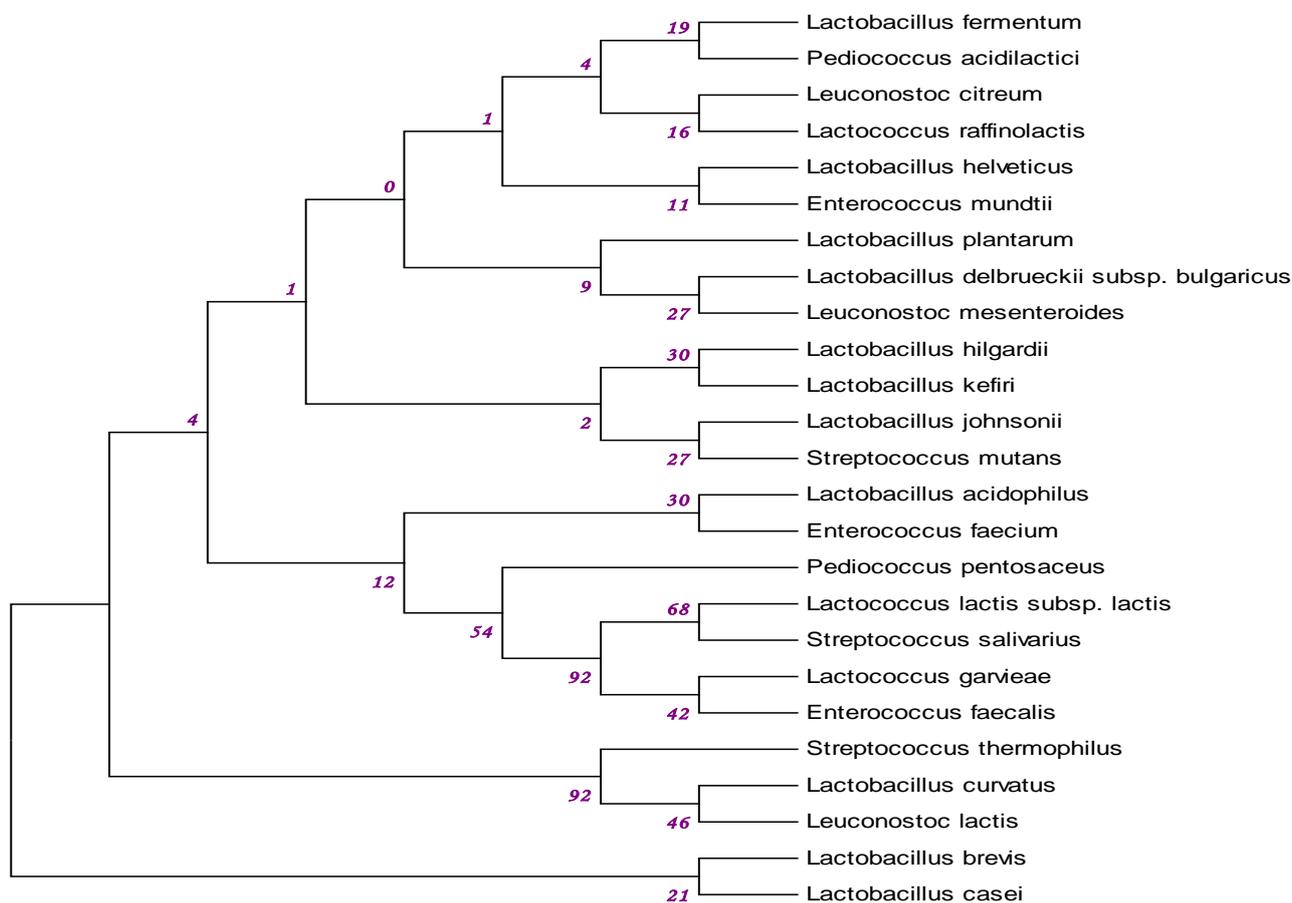


Fig2. Phylogenetic tree of the lactic acid bacteria was prepared by Neighbor-Joining method (boot strap analysis).

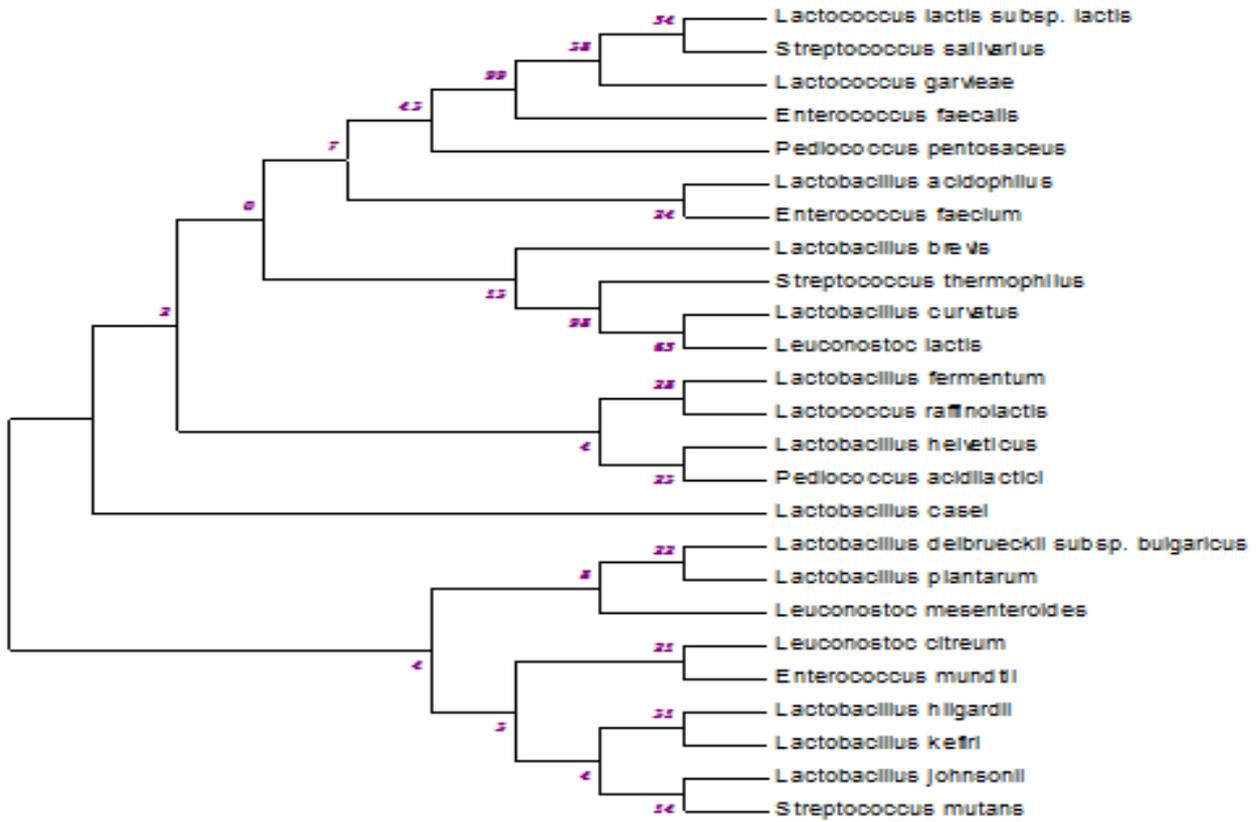


Fig3. Phylogenetic tree of the lactic acid bacteria was prepared by UPGMA method (boot strap analysis).

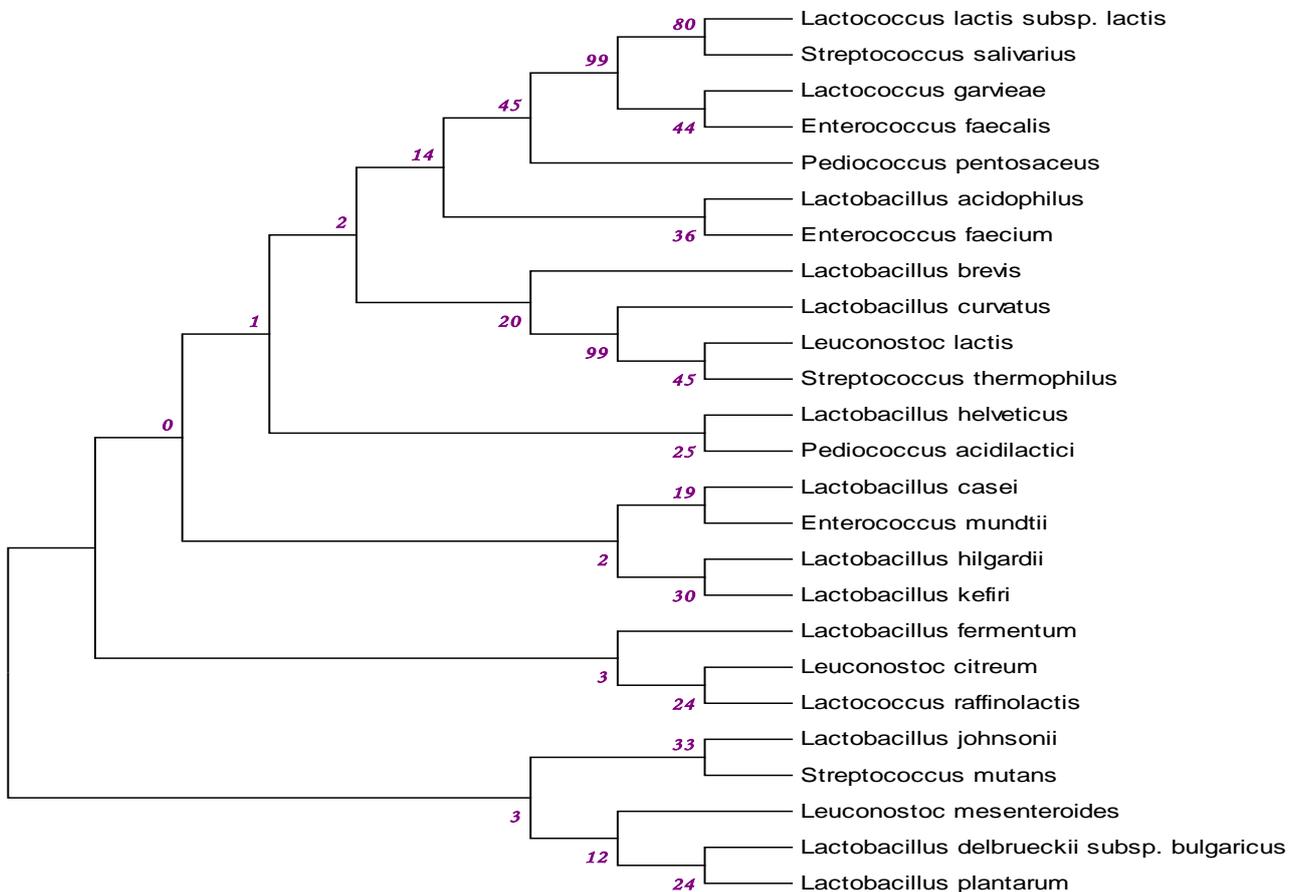
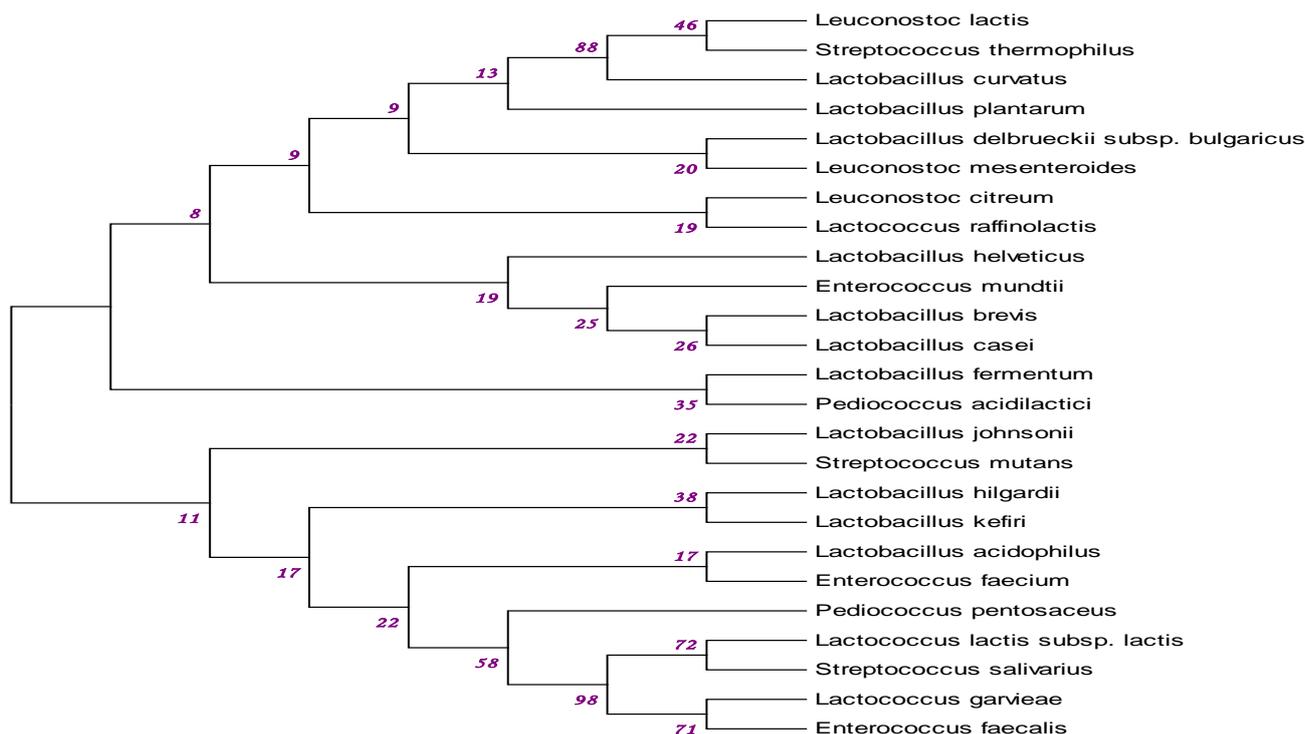


Fig4: phylogenetic tree of the lactic acid bacteria was prepared by Maximum Parsimony method (Boot strap method)



According to the percents similarity between the species of first clade, *Lactococcus lactis subsp lactis*, *Streptococcus salivarius*, *Lactococcus garvieae* and *Enterococcus faecalis* shows the highest similarity in the UPGMA methods is 99%. of the four closely related species *Streptococcus salivarius* is used as probiotic, and causes the production of class I bacteriocin (Table 1) and other one is *lactococcus garvieae* is present in raw milk and produces class II bacteriocin and *Enterococcus faecalis* mostly found in whey and causes the production of class I bacteriocin and the *Lactococcus lactis subsp. lactis* found in many dairy products and causes the production of class II bacteriocin. In the second clade the three species are present as *Streptococcus thermophilus*, *Lactobacillus curvatus* and *Leuconostoc lactis* of those only *Streptococcus thermophilus* belongs to class I bacteriocin while other two related to Class II bacteriocin.

As class I bacteriocin are the lantibiotic (lanthionin contains peptide) and heat labile while the class II bacteriocin are non lantibiotic containing membrane active peptide and heat stable. So the class II bacteriocin more effective then the class I bacteriocin

The other similarity also shows with the UPGMA method is 99% within the species *Streptococcus thermophilus*, *Lactobacillus curvatus* and *Leuconostoc lactis*. *Streptococcus thermophilus* found in fermented milk, yogurt and cheese produce the class I bacteriocin. *Leuconostoc lactis* found in dairy products as fermented milk, cheese, yogurt, butter milk and produces class II bacteriocin and *Lactobacillus curvatus* also found in butter milk and produce class II bacteriocin [22]. So the further confirmation by the wet lab analysis can be done to confirm the action of all these lactic acid bacteria on the pathogen and to set the more alternative food preservative against the pathogens where the more heat is required to produce milk products.

Table: 2 Comparison of the tree topology of 25 LAB species obtain from NJ, UPGMA, ML, MP methods.

S. No.	Topology	Phylogenetic analysis methods			
		NJ	UPGMA	ML	MP
1.	Taxa	25	25	25	25
2.	Branches	48	48	48	48
3.	Node	23	23	23	23
4.	Monophyletic	0	1	1	2
5.	Polyphyletic	5	4	5	3
6.	Monotomy	0	0	0	1
7.	Polytomy	0	0	0	0
8.	Dichotomy	9	10	11	8
9.	Root	0	1	0	0
10.	Out group	0	0	1	0

NJ= Neighbor-Joining method, MP= Maximum Parsimony, ML= Maximum Likelihood, UPGMA= Unweighted Pair Group method using arithmetic average.

IV. CONCLUSION:

Bioinformatics can be derived from two French words Bio-Biology, Informatique-Data processing, i.e., bioinformatics is collection of the data bases by which analyze the information related to the biology and computer science and solve the problems through the bioinformatics approaches. Bioinformatics is a vast area of the science and by using the bioinformatics study create a new programs and area, here examined the evolutionary analysis of 25 lactic acid bacterial species, to estimate the inherited relationship among the LAB those are found in milk and milk products and causes the production of bacteriocin. While predicting the phylogenetic tree comparison was made between the tree topology of the tree obtained from all the four methods, we have found that all the four methods showing the similarity in the number of branches-48, nodes-23, the difference was obtained from the monophyletic group, polyphyletic group, dichotomy, polytomy, monotomy and in root (Table 2). In this study we have found the best result within the UPGMA methods, it shows the highest bootstrap value and also shows the best analysis of the phylogenetic trees. On the basis of close similarity of the species found in phylogenetic analysis the wide spectrum of the alternative natural food preservative containing LAB can be achieved to get the bactericidal or bacteriostatics effect against the pathogenic bacteria. The combination of these closely related bacteria species can be used for treatment against the specific pathogen.

REFERENCES

[1] X. Airidengcaিকে, Chen, X. H. Du, W. H. Wang, J. C. Zhang, Z. H. Sun, W. J. Liu, L. Li, T. S. sun, and H. P. Zang. (2010). "Isolation and identification of cultivable lactic acid bacteria in traditional fermented milk of Tibet in China." *International Journal Dairy Technology*, 63: 437-444.

[2] S. F. Barefoot and T. R. Klaenhammer. (1983). "Detection and activity of lactacinB, a bacteriocin produced by *Lactobacillus acidophilus*." *Applied Environment microbiology*, 45: 1808-1815.

[3] F. S. L. Brinkman and D. D. Leipe. (1993). "Phylogenetic analysis." *National Center for Biotechnology Information*, 2: 323-385.

[4] J. Clevend, T. J. Montville, I. F. Nes, M. Chikindas. (2001). "Bacteriocin: safe, natural antimicrobials for food preservation." *International Journal of Food Microbiology*, 71: 1-20.

[5] P. O. Cotter, C. Hill and R. P. Ross. (2005). "Bacteriocins: developing innate immunity for food." *National Review Microbiology*, 3: 777-778.

[6] H. M. Dodd, N. Horn and M. J. Gasson. (1990). "Analysis of the genetic determinant for production of the peptide antibiotic nisin." *Journal general microbiology II*, 36: 555-566.

[7] U. Hechard, M. Dheibomez, Y. Cenatimpo and F. Letellier. (1992). "Antagonism of the lactic acid bacteria from goats' milk against pathogenic strains assessed by the "sandwich method." *Letter Applied Microbiology*, 11: 185-188.

[8] E. Izquierdo, E. Marchioni, D. Werner, C. Hasselmann and S. Ennahar. (2009). "Smearing of soft cheese with *Enterococcus faecium* WHE 81, a multi bacteriocin producer, aganist *Listeria Monocytogenes*." *Food Microbiology*, 26: 16-20.

[9] R. W. Jack, J. R. Tagg and B. Ray. (1995). "Bacteriocins of gram-positive bacteria." *Microbiology Review*, 59: 171-200.

[10] S. Kumar, K. Tamura, and M. Nei. (1993). "Manual for MEGA: Molecular Evolutionary Genetics Analysis Software." *University Park, PA: Pennsylvania State University*.

[11] J. M Kongo. (2013). "Lactic acid bacteria as starter-cultures for cheese processing: Past, Present and Future Developments." *InTech open science/openmind*, 1-20.

[12] S. Q. Liu, R. Holland and V. L. Crow. (2004). "Esters and their biosynthesis in fermented dairy products: a review." *International Dairy Journal*, 14: 923-945.

[13] C. Loseinkit, K. Uchiyama, S. Ochi, T. Takaoka, K. Nagahisa and S. Shioya. (2001). "Characterization of bacteriocin N 15 produced by *Enterococcus faecium* N 15 and cloning of the related genes." *Journal Bioscience Bioengineer*, 91: 390-395.

[14] M. Marbrouk, M. Hamdy, M. Mamdouh, M. Aboelfotoh and Y. M. Kadah. (2006). "BIOINFTool: bioinformatics and sequence data analysis in molecular biology using Mat Lab." *Proc. Cairo International Biomedical Engineering Conference*, 01-09.

[15] P. Neysen and DE Vuyst, L. (2005). "Kinetic and modelling of sourdough lactic acid bacteria." *Trends Food Science Technology*, 16: 95-103.

[16] M. Nei. (1987). "Molecular Evolutionary Genetics." *New York: Columbia University Press*.

[17] T. O'Keeffe, Hill and R. P. Ross. (1999). "Characterization and heterologous expression of the genes encoding enterocin a production, immunity, and regulation in *Enterococcus faecium* DPCII46." *Applied Environmental Microbiology*, 65: 1506-1515.

[18] E. Rodriguez, B. Gonzalis, P. Gaya, M. Nunez and M. Medina. (2000). "Diversity of bacteriocin produced by lactic acid bacteria isolated from raw milk." *International Dairy*, 10: 7-15.

[19] M. Rashid, K. Togo, M. Ueda, and T. Miyamoto. (2006). "Identification and characterization of dominant lactic acid bacteria isolated from traditional fermented milk Dahi in Bangladesh." *World Journal Microbiology Biotechnology*, 23: 125-133.

[20] A. Savadogo, C. A. Outtra, I. Bassole, and A. Traore. (2004). "Antimicrobial activities of lactic acid bacteria strains isolated from brukina faso fermented milk." *Pakistan Journal of Nutrition*, 3: 174-179.

[21] R. Sharma, B. S. Sanodiya, G. S. Thakur, P. Jaiswall, S. Pall, A. Sharma, and S.B. Prakash. (2013). "Characterization of lactic acid bacteria from raw milk samples of cow, goat, sheep, camel and buffalo with special elucidation to lactic acid production." *British Microbiology Research Journal*, 3: 722-756.

[22] S. Thokchom and S. R. Joshi. (2012). "Probiotic and bacteriocin efficacy of lactic acid bacteria from traditionally fermented foods: a review." *Journal of Science and Technology: Biological and Environment Sciences*, 10: 142-155.

[23] P. Vandamme, B. Plot, M. Gillis, P. De vos, K. Kersters and J. Swings. (1996). "Polyphasic taxonomic, a consensus approach to bacterial systematic." *Microbiology Review*, 60: 407-438.

[24] J. Xiong. (2006). "Book on essential bioinformatics." *Cambridge University Press, USA, I: 127-128*