BACTERIAL EMPIRE
2021, VOL. 4, NO. 3, e248

# ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF *Lactobacillus fermentum* EIPW5A ISOLATED FROM WHEY

*Dipanwita Bhattacharjee and Barun K Bhattacharyya*

***Address (es):***
Biotechnology - Research and Development, East India Pharmaceutical Works Ltd. 119, Biren Roy Road (West), Kolkata - 700061, INDIA.

*Corresponding author: microbio@eastindiapharma.org

https://doi.org/10.36547/be.248

**ABSTRACT**

The probiotic organisms are now used widely for different clinical indications. In an attempt to isolate a good probiotic strain for therapeutic applications, we have screened several isolates having probiotic attributes. The essential probiotic characters such as lactic acid production, antimicrobial activity, acid and bile tolerance, vitamin $B_{12}$ production and antibiotic resistance pattern were considered as parameters for screening of probiotic bacteria from its natural habitats. Considering the said probiotic properties the strain EIPW5A was selected for the present study. The organism was identified as *Lactobacillus fermentum* based on its morphological, biochemical, physiological characters and 16S rRNA gene sequencing results.

**Keywords:** *Lactobacillus fermentum*, identification, probiotic, acid and bile tolerance, Vitamin $B_{12}$, Cholesterol lowering property

## INTRODUCTION

The illness caused by compromised gut microflora due to gastrointestinal infections, irritable bowl syndrome and antibiotic induced diarrhea have increased the conscious habit of probiotic consumption. There are about 1000 different species of bacteria residing in the colon. The bacterial population in the tissue of colon comprised of approximately $10^{11}$ - $10^{12}$ CFU/g of bacteria **(Slavin., 2013)**. Normally, there is a balance, exist between pro-health and anti-health organisms in the gut. However, when this delicate ecological balance is perturbed by environmental and physiological factors, predisposition to infectious and immunoinflammatory disease is enhanced. Administration of specific strains with probiotic attributes is an option to optimize gut microflora. Majority of these probiotic (Greek meaning "for life") organisms are the members specifically from the genera *Lactobacillus*, *Lactococcus* and *Bifidobacterium* (**McFarland & Elmer, 1997; Kaur et al., 2002**). As advocated by WHO (World Health Organization) these organisms should be considered as important components of gastrointestinal microflora and generally recognized as safe (GRAS).

The origin of most of the established probiotic organisms and their formulations in the market are limited to Western countries and Japan. This limitation coupled with patenting and IPR issues also restrict their universal applications in commercial purposes. In a country like India, the market for probiotic is still in infancy, due to inaccessibility to the established authentic probiotic cultures. At the same time as the efficacy of the probiotic organisms depend on the regional variation of human population, so there is a need to have our own collection of well defined indigenous probiotic microorganisms after proper characterization. These strains should work in a much better way as our ecology of intestinal microflora does not match with the Western or Japanese counterpart.

In an attempt to search for such a potential probiotic organism, we have screened a strain (EIPW5A) of *Lactobacillus fermentum*. This strain was isolated from whey and has promises to be a good candidate of probiotic organism fulfilling all the statutory criteria **(WHO., 2001)**.

## MATERIALS AND METHODS

### Isolation and identification of bacterial isolate

For the isolation of probiotic strains, samples were obtained from whey of different sweet shops. These samples were cultured on MRS (**De Man et al., 1960**) agar plates and incubated at 37°C for 24 hrs. Among the few selected strains, EIPW5A was selected for syudy of its probiotic characters. The identification experiments were carried out both phenotypically and genotypically. The phenotypic characterization was done morphologically, physiologically and biochemically according to the Bergey's Manual of Systematic Bacteriology, **(Scheifer.,2009)**.

### Genotypic identification

The 16S rRNA sequencing and BLAST analysis for genotypic identification were done at IMTECH, Chandigarh, India. The 16S rRNA sequence was deposited at NCBI. A phylogenetic tree analysis was also done with other related speceies of *Lactobacillus* by using "One Click" mode of Phylogeny Analysis. (**http://www.phylogeny.fr/version2 cgi/index.cgi** ).

The following criteria were used to screen and select the strain EIPW5A as a good probiotic organism.

### Antibiotic susceptibility test

The antibiotic susceptibility patterns of the isolates were determined by using the agar diffusion method in MRS agar media using different clinically used antibiotics (**Tinrat et al., 2011**).

### Estimation of lactic acid

Production of D- and L-lactic acids were estimated in MRS medium after 24 hrs fermentation at 37°C. The lactic acid produced was determined using enzymatic method using lactate dehydrogenase and NAD **(Gutmann and Wahlefeld., 1974) .**

### Antimicrobial activity against pathogenic organisms

For the detection of antimicrobial activity of the isolate EIPW5A against pathogenic organisms, four test microorganisms (*Escherichia coli* NCTC9002, *Salmonella abony* NCTC6012, *Staphylococcus aureus* ATCC6538, *Pseudomonas aeruginosa* NCTC6750) were considered. The agar cup (9 mm, diameter) assay was performed (**Jack et al.,1995**) in nutrient agar medium using cell free supernatant and neutralized cell free supernatant (after fermentation) of isolate EIPW 5A as antibacterial test solution. The inhibition zones were recorded as the mark of antibacterial activity.

### Acid tolerance

The acid tolerance of the isolate EIPW5A was determined by exposing the stationary phase cells of the organism in MRS broth (**De Man et al., 1960**) previously adjusted to pH 3, 4, 5 and 6.5 with 6N HCl (**Sirilun et al., 2010**). The cells of isolate EIPW5A were kept at above said media of different pH for 1 h at 37°C. Acid tolerance was determined by comparing the final plate count of different pH values (after the exposure) against the control (pH 6.5) plate count.

### Bile tolerance

The bile tolerance of isolate EIPW5A was measured by using conjugated bile oxgall (SIGMA). The stationary phase cells of the isolate strain EIPW5A were subjected to expose different concentration of oxgall (0.5%, 1%, 2% and 3%) containing MRS broth (**Pereira et al., 2003**).The organism was incubated for 1 h at 37°C (**Sirilun et al., 2010**). The bile tolerance was determined by comparing the final plate count at different concentration of oxgall (after 1 h exposure) against control (without bile) plate count.

**Qualitative detection of vitamin $B_{12}$ production**

Preparation of cell free extract: In order to analyze the production of vitamin $B_{12}$ by the isolate EIPW5A, fermentation was done using vitamin $B_{12}$ assay (B12 free) medium (DIFCO). The fermentation was carried out at $37^0$C for 24 h. The cell free supernatant was used as vitamin $B_{12}$ containing test solution.

The agar cup assay using *Salmonella enterica* seovar *typhimurium* AR2680 (**Taranto et al.,2003**) was done for detection of vitamin $B_{12}$ in cell free supernatant of fermented broth of isolate EIPW5A. The strain AR2680 was grown in TY (**Atlas., 2010**) medium anaerobically. The cells were then harvested with 0.1M phosphate buffer (pH 7) and resuspended in original volume. These cells were then mixed with minimal agar medium (**Davis & Mungioli., 1950**) and plated. After solidification three wells (9 mm diameter) were made in each plate. The cell free fermented broth of EIPW 5A (test solution), standard vitamin $B_{12}$ solution (positive control) and uninoculated vitamin $B_{12}$ free medium (negative control ) were loaded in each of the three wells respectively of the individual plate. The diameter of growth area surrounding the well containing standard vitamin $B_{12}$ solution was compared for the detection of vitamin $B_{12}$ with the growth area surrounding the well containing cell free extract of isolate EIPW5A (**Taranto et al., 2003**).

**Cholesterol lowering property**

The isolate EIPW5A was analysed for its in vitro cholesterol lowering property. The strain EIPW5A was grown in MRS broth (**De Man et al., 1960**) supplemented with 0.1% (w/v) oxgall (SIGMA) and cholesterol (70 µg $mL^{-1}$; SRL, India). This medium was inoculated (1% v/v) with isolate EIPW5A and incubated anaerobically. The cells from the fermented broth were removed by centrifugation. The total amount of cholesterol present in cell free extract and uninoculated medium were determined (**Rudel & Morris., 1973**).

**RESULTS**

After screening strain EIPW5A was selected for identification and evaluation of probiotic properties. The strain EIPW5A was isolated from whey. The isolate was identified phenotypically and genotypically. It was found to be rod shaped, nonsporulating, nonmotile, Gram positive in nature (table1A and 1B). It could grow well at $37^0$C temperature as well as at a pH 5 to 6.5 in MRS medium and MRS medium supplemented with upto 2% NaCl. However it failed to grow at $15^o$C . The strain was found catalase negative and indole negative. It could produce lactic acid and hydrolyse casein but could not break down gelatin (table 2). In addition, the strain was able to ferment glucose, mannose, raffinose, ribose, sucrose, galactose but not arabinose, cellobiose, maltose, trehalose, rhamnose (table 3). Considering the above characteristics and following Bergey's Manual of Systematic Bacteriology (**Schleifer., 2009**), the strain EIPW5A was designated as *Lactobacillus fermentum*.

**Table 1A** Colony morphology of strain EIPW5A

| Strain no. | Configuration | Margin | Elevation | Surface | Pigment | Opacity |
|---|---|---|---|---|---|---|
| EIPW5A | Circular | Entire | Convex | Smooth | Absent | Opaque |

**Table 1B** Cell morphology of strain EIPW5A

| Strain no. | Shape | Size | Arrangement | Spore | Mobility | Gram character |
|---|---|---|---|---|---|---|
| EIPW 5A | Rod | 2-3 µm | Singly,in pairs and in short chains | Non sporulating | Non motile | Gram positive |

**Table 2** Physiological and biochemical characteristics of EIPW5A

| Strain no. | Physiological characteristics | | | | | Biochemical characteristics | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Growth under aerobic condition | Optimum growth temperature | Growth at 15°C | Growth pH | NaCl tolerance | Catalase test | Indole test | Nitrate reduction | Acid production | Casein hydrolysis | Gelatin hydrolysis |
| EIPW 5A | Positive | 37°C | Negative | 5-7 | Upto 2% | Negative | Negative | Negative | Positive | Positive | Negative |

**Table 3** Carbohydrate utilization pattern of the strain EIPW5A

| Carbon Source | Test Results |
|---|---|
| Arabinose | - |
| Cellobiose | - |
| Glucose | +++ |
| Galactose | +++ |
| Maltose | - |
| Mannose | +++ |
| Melizitose | - |
| Melibiose | + |
| Raffinose | +++ |
| Ribose | +++ |
| Sucrose | +++ |
| Trehalose | - |
| Xylose | + |
| Rhamnose | - |

**Legend:** No growth, +, Feeble growth ,+++, Good growth experiment was done in duplicate set.

This phenotypic identification was further validated and confirmed by identifying the isolate EIPW5A genotypically using 16S rRNA gene sequencing. The sequence of 16S rRNA from the strain EIPW5A was aligned with the nucleotide BLAST programme. The results showed that isolate EIPW 5A may be *Lactobacillus fermentum* and closely related with the 16S rRNA gene sequence of *Lactobacillus fermentum* CECT562 (99% homology). It was also found (Figure 1) that phylogenetically the isolate EIPW5A falls within the *Lactobacillus reuteri* group (**Schleifer., 2009**). The 16S rRNA sequence was deposited at NCBI (Banklt) and accession number of the strain was obtained (KF932274).

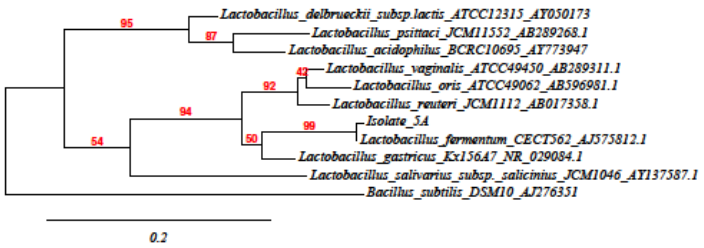


**Figure 1** Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain EIPW5A with other related strains of *Lactobacillus.*

The antibiotic susceptibility test of isolate EIPW5A showed that the organism was sensitive to the majority of the clinically used antibiotics. The strain

EIPW5A was found sensitive to penicillin, ampicillin, amoxycilin which are categorized by property of inhibiting the synthesis of prokaryotic cell wall. The isolate EIPW5A also showed sensitivity to antibiotics like chloramphenicol and tetracycline which were classified by the property of inhibiting protein synthesis. However the strain EIPW5A was found resistant to aztreonam (table 4).

**Table 4** Antibiotic sensitivity of the strain EIPW5A

| Antibiotic | Sensitivity |
|---|---|
| P (2 units) | S |
| A (10µg) | S |
| AM (10µg) | S |
| S (25 µg) | S |
| NX (10 µg) | S |
| OF (5 µg) | S |
| LE (5 µg) | S |
| CF (5 µg) | S |
| T (30 µg) | S |
| V (30 µg) | S |
| C (30 µg) | S |
| AO (30 µg) | R |

**Legend:** Penicillin (P), Ampicillin (A), Amoxycillin (AM), Streptomycin (S), Norfloxacin (NX), Ofloxacin (OF), Levofloxacin (LE), Ciprofloxacin (CF), Tetracycline (T), Vancomycin (V), Chloramphenicol (C), Aztreonam (AO) The experiment was done in duplicate set.

Enzymatic method used to estimate L- & D- lactic acid showed that EIPW5A produced 4.89 g/l of L- lactic acid and 3.9 g/l of D- lactic acid. The cell free supernatant of isolate EIPW5A showed inhibitory activity against the four pathogenic strains tested. The degree of inhibition (diameter of inhibition zone) was similar in case of four test organisms (table 5). However, when antibacterial activity assay was done using neutralized (pH 7) cell free extract (of strain EIPW5A), no inhibition zone was recorded. So, it is obvious that the antibacterial activity of the strain EIPW5A cell free extract was due to the organic acid (lactic acid) produced during fermentation.

**Table 5** Antibacterial activity of strain EIPW5A against test organisms

| Test organisms | Antibacterial activity (diameter of inhibition zone in mm) / 660 µg of total lactic acid) |
|---|---|
| *Escherichia coli* NCTC9002 | 13 ± 0.2 |
| *Salmonella abony* NCTC6012 | 14 ± 0.2 |
| *Staphylococcus aureus* ATCC6538 | 13 ± 0.3 |
| *Pseudomonas aeruginosa* NCTC6750 | 14 ± 0.3 |

**Legend:** All the experiments were done in duplicate sets.

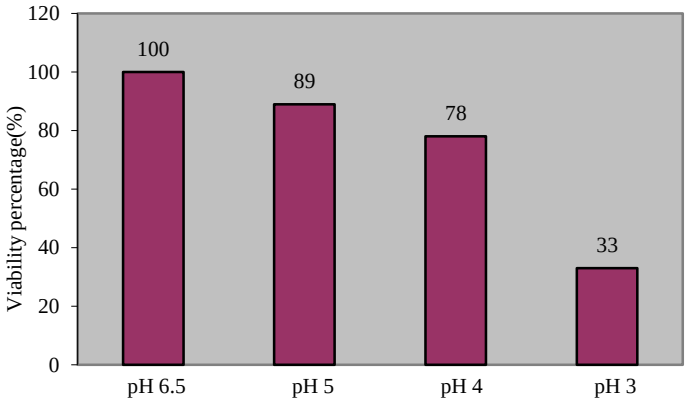


**Figure 2** Effect of exposure to different pH on viability of strain EIPW5A
**Legend:** All the experiments were done in duplicate sets.

The effect of lower pH on the isolate EIPW5A was evaluated (Figure 2). The strain showed tolerance to pH 3-5 for one hour with variations in percentage of viability. The viable count of EIPW5A in lower pH showed good acid tolerance upto pH 4 with more than 78% viability after incubation for 1 h at 37°C.

The bile tolerance of isolate EIPW5A was determined by exposing the strain to different concentration of oxgall for 1 h at $37^0$C. It was found that there was no log reduction in viable cell count (table 6) in case of 0.5% ($8X10^6$ CFU $ml^{-1}$) to 1.5% ($4.9X10^6$ CFU $ml^{-1}$) oxgall concentrations which indicates better bile tolerance. However at concentration of oxgall 2% ($2.2X10^6$ CFU $ml^{-1}$) and above, viability of organism was found much lower.

**Table 6** Effect of exposure to different concentrations (%) of oxgall on viability of strain EIPW5A

| Oxgall concentration (%) | Viable count (CFU/ml) |
|---|---|
| 0 (control) | $2.1 \pm 0.2 \times 10^8$ |
| 0.5 | $8.0 \pm 0.7 \times 10^6$ |
| 1.0 | $5.7 \pm 0.5 \times 10^6$ |
| 1.5 | $4.9 \pm 0.4 \times 10^6$ |
| 2.0 | $2.2 \pm 0.2 \times 10^6$ |
| 2.5 | $1.5 \pm 0.1 \times 10^6$ |

**Legend:** All the experiments were done in duplicates. CFU: Colony Forming Units

The qualitative detection of vitamin $B_{12}$ production was done by agar cup assay using *Salmonella enterica* serovar *typhimurium* AR2680 (**Taranto et al., 2003**). It was found that the strain AR2680 grows surrounding the well loaded with cell free fermentation broth of isolate EIPW5A (test solution), it also grows surrounding the well loaded with vitamin $B_{12}$ standard solution (positive control). However no growth was found surrounding the well containing uninoculated vitamin $B_{12}$ free media (Figure 3). This indicates that the cell free fermented broth may contains vitamin $B_{12}$ or its analogues.

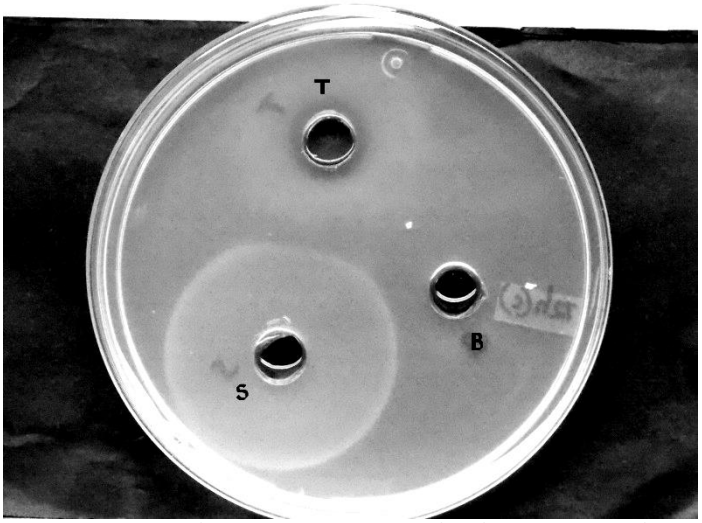


**Figure 3** Detection of Vitamin B12 in the cell free fermented broth of strain EIPW5A by using *S. enterica* serovar *typhimurium* AR2680.
**Legend:** T, culture extract from *L. fermentum* EIPW5A; S, standard cyanocobalamin; B, uninoculated Vitamin B12 free medium.
The experiment was done in duplicate set.

The strain EIPW5A was tested for removal of cholesterol during growth. In this experiment cells (isolate EIPW5A) were inoculated in the medium supplemeted with cholesterol (70µg $ml^{-1}$) and oxgall (0.1% w/v). After 16 h anaerobic growth at $37^0$C, it was found that the cholesterol concentration in the cell free extract was lower than the initial (65 µg $ml^{-1}$). This indicates that the organism EIPW5A can reduce cholesterol (7%) during growth.

**DISCUSSION**

Probiotics have been exploited for centuries in food and pharmaceutical formulations as a source to promote good human health. The essential criteria for the selection of a potential probiotic organism are resistance to gastric acidity, bile resistance, antimicrobial activity against potentially pathogenic bacteria, cholesterol removal and vitamin production (**Ouwehand et al., 1999/WHO 2001** ). Generally the probiotic organisms are used to isolate from their own habitat. The probiotic strain EIPW5A was isolated from whey and identified as *Lactobacillus fermentum* based on phenotypic and genotypic characters. It was found to be gram positive, rod shape, lactic acid producing, catalase and indole negative and can utilize galactose, mannose, raffinose, ribose and sucrose. The 16S rRNA gene sequence of isolate EIPW5A was closely related to type strain *Lactobacillus fermentum* CECT562 (99% similarity) and phylogenetically located within the defined cluster of *Lactobacillus reuterii* group (**Schleifer., 2009**). Generally 16S rRNA gene sequence analysis shows the representative strains from well defined cluster with their type strains in a phylogenetic tree. It was also important that the phenotypic properties and molecular techniques should be accurately combined together to identify any microorganisms at the species level.

The antibiotic resistance pattern is an important character for screening a probiotic organism. The antibiotic resistance is usually an intrinsic property that is chromosomally encoded and nontransmissible (**Zhou et al., 2000**). But as the probiotic organisms are now widely used in food and pharmaceutical industry, the safety assessment for commercial application is now obligatory (**Bernadeau et al., 2008**). More specifically the focus is on transferable antibiotic resistance in novel commercial strains (**Vankerchoven et al., 2008**). It was observed that isolate EIPW5A is sensitive to majority of clinically used antibiotics. The results showed that the strain EIPW5A is sensitive to vancomycin and resistant to aztreonam and nalidixic acid. Vancomycin is of major concern as it is the one of the last antibiotics broadly efficacious against clinical infections caused by multidrug resistant pathogens (**Lavanya et al., 2011**).

The antibacterial property of the isolate EIPW5A was evaluated against four pathogenic strains of bacteria. It was observed that lactic acid was responsible for antibacterial acitivity of the strain EIPW5A. The antimicrobial effect of weak organic acids is principally produced by the undissociated molecules through the acidification of cytoplasm, destruction of transmembrane proton motive force and loss of active transport of nutrient through membrane (**Ray., 1992**).

The probiotic bacteria which are delivered orally must have acid and bile tolerance to survive during transit in the human gastrointestinal tract. Survival at lower pH (**Usman., 1999**) and bile salt (oxgall) concentration 0.15 to 0.3% (w/v) (**Suskovic et al., 2000**) is considered optimal acid and bile tolerance for probiotic strains. In this study *Lactobacillus fermentum* EIPW5A could survive well during 1 h exposure at lower pH (3-4) and 1.5% bile (oxgall).

Physiologically we have a requirement for vitamin $B_{12}$ and suboptimal intake can cause different diseases like neuropathy, birth defects, anaemia, cancer etc (**FAO., 2004**). Isolate EIPW5A can synthesize vitamin $B_{12}$ or its analogue extracellularly which was qualitatively determined by using the organism *Salmonella enterica* serovar *typhimurium* AR2680. The strain AR2680 needs cobalamin to grow in the minimal medium due to cobalamin independent methionine synthase (Met E) mutation. Therefore, the only way to synthesize methionine is through the cobalamin dependent methionine synthase (Met H). Since the strain AR2680 also possess a mutation in cbiB gene (coding for cobinamide synthase), exogenous cobalamin (or a late precursor) has to be added to the medium for its growth (**Taranto et al., 2003**). It was found that cell free extract from EIPW5A can able to correct the cobalamin requirement of strain AR2680.

One of the most important health benefit of probiotic organisms is the reduction of serum cholesterol as evidenced from results of different clinical studies (**Pereira & Gibson., 2003**). The elevated serum cholesterol is one of the major risk for coronary heart diseases (**Levine et al., 1995**). It was reported that 1% reduction of serum cholesterol causes a 2% reduction of the risk for coronary heart diseases (**Hjermann et al., 1981**). The possible mechanisms are assimilation of cholesterol by growing cells, binding of cholesterol to cellular surface, incorporation of cholesterol to cellular membrane, deconjugation of bile via bile salt hydrolase, coprecipitation of cholesterol with deconjugated bile and production of short chain fatty acids by oligosaccharides (**Kumar et al., 2012**). In this report we have found that the isolate EIPW5A can remove cholesterol from media during growth.

So from this study considering the probiotic characteristics of isolate EIPW5A, it can be concluded that the probiotic organism *Lactobacillus fermenum* EIPW5A is a promising candidate to be used as therapeutic agent.

**Acknowledgements:** Authors are thankful to management of East India Pharmaceutical Works Limited, Kolkata for providing facilities and encouragement. Authors also convey a special note of thanks to Dr. Evelyne Deery, University of Kent, Biosciences, Canterbury, UK for providing the strain *Salmonella enterica* serovar *typhimurium* AR2680, IMTECH (MTCC), Chandigarh, India for doing the genotypic identification and NCBI for providing accession number for the 16S rRNA gene sequence. There is no conflicts of interest what so ever among the authors.

**REFERENCES**

Atlas., R. M. (2010). In: Handbook of microbiological media, 4th (Ed.), (pp.1856). Washington: CRC Press, Taylor & Francis.

Bernardeau, M., Vernoux, J. P., Henri-Dubernet, S. & Guegueen, M., (2008). Safety assessment of dairy microorganisms: The *Lactobacillus* genus. *Int J Food Microbiol*,126 (3), 278-285. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.08.015.

Davis, B. D. & Mungioli, E. (1950). Mutants of *E. coli* requiring methionine of $B_{12}$. *J Bacteriol*, 60, 17-28.

De Man, J. C. Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J Appl Bacteriol*, 23, 130-135. http://dx.doi.org/ 10.1111/j.1365-2672.1960.tb00188.x.

Food and Agricultural Organization, World Health Organization (2004). Vitamin and mineral requirements in human nutrition. 2nd ed. Geneva: WHO.

Gutmann I. and Whlefeld A. W. (1974). Methods of Enzymatic Analysis. 2nd Edition. Vol. 3. Academic Press, New York.

Hjermann, I., Byre, K., & Holme, I., (1981). Effect of diet and smoking intervention on the incidence of coronary heart diseases. *Lancet*, 2 (8259), 1303-1310. http://dx.doi.org/10.1016/s0140-6736(81)91338.

Jack, R. W. Tagg, J. R. & Ray, B. (1995). Bacteriocins of gram positive bacteria. *Microbiol Rev*, 59, 171-200.

Kaur, I. P. Chopra, K. & Saini, A. (2002). Probiotics: potential pharmaceutical applications. *Eur J Pharma Sci*, 15, 1-9. http://dx.doi.org/10.1016/S0928-0987(01)00209-3.

Kumar, M. Nagpal, R. Kumar, R. ……Yadav, H. (2012). Cholesterol lowering probiotics as potential biotherapeutics for metabolic diseases. *Exp Diabetes Res*, 1-14. http://dx.doi.org/10.1155/2012/902917.

Lavanya, B. Sowmiya, S. Balaji, S. & Muthuveltan., B. (2011). Screening and characterization of lactic acid bacteria from fermented milk. *British J Dairy Sci*,2(1), 5-10.

Levine, G. N. Keaney, J. F. & Vita, J. A. (1995). Cholesterol reduction in cardiovascular disease. Clinical benefits and possible mechanisms. *N Engl J Med*,332, 512-521. http://dx.doi.org/10.1056/NEJM199502233320807.

McFarland, L. V. & Elmer, G. W. (1997). Pharmaceutical probiotics for the treatment of anaerobic and other infections. *Anaerobe*, 3, 73-78. http://dx.doi.org/10.1006/anac.1996.0062.

Ouwehand, A. C. Kirjavainen, P.V. Shortt, C. & Salmien, S. (1999). Probiotics: mechanisms and established effects. *Int Dairy J*,9, 43-52. http://dx.doi.org/10.1016/50958-6946(99)00043-6.

Pereira, D. I. A. McCartney, A. L. & Gibson, G. R. (2003). An in vitro study of the probiotic potential of a bile salt hydrolyzing *Lactobcillus fermentum* strain, and determination of its cholesterol lowering properties. *Appl Env Microbiol*, 69, 4743-4752. http://dx.doi.org/10.1128/AEM.69.8.4743-4752.2003.

Ray, B. (1992). Acetic, propionic and lactic acids of starter culture bacteria as biopreservatives. In Food biopreservatives of microbial origin, (pp 103-136). [Ray B and Daeschel M, editors]. Florida: CRC Press. Boka Raton.

Rudel, L. L. & Morris, M. D. (1973). Determination of cholesterol using o-pthalaldehyde. *J Lipid Res*,14, 364-366.

Schleifer, K. H. (2009). Lactobacillaceae. In: *Bergey's Manual of Systematic Bacteriology* 2nd (Eds.), (vol. 2). New York: Springer.

Sirilun, S. Chaiyasut, C. Kantachote, D. et al (2010). Characterization of non human origin probiotic *Lactobacillus plantarum* with cholesterol lowering property. *African J Microbiol Res*, 10, 994-1000.

Slavin, J. (2013). Fibre and probiotics : Mechanisms and health benefits. *Nutrients*, **5**, 1417-1435. http://dx.doi.org/10.3390/nu5041417.

Suskovic, J. Kos, S. Matosic, S. & Besendrofer, V. (2000). The effect of bile salt on survival and morphology of a potential probiotic strain *Lactobacillus*

*acidophilus* M92. *World J Microbiol Biotechnol*,16, 673-678. http://dx.doi.org/10.1023/A:1008909505651.

Taranto, M. P. Vera, J. L. Hugenholtz, J….. Sesma, F. (2003). *Lactobacillus reuteri* CRL1098 produces cobalamin. *J Bacteriol* , 185, 5643-5647. http://dx.doi.org/10.1128/jb185.18.5643-5647.203.

Usman, H. A. (1999). Bile tolerance, taurocholate deconjugation and binding of cholesterol by *Lactobacillus gasseri* strains. *J Dairy Sci*, 82 (2), 243-248.

Vankerchoven, V. Huys, G. Vancanneyt, M. Yacl ,C. & Goossens, H. (2008). Biosafety assessment of probiotic used for human consumption: Recommendation from the EU-PROSAFE project. *Trends Food Sci Tech*, 19 (2), 102-114. http://dx.doi.org/10.1016j.tifs.2007.07.013.

Yu, J. Gao, W. Qing, M.…. Zhang, H. (2012). Identification and characterization of lactic acid bacteria isolated from traditional pickles in Sichuan. *China J Gen Appl Microbiol*,58, 163-172. http://dx.doi.org/10.2323/J9am58.163.

Zhou, J. S. Shu, Q. Rutherford, K. J. …. Gill, H. S. (2000). Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN97 and *Bifidobacterium lactis* HN019 in BALB/c mice. *Int J Food Microbiol*,56, 87-96. http://dx.doi.org/10.1016/s0168-1605(00)00219-1.