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ISOLATION, SCREENING, CHARACTERIZATION AND DETERMINATION OF B-COMPLEX VITAMINS BY *LACTOBACILLUS* STRAINS FROM DIFFERENT SOURCES OF MEAT

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ABSTRACT

In this study, isolation, screening, characterization, antibiotic susceptibility tests, curing & Determination of B-complex vitamin production by potent probiotic *Lactobacillus* strains from different sources of meat was analysed. 250 grams of intestine parts of goat meat and beef each were transferred to MRS broth to screen the potent probiotic *Lactobacillus* strains. Initially 57 strains were isolated and confirmed by gram`s staining and catalase tests. After that, 51 strains selected for further biochemical tests to confirm all were Lactobacillus. Later, 41 strains were selected for antibiotic susceptibility tests by using three different classes of antibiotics viz., tetracycline, ampicilin, and streptomycin. Those strains which showed resistant to all three antibiotics used, further selected for curing of plasmid DNA and again antibiotic susceptibility tests was performed by the same antibiotics used before. Finally, only 5 strains were selected as potent probiotic *Lactobacillus and* unfortunately all strains from Beef intestine derived. Then, determination of B-Complex vitamin production of those selected 5 potent probiotic *Lactobacillus* by thin layer chromatographic method; Spectroflurophotometric method and RP-HPLC method were performed. From those methods confirmed as, all the five *Lactobacillus* strains were producing only riboflavin among the B-Complex vitamins, in trace amounts when compared to standard riboflavin and control (media without *Lactobacillus*). Those five isolated potent probiotic strains of *Lactobacillus* were named as B-01; B-06; B-12; B-21 & B-23. (The term B refers *Lactobacillus* strains isolated from Beef intestine and numeric value refers particular strain).

INTRODUCTION: Probiotics have been defined as “live microbial food supplements which beneficially affect host by improving the intestinal microflora balance” or more broadly as “living microorganisms, which upon ingestion exert health benefits beyond inherent general nutrition”. There are, more recently, increasing experimental and clinical data to support the use of proven probiotic organisms in prevention and treatment of many gastrointestinal disorders. These include inflammatory bowel disease, infectious and antibiotic related diarrheas, and post – resection disorders including pouchitis. *Lactobacillus* is commercially important bacterium with wide variety of applications, both in food industry and as a probiotic agent for the improvement of human health [1, 2]. The long history of its use in food industry and its “generally regarded as safe ”(GRAS) status render it a promising bacterial strain for genetic modification [1].

Genetic modifications to *Lactobacillus* strains are normally targeted toward the improvement or augmentation of specific strain characteristics, such as the production of compounds antagonistic to common food pathogens [3-6]. Attempts have also been made to transform *Lactobacillus* strains into delivery vehicles for biological compounds, particularly for the delivery of enzymes in to the gut of the host, thereby enhancing digestion [7].Under ideal conditions, these modified strains would benefit the host.

However, under natural conditions, the performance of the modified *Lactobacillus* strains is frequently affected by indigenous plasmid. Especially in cases in which the manipulations are plasmid mediated. Incompatibility between the indigenous plasmids and the introduced plasmids is one of the principle factors contributing to plasmid instability within a host [8]. These plasmids may not only interfere with the stability of the recombinant plasmid, but also harbor undesirable traits, e.g., antibiotic resistance [8]. Thus, before genetic modification studies can be considered with such a strain, it becomes necessary to eliminate such indigenous plasmids.

Here, the aim of this study is to investigate the B-complex vitamin production by *Lactobacillus* strains which are isolated from different sources of meat**.** Meat is defined as the parts of livestock muscles that is skeletal or is found in the tongue, diaphragm, heart, oesophagus with or without the accompanying of overlaying fat and the portion of the bone, skin, sinew, nerves and blood tissue that normally accompany the muscle tissue and are not separated from it in the process of dressing [9]. Here, the meat portion taken is the cutted pieces of goat intestine and beef intestine for analysis of B-complex vitamin production by *Lactobacillus* strains.

MATERIALS AND METHODS:

Isolation of Lactic Acid Bacteria from Goat and Beef Intestine:

Steps involved:

1. Meat portion of goat intestine and beef intestine were taken separately and put in MRS broth for 5 minutes to isolate *Lactobacillus.*
2. Then transferred the *Lactobacillus from* broth to the petri plates having MRS agar media with the help of loop.
3. Allow for 24 hrs incubation to get clear colonies of *Lactobacillus* strains.
4. Then transfer the single colony of *Lactobacillus* strains to the boiling tubes having MRS broth with help of tooth pick and kept in shaker for 24 hrs.

After 24 hrs, *Lactobacillus* strains were transferred to the petri plates having MRS agar media with help of loop to get individual strains of *Lactobacillus* [5].

Preliminary & Biochemical tests to confirm LAB: Preliminary studies like Gram's staining and catalase test was performed to confirm isolated species were Lactobacillus. Later, Biochemical tests like Gas Production Test, Arginine Test, and Growth at 45^{o}C, 15^{o}C, and 10^{o}C temperatures were performed to confirm exactly lactobacillus species [5].

Antibiotic susceptibility tests before and after curing: Streptomycin, Ampicilin, Tetracycline were taken in the range of 10 mcg/disc, 10 mcg/disc, 30 mcg/disc respectively from HIMEDIA for antibiotic susceptibility test. Then MRS broth contained 41 strains were individually transferred to MRS Agar plates by spread plate technique & kept in incubator at 37^{o}C for 24 hrs. Next day, Zone of inhibition for those antibiotics was measured according to the standard zone size interpretative chart given by HIMEDIA [9, 10, 28].

Curing of Plasmid DNA: Curing of plasmid DNA (means removal of plasmid DNA) can be done by using various curing agent like Ethdium bromide (Etbr), Novobiocin, acriflavin, SDS (Sodium dodecyl lauryl sulphate [6, 24]. Here, Ethdium bromide (Etbr) was used as curing agent to cure the plasmid DNA in the concentration of 12 micro liter/ml of organism culture (Posono et*. al.,)* and kept incubation overnight. Next day, isolation of plasmid DNA was carried out by agrose gel method to confirm whether curing has happened or not? Then again antibiotic susceptibility tests by those antibiotics were used before. The procedure followed for isolation of plasmid DNA was alkaline lysis method. Finally 5 isolates were selected as potent probiotic [27, 30].

Determination of B-complex vitamin production by 5 isolated potent probiotic *Lactobacillus* strains:

- **By Thin Layer Chromatographic Method (separation of B-complex vitamins):** Using TLC method, Separation of B-Complex vitamins production from 5 isolated Lactobacillus strains against standards from HIMEDIA such as Vitamin B-1; B-2; B-12 & Folic acid [22, 26, 29]. The Stationary phase used was Silica gel coated glass plate and Mobile phase used was n-propanol-butanol, water and ammonia in the ratio of 7: 5: 1: 2. The Rf value (in cm) is calculated by using formula [16, 14]:

$$\frac{\text{Distance travelled by solute front}}{\text{Distance travelled by solvent front}}$$

- **By Spectroflurophotometry Method (Quantitative Analysis of Riboflavin):** This method involves the spectral analysis of riboflavin excitation wavelengths measured at 440 nm and emission wavelengths measured at 510 nm.Quantitative analysis of Riboflavin at wavelength of 440-510 nm was analyzed [20, 23].

- **By RP-HPLC method (qualitative analysis of riboflavin):** The RP- HPLC system of Shimadzu was used consisted of RP column. A solvent System of 0.1% Ortho phosphoric acid, Acetonitrile, Water in the ratio of (0.1: 9: 91 v/v) were used. The sample flow rate of 0.1 ml / min was injected. From the chromatogram obtained, Retention time was observed for all the samples injected and compared with standard riboflavin [17, 25, 11].

RESULTS AND DISCUSSION:

Preliminary & Biochemical tests to confirm LAB: Preliminary Tests such as Gram`s staining, Catalase tests and Biochemical tests like gas production tests, Arginine tests ,Growth at different temperatures 45°C, 15°C, 10°C were performed and confirmed as 41 strains (21 from Goat & 20 from Beef) were Lactobacillus according to the following flow chart [5];

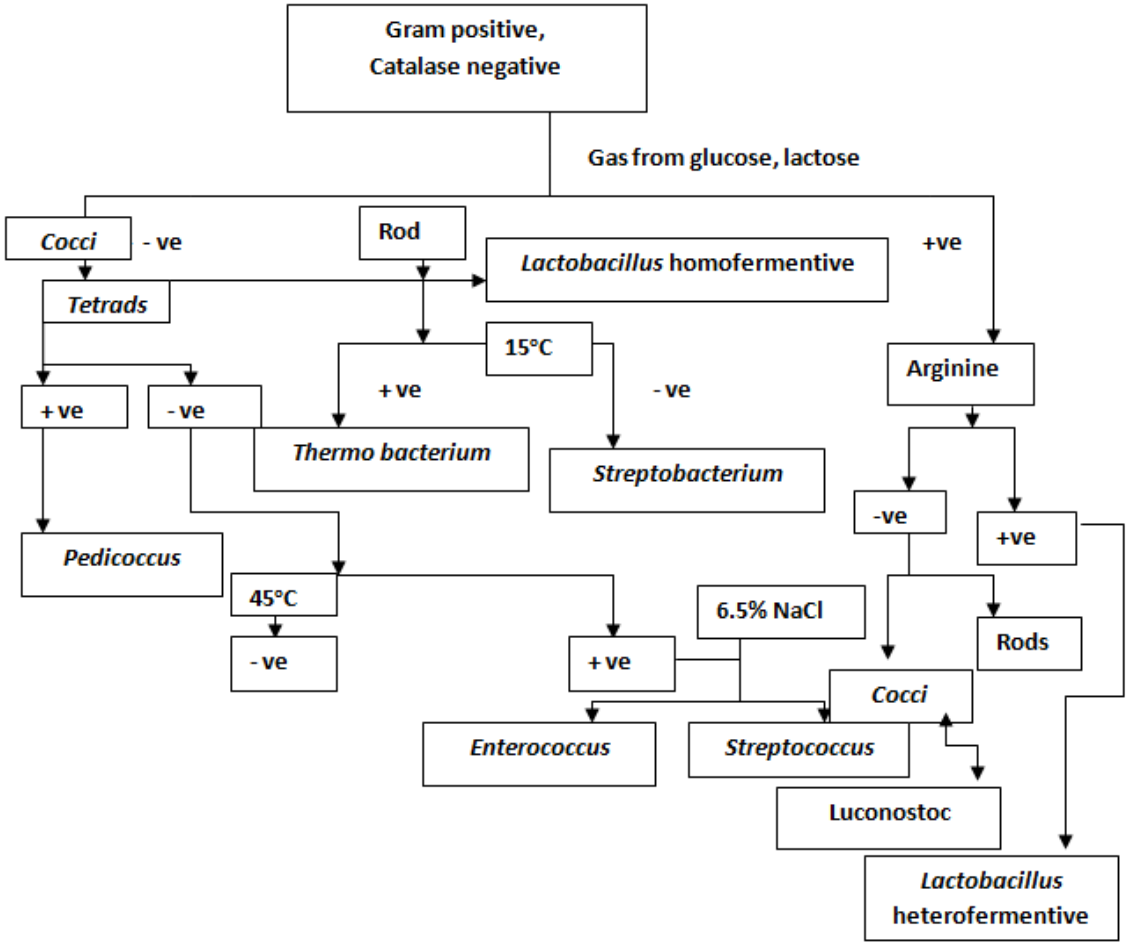

FIG. 1: LACTOBACILLUS CONFIRMATION CHART (*BERGEY'S MANUAL,* BUCHANAN & GIBBONS, 1974)

Probiotic Characteristics:

- **Antibiotic susceptibility tests before curing:**

TABLE 1: ISOLATED STRAINS FROM GOAT INTESTINE:

Strains	Streptomycin (10mcg/disc) zone of inhibition in mm diameter	Ampicilin (10mcg/disc) Zone of inhibition in mm diameter	Tetracycline (30mcg/disc) Zone of inhibition in mm diameter
G-02	1	20	28
G-03	1	22	19
G-05	0	18	23
G-06	0	15	39
G-07	0	17	38
G-12	**0**	**0**	**0**
G-13	**0**	**14**	**0**
G-14	0	20	31
G-15	**0**	**20**	**16**
G-16	0	27	27
G-17	0	18	19
G-18	0	12	19
G-19	**0**	**16**	**18**
G-20	0	16	29
G-21	0	0	26
G-22	**0**	**15**	**10**
G-23	21	22	26
G-25	22	20	30
G-26	0	20	20
G-27	0	19	19
G-28	0	18	22

TABLE: 2 ISOLATED STRAINS FROM BEAF INTESTINE

strains	Streptomycin (10mcg/disc) zone of inhibition in mm diameter	Ampicilin (10mcg/disc) Zone of inhibition in mm diameter	Tetracycline (30mcg/disc) Zone of inhibition in mm diameter
B - 01	**0**	**17**	**0**
B - 02	11	20	28
B - 03	18	22	19
B - 04	**0**	**19**	**16**
B - 05	20	18	23
B - 06	**0**	**24**	**0**
B - 08	**0**	**16**	**16**
B - 09	11	16	20
B - 12	**0**	**13**	**14**
B - 13	**0**	**17**	**17**
B - 16	0	15	22
B - 17	**0**	**20**	**16**
B - 18	21	22	28
B - 19	0	20	36
B - 20	22	23	34
B - 21	**0**	**15**	**14**
B - 22	0	30	11
B - 23	**0**	**10**	**15**
B - 25	17	27	31
B - 28	**0**	**15**	**15**

TABLE -3 STANDARD ZONE OF INHIBITION INTERPRETATIVE CHART (FROM HIMEDIA)

Name of antibiotics	symbol	Disc content	Resistant mm in diameter or less	Intermediate mm in diameter	Sensitive mm in diameter or more
Streptomycin	S	10mcg/disc	0	0	0
Ampicilin	A	10mcg/disc	28	0	29
Tetracycline	T	30mcg/disc	18	19-22	23

- **Antibiotic susceptibility tests after curing:**

TABLE: 4 STRAINS ISOLATED FROM GOAT:

strains	Streptomycin (10mcg/disc) zone of inhibition in mm diameter	Ampicilin (10mcg/disc) Zone of inhibition in mm diameter	Tetracycline (30mcg/disc) Zone of inhibition in mm diameter
G-12	0	20	20
G-13	0	19	22
G-15	0	17	20
G-19	0	26	25
G-22	0	20	22

TABLE: 5 STRAINS ISOLATED FROM BEEF

Strains	Streptomycin (10mcg/disc) zone of inhibition in mm diameter	Ampicilin (10mcg/disc) Zone of inhibition in mm diameter	Tetracycline (30mcg/disc) Zone of inhibition in mm diameter
B - 01	**0**	**17**	**0**
B - 04	**0**	**29**	**26**
B - 06	**0**	**24**	**0**
B - 08	**0**	**16**	**29**
B - 12	**0**	**13**	**14**
B - 13	**0**	**17**	**27**
B - 17	**0**	**20**	**26**
B - 21	**0**	**15**	**14**
B - 23	**0**	**10**	**15**
B - 28	**0**	**15**	**25**

According to the results obtained above, compared with standard zone size interpretative chart given by HIMEDIA (which published by CLSI , formerly NCCLS standards) explained in table 3. Antibiotic susceptibility tests after curing the *Lactobacillus* strains isolated from goat sample didn't obey to standards whereas 5 *Lactobacillus* strains isolated from beef sample obeys standards (selected only resistant values for all the three antibiotics used) which were shown in grey colour background selected as potent probiotics.

Finally, after above screening and characterization methods, only 5 strains of isolated lactobacillus were selected as potent probiotic shown below in table:6 and further determination of vitamin production by thin layer chromatographic methods, and Spectrofluro-photometric methods and HPLC method were done.

TABLE 6: POTENT PROBIOTIC OF 5 STRAINS OF ISOLATED LACTOBACILLUS

B - 01
B - 06
B - 12
B - 21
B - 23

Determination of B-Complex Vitamin Production by 5 Isolated Potent Probiotic *Lactobacillus* Strains:

- **By Thin layer chromatographic methods:** The B-Complex vitamins were analyzed by thin layer chromatographic method which were produced by 5 isolated strains of *Lactobacillus*. Compared with Rf values of standards all the sample`s Rf values match with Riboflavin Rf value exactly, and also it vareies from control . So isolated 5 potent *Lactobacillus* strains producing only B-complex (Riboflavin) vitamins.

- **By Spectroflurophotometric Methods:**

TABLE 7: QUANTIFICATION OF RIBOFLAVIN VITAMIN BY SPECTROFLUROPHOTOMETRIC (@ 440 -510 nm)

Name	Fluorescent intensity of emission data (FI)	Concentration of riboflavin in (µ gm/ml)
Standard (Riboflavin) - 01	073 .14	0.2
Standard - 02	150 .78	0.4
Standard - 03	240 .12	0.6
Standard - 04	342 .16	0.8
Standard - 05	463 .17	1.0

Control(media without organism)		220.059	0.56
Sample	B- 01	344.148	0.79
Sample	B- 06	260.35	0.59
Sample	B- 12	336.698	0.77
Sample	B- 21	326 .31	0.75
Sample	B- 23	385.952	0.88

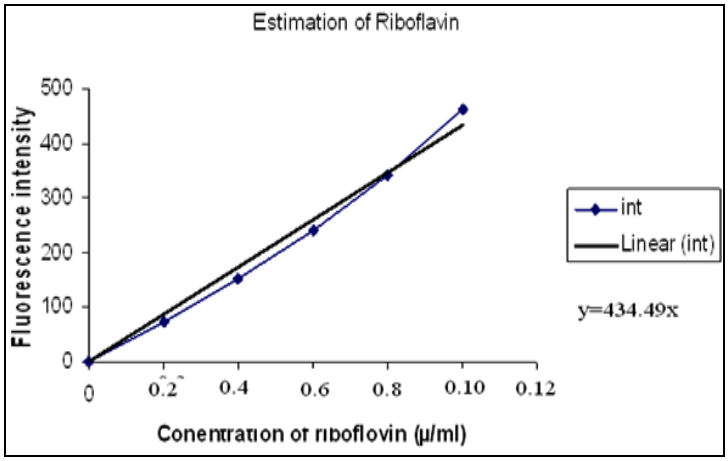

FIG. 2: STANDARD GRAPH FOR ESTIMATION OF RIBOFLAVIN BY SPECTROFLUROPHOTOMETRY

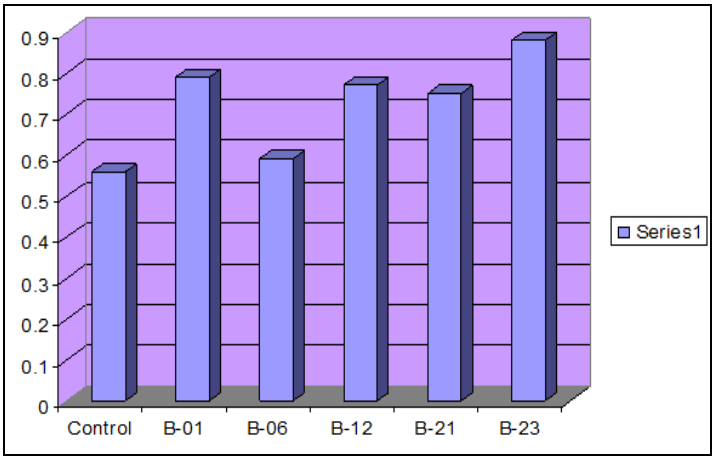

FIG. 3: COMPARISONS BETWEEN CONCENTRATION OF RIBOFLAVIN (µgm/ml) PRESENT IN SAMPLES AND CONTROL

From the standard graph for estimation of riboflavin obtained above (**Fig. 2**), the unknown concentration of riboflavin present in control and samples of B-1; B-6; B-12; B-21; B-23 were calculated as follows :

According to equation,

$$y = mx+c$$

But we need x, So, x = y/m (since, c = negligible); For control, y = 220.059; m= 434.49; then 220.059/434.49= 0.56 µgm/ml

Like wise, unknown concentration of riboflavin in samples as follows:

B- 01 = 0.79

B-06 = 0.59

B-12 = 0.77

B-21 = 0.75

B-23 = 0.88

Comparatively, all the 5 strains producing riboflavin is more than control. The average amounts of riboflavin produced by those strains were 0.20 µg/ml more than control shown in **Fig. 3**.

- **By RP- HPLC -Qualitative Analysis of Riboflavin:** After the result obtained from spectroflurophotometry, further RP-HPLC (Shimadzu LC solution Analysis Report) method was performed to determine the riboflavin production by isolated Lactobacillus strains .Among the 5 isolated strains, only the 2 strains viz., B-01 & B-23 which showed comparatively high concentration of riboflavin production (shown in Fig. 3) were further taken for qualitative analysis of riboflavin by RP-HPLC method **Fig. 4** Chromatogram shows for standard riboflavin (HIMEDIA) compared with sample B-1(**Fig. 5**) and B-23 (**Fig. 6**)

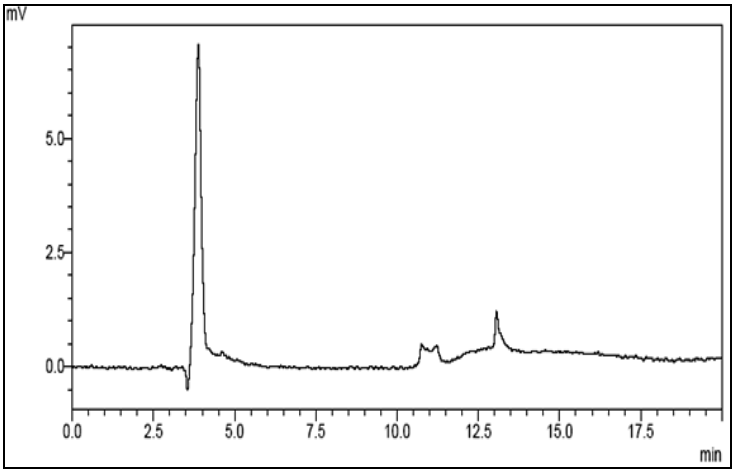

FIG. 4: CHROMATOGRAM SHOWS FOR STANDARD RIBOFLAVIN (HIMEDIA)

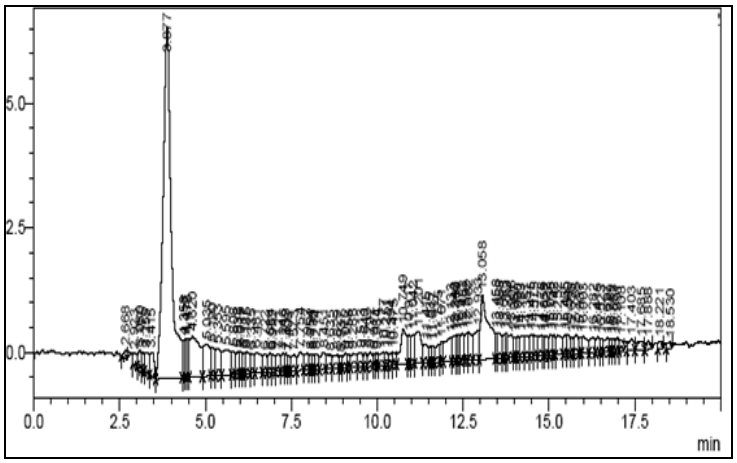

FIG. 5: CHROMATOGRAM SHOWS FOR SAMPLE B-01

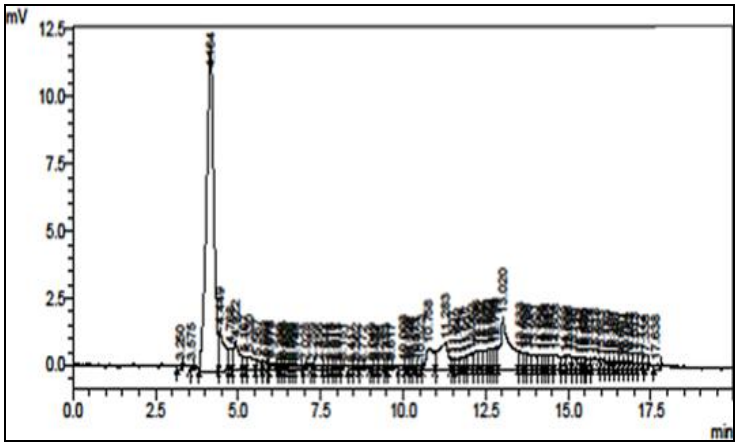

FIG. 6: CHROMATOGRAM SHOWS FOR SAMPLE B-23

From the chromatogram obtained by RP-HPLC method, retention time of both standard and samples were same. This reveals riboflavin were producing by isolated strains of *Lactobacillus.* So, these strains were more beneficial to human.

CONCLUSION: Probiotics plays a vital role in giving the beneficial to human by its eminent activities like role of cholesterol reduction, in the treatment of irritable bowel syndrome, anti cancer activities , antibiotic associated diahorrea and many more.

So, from these experiments the potent probiotic strains from different sources of meat viz., B-01, B-06, B-12, B-21, B-23 were screened, isolated, characterized, antibiotic susceptibility tests before and after curing of plasmids reveals they were potent Probiotics. Determination of B-complex vitamins produced by these strains concluded, these strains having ability to produce only the vitamin riboflavin, which more important to human. The isolated Probiotics strain having the riboflavin production is warmth welcome to neutraceutical field.

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