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In Vitro Inhibition of Pathogenic Bacteria Through Probiotics Lactobacillus Metabolites

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ABSTRACT
Bowel or gastro-intestinal infections are the most prevalent problem amongst all age group people. It's primarily caused by bacteria & virus. Probiotics or good bacteria are the desirable solution for these types of infections. This study was based on the antimicrobial efficiency of probiotic lactobacillus strains against pathogens. Lactobacillus strains isolated from dairy curd. It's essential extra cellular metabolites were extracted by fermentation process & then tested against Salmonella Typhiimurium & Enterococcus faecalis that are responsible for bowel infection. Results showed positive potential of Lactobacillus metabolites. These metabolites were further investigated on different tolerance that eventually showed inhibition against Salmonella Typhiimurium than Enterococcus faecalis. This can give a way to develop more natural medicament for competing gastrointestinal infection.

Keywords: Bowel Infection, Probiotics, Metabolites, Salmonella Typhiimurium, Enterococcus faecalis

INTRODUCTION:-

Probiotics are live microorganisms that, when consumed in sufficient amounts by the host, confer health benefits.(1) Recent studies claimed that probiotics exhibit an indispensable role in the treatment of diseases such as diarrhoea, allergy, diabetes, hypertension, cancer, and genetic disorders and also boost immunity. The probiotic microorganism should be acid-resistant, bile-tolerant, non-carcinogenic, non-pathogenic, adhere to host epithelial tissue, enrich the intestinal micro flora, (3) reduce pathogenic adherence, and able to produce of secondary metabolites antagonistic to pathogen microorganisms. Bowel infection or Gastroenteritis is inflammation of the stomach and intestines. This can cause symptoms ranging from mild to severe. A virus, bacteria, or parasite can cause gastroenteritis. When it's caused by a type of bacterium, it's known as bacterial gastroenteritis. Lactobacilli species produces antimicrobial peptides (e.g. bacteriocins), organic acids (e.g. acetic acid and lactic acid), and other metabolites (e.g. hydrogen peroxide), which are reported to inhibit the growth of pathogenic bacteria. (4)

OBJECTIVE:-

To isolate and characterize Lactobacillus bacteria from dairy curd, evaluate their physiological properties, and investigate the antimicrobial activity of their metabolites against bowel infection-causing pathogens.

METHODOLOGY

1. Isolation of Lactobacillus bacteria

The isolation of Lactobacillus species from local dairy curd was performed. MRS media was prepared, and serial dilutions of the curd sample were carried out. The diluted samples were then inoculated on MRS agar plates. The plates were incubated for 24 hours.

2. Morphological Studies:-For morphological studies, bacterial samples were counted on colony counter machine and then calculated using the appropriate formula. Subsequently, their morphology was studied, where characteristics such as texture, shape, size, and color were observed. Furthermore, for internal morphology, Gram staining was performed to identify the bacteria's shape and type.

3. Biochemical characterisation: -

To identify the Lactobacillus bacteria, biochemical characterization tests were conducted.

Skimmed milk test: - The Skimmed Milk Test was performed to determine the ability of Lactobacillus bacteria to ferment lactose and produce acid. In this test, the isolated bacteria were inoculated into skimmed milk and incubated at a suitable temperature.

Catalase test: - The Catalase Test was performed to determine the presence or absence of catalase enzyme in Lactobacillus bacteria. In this test, a small amount of bacterial culture was added to a slide and a few drops of hydrogen peroxide (H2O2) were added.

Carbohydrate Fermentation test:-The Carbohydrate Fermentation Test was performed to determine the ability of Lactobacillus bacteria to ferment various carbohydrates. In this test, the isolated bacteria were inoculated into tubes containing different carbohydrate sources, such as glucose, lactose, sucrose, and others, and incubated at a suitable temperature.

4. Fermentation Process: -

The fermentation process was initiated by inoculating the bacterial sample into MRS broth. Each container held 40 ml of MRS broth, into which the samples were inoculated. The containers were then capped and allowed to undergo fermentation for approximately 15 days. This fermentation process was conducted as batch fermentation.

5. Extraction of Metabolites:-

After fermentation, heat the medium at 100^0C on boiling water bath for 20 minutes then filter or centrifuge the medium for cell harvesting. Then add 20% TCA in equal volume then keep the sample in deep freezer for overnight to precipitate the produce enzyme/secondary metabolite. For the activation of enzymes use fractional method at different temperature.

6. Anti Microbial Activity: -

The antimicrobial activity of the metabolites produced by the bacterial samples was evaluated against pathogens such as Salmonella Typhi and Enterococcus faecalis. Muller-Hinton media were prepared, and then the pathogens were inoculated onto the media. Discs were dipped in the metabolites and then placed on the inoculated pathogens. The plates were incubated for 24 hours to observe the antimicrobial activity of the metabolites.

OBSERVATION:-

Morphological Analysis of Lactobacillus Analysis

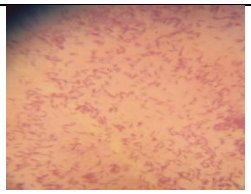
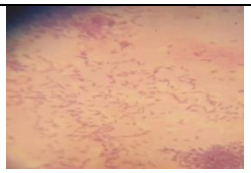
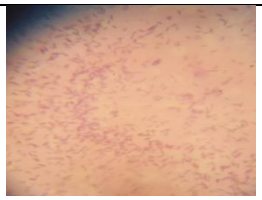
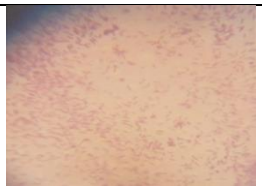
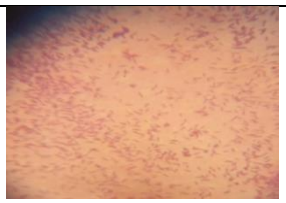
S.No	**Sample No.**	**Colony/Cell type**	**Color**	Gram Staining	CFU/ML
1	Mixed culture	Colonies were Irregular & Filamentous	off White		Mixed colony
2	S5	Colonies were Round with slimy texture. Some colonies were filamentous	White		110,240
3	S4	Colonies were round & filamentous	White & off white		35,916
4	S3	Colonies were irregular & filamentous	white		1,488
5	S2	Colonies were round, irregular & filamentous	White & off white		335

Table:-1 External & Internal Morphological study of bacterial isolates.

BIOCHEMICAL ANALYSIS

Test	**S1 2**	**S1 3**	**S2 2**	**S2 3**	**S3 3**
Catalase test	-	-	-	-	-
Citrate utilization test	+	-	-	+	+
Carbohydrate test	+	+	+	+	+
Litmus Milk test	+	+	+	+	+
Skimmed milk test	+	-	+	+	-
Carbohydrate fermentation test (Glucose, Sucrose, Lactose)	+	+	+	+	+
Indole Test	-	-	-	-	-
MRVP Test	-	-	-	-	-

Table:- 2 Biochemical Analysis of isolated bacterial samples

Experimental Conditions

1. Salinity level: 0.5% to optimize growth and metabolic activity of isolated bacterial extract.
2. pH 4 to favor antimicrobial metabolite production.
3. Fermentation: 40ml of culture, batch fermentation for 15 days.

Post-Fermentation Process

1. Heat treatment: 100°C, 20 minutes, boiling water bath.
2. Cell harvesting: Filtration or centrifugation.
3. Enzyme/secondary metabolite precipitation: 20% TCA, overnight, -20°C deep freezer.
4. Enzyme activation: Fractional method at various temperatures (25^0C,50^0C, 75^0C, 100^0C)

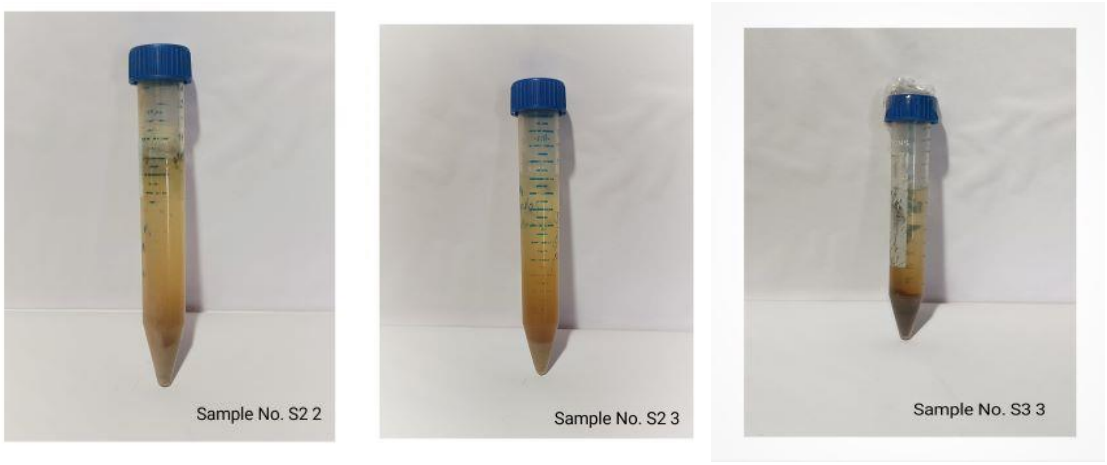


Fig: 1 Metabolites of isolated bacteria (Sample $S^2$2, S^2, $S^3$3)

Step 6: Antimicrobial Activity Testing

The extracted metabolites were tested for their antimicrobial activity against two bowel infection-causing pathogens:

- S. typhiimurium (Salmonella typhiimurium)
- E. fecalis (Enterococcus faecalis)

Disk Diffusion Method

The antimicrobial activity of the metabolites was evaluated using the disk diffusion method. This method involves placing a disk impregnated with the metabolites on an agar plate inoculated with the test microorganisms. The zone of inhibition around the disk indicates the antimicrobial activity of the metabolites.

Anti Microbial activity of isolated lactobacilli metabolites against E.Fecalis

Fig:2 Antimicrobial activity of bacterial isolates (Sample $S^2$2, S^2, $S^3$3,) against Enterococcus faecalis by disc diffusion method.

Zone of inhibition at different temperature isolated lactobacillus metabolites with Enterococcus faecalis

Sample	25*C	50*C	75*C	100*C
S^2 2	.7mm	.8mm	.7mm	.5mm
S^2 3	.5mm	.5mm	.5mm	.7mm
S^3 3	.7mm	blank	.7mm	.5mm

Table:-3 (Sample $S^2$2, S^2, $S^3$3,) showed following inhibition against Enterococcus faecalis

Observation: - The antimicrobial activity of the samples varies with temperature. S2 2 showed optimal activity at 50°C, while S2 3 showed increased activity at 100°C. S3 3 showed consistent activity across 25°C and 75°C.

Anti Microbial activity of isolated lactobacilli metabolites against Salmonella typhiimurium

Fig:3 Antimicrobial activity of bacterial isolates (Sample $S^2$2, S^2, $S^3$3,) against Salmonella typhiimurium by disc diffusion method.

Zone of inhibition at different temperature isolated lactobacillus metabolites with Salmonella typhiimurium

Sample	25*C	50*C	75*C	100*C
S^2 2	12mm	13mm	14mm	15mm
S^2 3	blank	blank	blank	blank
S^3 3	blank	blank	blank	blank

Table:-4 (Sample $S^2$2, S^2, $S^3$3,) showed following inhibition against Salmonella typhiimurium

Observation: - S2 2: The zone of inhibition increased with temperature, indicating that the antimicrobial activity of S2 2 is temperature-dependent. The results suggest that S2 2 has antimicrobial activity that increases with temperature. Whereas S2 3 and S3 3 No zone of inhibition was observed at any temperature, indicating that these samples may not have antimicrobial activity hence do not show antimicrobial activity at the tested temperatures.

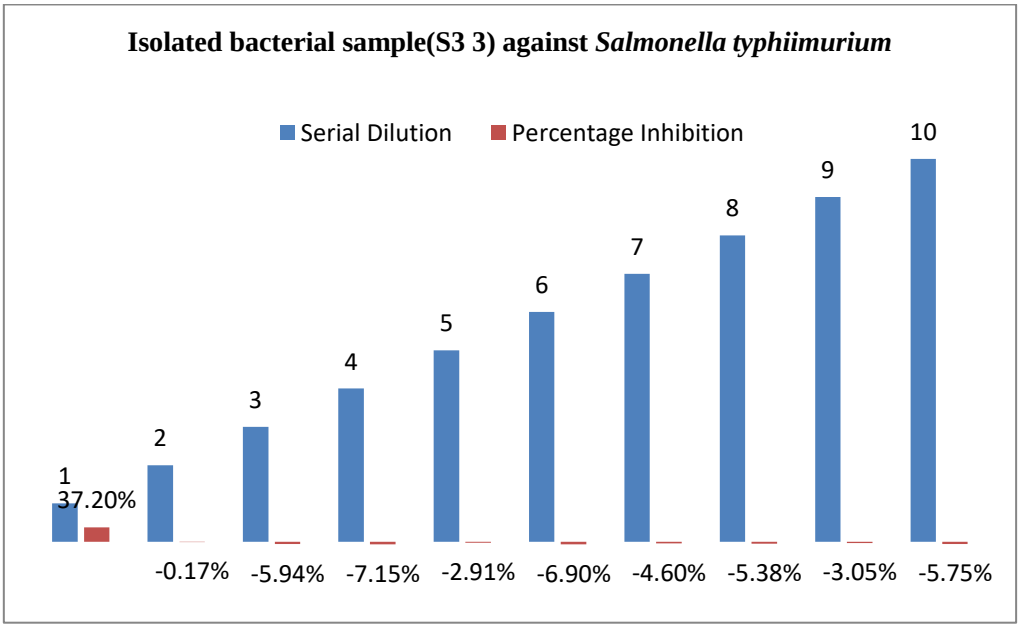
MINIMUM INHIBITORY CONCENTRATION

After confirming the antimicrobial activity of the bacterial extracts against Salmonella typhiimurium, the next step was to determine the Minimum Inhibitory Concentration (MIC). The MIC is the lowest concentration of the extract that can inhibit the growth of the test microorganism.

Bacterial Extract S3 3against Salmonella Typhiimurium

Serial Dilution	Initial Absorbance	Final Absorbance	Obtained Value	Initial Absorbance	Obtained Value/Initial Value	*100
1	1.188	0.746	0.442	1.188	0.372053872	37.20539
2	1.188	1.19	-0.002	1.188	-0.001683502	-0.16835
3	1.188	1.258666667	-0.0706667	1.188	-0.059483726	-5.94837
4	1.188	1.273	-0.085	1.188	-0.071548822	-7.15488
5	1.188	1.222666667	-0.0346667	1.188	-0.029180696	-2.91807
6	1.188	1.27	-0.082	1.188	-0.069023569	-6.90236
7	1.188	1.242666667	-0.0546667	1.188	-0.046015713	-4.60157
8	1.188	1.252	-0.064	1.188	-0.053872054	-5.38721
9	1.188	1.224333333	-0.0363333	1.188	-0.030583614	-3.05836
10	1.188	1.256333333	-0.0683333	1.188	-0.057519641	-5.75196

Table:- 5 Bacterial Extract S3 3against Salmonella Typhiimurium



Graph:- 1. Isolated bacterial sample (S3 3) against Salmonella typhiimurium

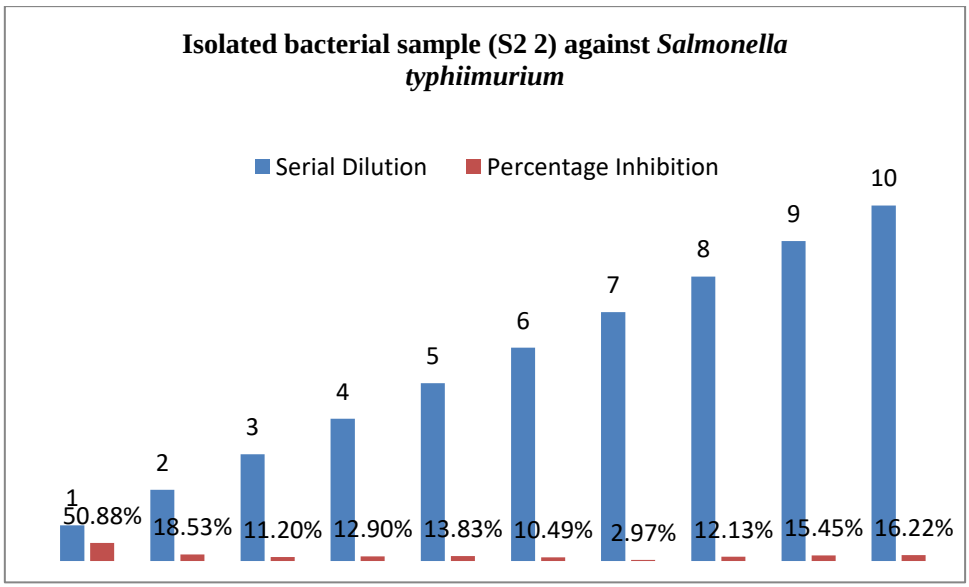
Observation: - The highest percentage inhibition was observed in dilution 1 (37.20%).The lowest percentage inhibition was observed in dilution 2 (-0.17%). Most dilutions showed a negative percentage inhibition, indicating a decrease in absorbance.

Bacterial Extract No. S2 2 against Salmonella Typhiimurium

Serial Dilution	Initial Absorbance	Final Absorbance	Obtained Value	Initial Absorbance	Obtained Value/Initial Value	*100
1	1.255	0.616333	0.638667	1.255	0.508898	50.88977

2	1.255	1.022333	0.232667	1.255	0.185392	18.53918
3	1.255	1.114333	0.140667	1.255	0.112085	11.2085
4	1.255	1.093	0.162	1.255	0.129084	12.90837
5	1.255	1.081333	0.173667	1.255	0.13838	13.83798
6	1.255	1.123333	0.131667	1.255	0.104914	10.49137
7	1.255	1.217667	0.037333	1.255	0.029748	2.974768
8	1.255	1.102667	0.152333	1.255	0.121381	12.13811
9	1.255	1.061	0.194	1.255	0.154582	15.45817
10	1.255	1.051333	0.203667	1.255	0.162284	16.22842

Table:- 6 Bacterial Extract No. S2 2 against Salmonella Typhiimurium



Graph:- 2 Isolated bacterial sample (S2 2) against Salmonella typhiimurium

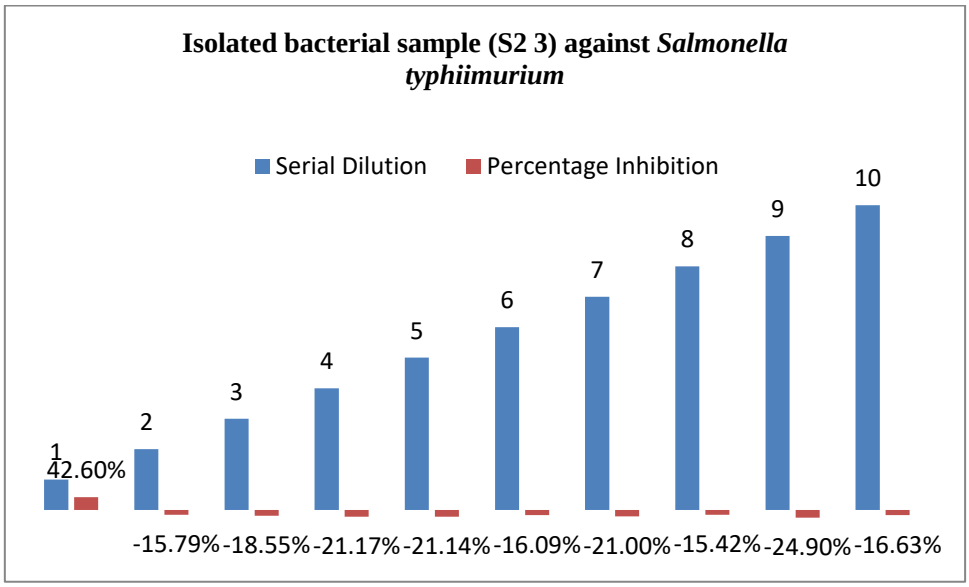
Observation: - The highest percentage inhibition was observed in dilution 1 (50.89%). The lowest percentage inhibition was observed in dilution 7 (2.97%). Most dilutions showed a moderate to high percentage inhibition, indicating that the bacterial extract has antimicrobial activity against Salmonella Typhiimurium.

Bacterial Extract No. S2 3 against Salmonella typhiimurium

Serial Dilution	Initial Absorbance	Final Absorbance	Obtained Value	Initial Absorbance	Obtained Value/Initial Value	*100
1	0.938	0.538333	0.399667	0.938	0.426084	42.60839
2	0.938	1.0862	-0.1482	0.938	-0.158	-15.7996
3	0.938	1.112	-0.174	0.938	-0.1855	-18.5501
4	0.938	1.136667	-0.19867	0.938	-0.2118	-21.1798

5	0.938	1.136333	-0.19833	0.938	-0.21144	-21.1443
6	0.938	1.089	-0.151	0.938	-0.16098	-16.0981
7	0.938	1.135	-0.197	0.938	-0.21002	-21.0021
8	0.938	1.082667	-0.14467	0.938	-0.15423	-15.4229
9	0.938	1.171667	-0.23367	0.938	-0.24911	-24.9112
10	0.938	1.094	-0.156	0.938	-0.16631	-16.6311

Table:- 7 Bacterial Extract No. S2 3 against Salmonella typhiimurium



Graph:- 3 Isolated bacterial sample (S2 3) against Salmonella typhiimurium

Observation:- Dilution 1 showed Highest percentage inhibition: of 42.61%. Negative percentage inhibition: Dilutions 2-10 showed negative % inhibition, indicating an increase in bacterial growth.

RESULTS:-

The results of this study demonstrate the antimicrobial activity of bacterial extract S2 2 against Salmonella typhiimurium. The zone of inhibition increased with temperature, indicating that the antimicrobial activity of S2 2 is temperature-dependent. The highest percentage inhibition was observed in dilution 1, with a % inhibition of 42.61% and 50.89% in two separate experiments. In contrast, S2 3 and S3 3 did not show significant antimicrobial activity against Salmonella typhiimurium, with no zone of inhibition observed at any temperature. The Minimum Inhibitory Concentration (MIC) of S2 2 against Salmonella typhiimurium, was observed at dilution 1, indicating that this extract has potent antimicrobial activity against this pathogen. Overall, the results of this study suggest that bacterial extract S2 2 has significant antimicrobial activity against Salmonella typhiimurium, and may be a potential candidate for the development of new antimicrobial agents.

Key Findings:

1. Antimicrobial activity: S2 2 showed significant antimicrobial activity against Salmonella typhiimurium

2. Temperature-dependent activity: The antimicrobial activity of S2 2 increased with temperature.
3. MIC: The MIC of S2 2 against Salmonella typhiimurium was observed at dilution 1.
4. No activity: S2 3 and S3 3 did not show significant antimicrobial activity against Salmonella typhiimurium

DISCUSSION:-

The antimicrobial properties of Lactobacillus species have been extensively studied, highlighting their importance in food safety and potential as probiotics and natural antimicrobial agents (FAO/WHO, 2002; Servin, 2004; Makinen et al., 2006). While various Lactobacillus species have been shown to exhibit antimicrobial activity, the current study focuses on the specific findings of Lactobacillus bacteria isolated from dairy curd, particularly the antimicrobial activity of S2 2 against Salmonella typhiimurium. Additionally, the temperature-dependent activity of S2 2 suggests that its antimicrobial compounds may be more effective at higher temperatures.

The comparison of these findings with existing research reveals research gaps and limitations, including the need to characterize the antimicrobial compounds produced by Lactobacillus species (Atanasova et al., 2011), optimize their production, and investigate their therapeutic potential in preventing and treating gastrointestinal infections (Gill et al., 2000). These gaps suggest areas for future research, including the investigation of different Lactobacillus species, optimization of antimicrobial compound production, and exploration of therapeutic applications. Overall, the current study contributes to the existing literature and identifies directions for future research.

CONCLUSION:-

The present study successfully isolated and characterized Lactobacillus bacteria from dairy curd. The isolated bacteria were identified based on their morphological, biochemical, and physiological characteristics. The results showed that the isolated Lactobacillus bacteria exhibited antimicrobial activity against Salmonella typhiimurium and Enterococcus faecalis two bowel infection-causing pathogens. The metabolites extracted from the fermented Lactobacillus bacteria S2 2, S2 3, S3 3, showed inhibitory effects against the test microorganisms, indicating their potential as natural antimicrobial agents. This study highlights the importance of Lactobacillus bacteria as probiotics and their potential applications in the prevention and treatment of gastrointestinal infections. Further studies are recommended to explore the therapeutic potential of Lactobacillus metabolites and their mechanisms of action.

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