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Research Article

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Effects of *Lactobacillus* isolated from the stool of healthy infants and yoghurt on the growth of some pathogenic bacteria

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Abstract This study aims to expose the effects of inhibitory substances produced by Lactobacillus isolated from the stool of healthy infants and yoghurt on the growth of some intestinal pathogenic gram-positive and gram-negative bacteria, including Salmonellae, Staphylococcus aureus, and Escherichia coli. The probiotic actions of some lactic acid bacteria (LAB) are presently the only choice available for replacing the antibiotics universally used due to their ability to enhance the growth and the health of animals and maintain normal microflora of the intestine through antagonistic activities against pathogens. After incubation for two to three days, Lactobacillus produces antibacterial compounds that inhibit the growth of these pathogens. These compounds mainly consisted of organic acids, undissociated acids, and bacteriocin. The inhibition zone against S. aureus ranged from 7 to 20 and 7 to 17 mm against Salmonella. Conversely, only three strains showed a lower inhibition against E. coli. This suggests that lactobacilli create substances that hinder the growth of these pathogenic bacteria.

Keywords *Lactobacillus*, Pathogenic bacteria, Antibiotic substances, Infant stool

1. Introduction

Lactic acid bacteria (LAB) strains have the potential to be promising due to their ability to produce bactericidal bioactive compounds that can effectively inhibit the growth of pathogens. Maragkoudakis et al. (2006) and Charlier et al. (2008) have described the advantageous effects of Lactobacilli, which include the ability to suppress both gram-negative and gram-positive pathogenic bacteria. Ensuring the continued effectiveness of probiotics' ability to kill microorganisms will validate their use in creating functional food that promotes the health of consumers (Eduardo et al., 2003). The *Lactobacillus* isolates studied previously displayed significant and easily observable antibacterial action against harmful microorganisms. Various strategies have been suggested to hinder the growth of harmful bacteria. Some of these strategies include: *Lactobacillus* isolates were found to produce antimicrobial compounds such as bacteriocins, hydrogen peroxide, and organic acids (Charlier et al., 2008; Lin et al., 2006; Hernandez and Cardell, 2005; Chen et al., 2009; Kmet and Lucchini, 1999; Makras et al., 2006; Schachtsiek et al., 2004). These agents work by fighting pathogens for adhesion sites and forming coagulates with them. Only a few studies have suggested that the synthesis of organic acids is the sole factor responsible for the antibacterial action of lactobacilli (Ogawa et al., 2001). *Lactobacilli* have been found to possess several anti-infective properties. These include their capacity to adhere to surfaces and prevent the attachment of pathogens, prevent the growth of pathogens, consume nutrients that would normally be available to pathogens, and regulate the host immune response and microenvironment, thereby reducing the risk of infection (Reid and Burton, 2002). Nevertheless, it should be noted that this statement is a broad generalization,

and not all characteristics may be necessary to hinder the spread of infection caused by every pathogen effectively. Various mechanisms have been suggested to explain the positive impact of probiotics (Prabhurajeshwar and Chandrakanth, 2017). The capacity of probiotic microorganisms to adhere to the intestinal mucosa is seen as crucial for the various beneficial impacts on health attributed to probiotics. The capacity to attach to epithelial cells and mucosal surfaces has been proposed as a significant characteristic of numerous bacterial strains employed as probiotics. Adherence is a crucial requirement for the establishment of probiotics in the intestinal cavity, giving them a competitive edge in this ecosystem (Prabhurajeshwar and Chandrakanth, 2017). Multiple studies have proposed that the capacity of beneficial microbes to come together and stick to surfaces helps them colonize the gut and create a protective barrier that hinders the development of harmful infections. The presence of beneficial microbes and the regulation of the gut immune system by these organisms are factors that prevent infection (Sherman et al., 2009). According to Prabhurajeshwar and Chandrakanth (2017), the T2 isolate of *Lactobacillus* was found to have the most dominance in evaluating the qualitative and quantitative aggregation and co-aggregation capacities of the collected *Lactobacillus* isolates. This study aimed to investigate the impact of inhibitory substances produced by *Lactobacillus*, isolated from the stool of healthy infants and yoghurt, on the growth of various intestinal pathogenic bacteria, including *Salmonellae*, *Staphylococcus aureus*, and *Escherichia coli* (*E. coli*).

2 Materials and Methods

2.1 Chemicals

The equipment, chemical reagents, and solutions used to perform the experiments of this study were analytical grades.

2.2 Isolation, transportation and storage

A total of 111 *Lactobacilli* samples were isolated and used in this investigation, with the majority being stool specimens and only a small number being from yogurt. The raw materials used as a source for isolated *Lactobacilli* are listed in Table 1. The collected specimens were preserved in normal saline and rapidly transported to the laboratory to prevent drying of the swab and decrease the possibility of bacterial infection or mortality, depending on the bacterial type.

Table 1: Raw materials used as sources for isolated *Lactobacilli*

No.	Type of specimens	Source
1	Milk	Dairy/animal
2	Yoghurts	Dairy
3	Spoiled food	Kitchen
4	Stool (healthy infants)	Babies
5	Vomiting	patient
6	Urine	patient
7	Sewage water	Sewage

2.3 Sterilization method

The culture medium was sterilized using autoclaving at a temperature of 121 ºC for a duration of 30 minutes. The glassware was sterilized through the process of subjecting it to a temperature of 180 ºC in an electric oven for a duration of 30 minutes. The workspaces, particularly the bench, were sanitized using ethanol and a flame.

2.4 Bacterial culture

Lactobacillus samples were cultivated on MRSA and MRS broth medium using a sterile loop in a sterile environment. The plates were thereafter placed in an incubator set at a temperature of 37 ºC for a duration of 2-3 days. *Staphylococcus aureus* samples were cultured on both mannitol salt agar and nutrient agar, and subsequently placed in an incubator at a temperature of 37 ºC for a period of 24 hours. *Escherichia coli* and *Salmonella* samples were grown on XLD agar, CLED, MacConkey agar, and DCA media, and kept in an incubator at a temperature of 37 ºC for 24 hours.

2.5 Bacterial identification

2.5.1 The Gram stain procedure

After the bacteria grew on the media, a Gram stain was conducted to examine the microscopic characteristics of each bacterium. A small amount of each bacterium was applied onto a clean slide and combined with a small amount of normal saline. The slide was thereafter subjected to many rounds of heating over a flame for fixing. Subsequently, the film was treated with crystal violet and subsequently washed with water to remove the stain. Afterwards, an iodine solution was added and left to react for a duration of one minute. Subsequently, it was removed and cleaned with water. As a result, the slide was covered with a layer of 95% acetone and then rinsed with water to eliminate the violet color. Afterward, the film was submerged in the counter-stain safranin and allowed to react for a duration of one minute. Meanwhile, the slide was placed in an airstream to accelerate the drying process. Immersion oil was applied in a small amount. The film was examined under a microscope.

2.5.2 Biochemical assays

2.5.2.1 The coagulase test

This test is utilized to distinguish Staphylococcus aureus from other staphylococci, with Staphylococcus aureus producing positive results while the others produce negative results. The procedure includes mixing Staphylococcus aureus with a minimal quantity of normal saline and subsequently introducing undiluted human plasma. Coagulase rapidly transforms plasma fibrinogen into fibrin within a 15-minute period, leading to the production of coagulated plasma.

2.5.2.2 Catalase Test

This test is utilized to differentiate between gram-positive bacteria and gram-negative bacteria. The method is mixing a small amount of either *Staph. aureus* or *Lactobacillus* with a small amount of hydrogen peroxide (H_2O_2) as catalase facilitates the breakdown of hydrogen peroxide into oxygen and water.

2.5.2.3 Triple sugar iron agar (TIS)

TIS is a method used to distinguish between different types of gram-negative bacteria, notably *Salmonella, E. coli, Shigella, Pseudomonas, and Proteus.* The process entails introducing each bacterium onto TIS agar and placing it in an incubator set at a temperature of 37 ºC for a period of 24 hours. After the duration of incubation, *E. coli* displays a yellow slant (indicating acidity) and a yellow butt (also indicating acidity) with the production of gas at the bottom. On the other hand, Salmonella exhibits a red slant (alkaline) and a yellow butt (acidic) with minimal or no gas formation.

2.6 Test of Inhibition

The antibacterial effectiveness of *Lactobacillus,* mainly obtained from the feces of healthy infants, as well as from yogurt and animal and dairy milk, against pathogenic strains was assessed using the methodology described by Prabhurajeshwar et al. (2017), with some modifications. *Lactobacillus* was cultured in test tubes using MRS broth. The culture was thereafter incubated at a temperature of 37 ºC for a duration of 2-3 days. Following the incubation period, the tubes were extracted from the incubator and positioned in a centrifuge apparatus, guaranteeing appropriate equilibrium. Subsequently, they were subjected to centrifugation at a speed of 4000 rpm for 10 minutes. Following centrifugation, the liquid portion (supernatant) was carefully transferred to separate tubes, while the solid portion (sediment) was discarded. A paper resembling an antibiotic was completely saturated with the liquid solution until it reached its maximum absorption capacity. After the paper was completely soaked, it was carefully placed on a culture medium containing *Salmonella*, *Escherichia Coli*, and *Staphylococcus aureus* bacteria. The plates were thereafter placed in an incubator set at a temperature of 37 °C for 24 hours. The presence of a well-defined area of inhibition around the paper indicates that antibacterial substances obtained from lactobacillus successfully prevent the growth of pathogens. Therefore, the influence is assessed by measuring the dimensions of the zone.

3. Results and Discussion

In this experiment, a total of 111 *Lactobacilli* samples were obtained, primarily from stool specimens and a minority from yogurt samples. The samples are divided into three equal partitions, with each partition

comprising 37 samples. Each one was utilized to fight specific pathogens, such as *Staphylococcus aureus*, *Salmonella*, and *Escherichia coli*. The study organized the data acquired from any bacterium by creating schedules that detailed and measured the inhibitory effects generated by substances produced by *Lactobacillus*. The good scores were represented by the (+ve) symbol, and negative values were signified by the (-ve) symbol. The measurement of the inhibition zone was conducted in millimeters (mm) by assessing the diameters of the area surrounding the disc. Most of the stool samples showed positive results with different degrees of inhibitory effectiveness. The most significant positive results were recorded (+++). The samples comprise three specimens: two stool samples designated as 10 and 27, and one yogurt sample designated as 18. The size of the inhibitory zone for each sample is 20 mm. Out of the remaining samples, 17 were classified as (++), indicating positive outcomes, while 16 were labeled as (+). Only one specimen exhibited a negative result, indicated by (-). The data definitely indicate that 97% of S. aureus demonstrated sensitivity to antibacterial medicines generated from *Lactobacillus* (Table 2).

Table 2: The inhibition zone produced by *Lactobacillus* against *Staphylococcus aureus*.

Number	Type of samples	Size of inhibition zone (mm)	Results
1	Stool	14	++
2	Stool	11	++
3	Stool	9	+
4	Stool	10	++
5	Stool	13	++
6	Stool	9	+
7	***Yoghurt***	7	+
8	Stool	19	++
9	Stool	12	++
10	Stool	20	+++
11	Stool	10	++
12	***Yughort***	9	+
13	Stool	15	++
14	Stool	14	++
15	Stool	11	++
16	Stool	9	+
17	Stool	14	++
18	***Yughort***	20	+++
19	***Yughort***	9	+
20	Stool	10	++
21	Stool	8	+
22	***Yughort***	9	+
23	Stool	**9**	+
24	Stool	10	++
25	Stool	7	+
26	Stool	9	+
27	Stool	20	+++
28	Stool	10	++
29	Stool	0	_
30	Stool	7	+
31	Stool	9	+
32	Stool	7	+
33	Stool	9	+
34	Stool	7	+
35	Stool	12	++
36	Stool	10	++
37	**Stool**	**10**	++

According to the data shown in Table 4, the feces and yogurt samples showed the most significant levels of inhibition against *Salmonella*. The markers were annotated with (++ & +), with twenty samples denoted by (++)

and thirteen by (+). Negative scores are represented by the symbol (-) and include three samples. *Salmonella* is susceptible to antibacterial agents, as is *S. aureus*, although to a lesser degree.

Table 3: The inhibition zone produced by *Lactobacillus* against *Salmonella*

Number	Type of sample	Size of inhibition zone (mm)	Result
1	Stool	7	+
2	Stool	7	+
3	Stool	12	++
4	Stool	12	++
5	Stool	9	+
6	Stool	10	+ +
7	***Yughort***	13	++
8	Stool	0	_
9	Stool	10	+ +
10	Stool	1 0	+ +
11	Stool	9	+
12	***Yughort***	17	++
13	Stool	9	+
14	Stool	11	++
15	Stool	10	++
16	Stool	12	++
17	Stool	7	+
18	***Yughort***	10	++
19	***Yughort***	8	+
20	Stool	10	++
21	Stool	7	+
22	***Yughort***	10	++
23	Stool	**0**	_
24	Stool	8	+
25	Stool	15	++
26	Stool	12	++
27	Stool	8	+
28	Stool	12	++
29	Stool	8	+
30	Stool	12	++
31	Stool	0	_
32	Stool	9	+
33	Stool	13	++
34	Stool	8	+
35	Stool	10	++
36	Stool	0	_
37	**Stool**	**13**	++

In addition, as listed in Table 4, most of the tested samples had negative results against *E. coli*, except for three samples: two yoghurt samples (12 and 19) and one stool sample (4), which tested positive. The antibacterial medications proved that the *E. coli* had resistance against substances produced by *Lactobacilli*.

Table 4: The inhibition zone produced by *Lactobacillus* against *E.coli.*

Number	Type of sample	Size of inhibition zone (mm)	Result
1	Stool	0	_
2	Stool	0	_
3	Stool	0	_
4	**Stool**	**7**	+
5	Stool	0	_
6	Stool	0	_
7	Yoghurt	0	_
8	Stool	0	_
9	Stool	0	_
10	Stool	0	_
11	Stool	0	_
12	Yoghurt	**9**	+
13	Stool	0	_
14	Stool	0	_
15	Stool	0	_
16	Stool	0	_
17	Stool	0	_
18	Yoghurt	0	_
19	**Yoghurt**	**7**	+
20	Stool	0	-
21	Stool	0	-
22	Yoghurt	0	-
23	Stool	0	-
24	Stool	0	-
25	Stool	0	-
26	Stool	0	-
27	Stool	0	-
28	Stool	0	-
29	Stool	0	-
30	Stool	0	-
31	Stool	0	-
32	Stool	0	-
33	Stool	0	-
34	Stool	0	-
35	Stool	0	-
36	Stool	0	-
37	Stool	0	-

Based on the data, each bacterium has exhibited greater variations compared to others. These differences may be attributed to variations in the mechanism of antibiotic resistance and the source from which the *Lactobacillus* is isolated. Even when the same *Lactobacilli* are isolated from different specimens, the inhibitory effects vary between different bacteria. For instance, the inhibitory effects of *Lactobacillus* from yogurt against *Salmonell*a are significantly greater than those against *Staph. aureus* and *E. coli*. According to the data in the tables, *Staph. aureus* is more vulnerable to antibacterial agents produced by *Lactobacillus*. *Salmonella* is moderately susceptible, while *E.coli* is slightly susceptible due to its resistance mechanisms and strains of *Lactobacillus.* Some strains of *Lactobacillus* may strongly inhibit *Staph. aureus*, but not *E.coli* or *Salmonella*, and vice versa. However, some previous studies reported that some strains of lactobacilli inhibited the growth of *E. coli*. There is research data conducted *in vitro* demonstrating that *Lactobacilli* have ability to prevent the growth and

attachment of uropathogenic *E. coli* to uroepithelial cells. This evidence was reported by McGroarty and Reid in 1988. As a result, there has been a decrease in the occurrence of infections in both animals and people (Reid and Burton, 2002). There is currently no direct evidence from experiments conducted *in vivo* to support the mechanisms of action. However, the available data indicate that the activity may be related to the competition for mannose and glycoprotein receptors that are used by the pathogens. Additionally, it is suggested that the activity may involve the killing of cells through the use of hydrogen peroxide and bacteriocin-like compounds (McGroarty and Reid, 1988; Reid and Burton, 2002).

4. Discussion

The purpose of this inhibitory experiment was to highlight the capacity of *Lactobacillus* to produce antibiotic substances that inhibit the proliferation and growth of several pathogenic bacteria, including *S. aureus*, *Salmonella*, and *E. coli*. Antimicrobial activity is a critical factor in determining the effectiveness and novelty of probiotics. The antimicrobial properties of all *Lactobacillus* isolates are maintained by the production of several compounds, including organic acids (such as lactic, acetic, propionic, and succinic acids), hydrogen peroxide, low molecular weight antimicrobial chemicals, and bacteriocins (Savadogo et al., 2004; Prabhurajeshwar and Chandrakanth, 2017). Probiotics such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* spp. have been found to effectively suppress the growth of many harmful microorganisms in the human intestines (Prabhurajeshwar and Chandrakanth, 2017). Differences in the antibacterial effects were apparent among these three microorganisms. The relative differences in susceptibility to antibacterial compounds produced by *Lactobacillus* may be attributed to genetic variability among them. Specifically, *S. aureus* is more susceptible to these agents, while *E. coli* has a lower susceptibility. Future research may offer clarification regarding variances in antibacterial effects. Furthermore, experimental studies have indicated that probiotic bacteria may have a preventive effect against the formation of colon tumors, in addition to their positive impact on diseases caused by an imbalance of gut microflora (Murry et al., 2004). In Osuntoki et al.'s 2008 investigation, *Lactobacillus* spp. obtained from fermented dairy products shown antibacterial properties against clinically significant pathogens including *Enterotoxigenic E. coli* (4.2 mm), *Salmonella typhimurium* (4.3 mm), and *Listeria monocytogenes* (5.0 mm). The isolates in the current investigation exhibit superior antibacterial efficacy compared to the isolates of *Lactobacillus spp*. The antagonistic activity of isolates against *Salmonella* and *Staph. aureus* was comparable to that of *Lactobacillus plantarum* and *Lactobacillus salivarius*, which were isolated by Murray et al. (2004) from a botanical probiotic. The study conducted by Gharaei-Fathabad and Eslamifar, 2011; and Grześkowiak et al. (2012), found that a particular strain of *Lactobacillus paraplantarum*, which was obtained from tea leaves, exhibited significant inhibitory effects against several bacteria including *S. typhii* (65 mm), *E. coli* (30 mm), *S. aureus* (56 mm), *E. faecalis* (55 mm), and *Citrobacter* spp. (60 mm). The study demonstrated that all selected *Lactobacillus* isolates exhibited significant antagonistic activity against three different test pathogens, as indicated in Tables 2, 3, and 4. This activity was attributed to the production of organic acids and low molecular weight antimicrobial substances by the *Lactobacillus* isolates.

5. Conclusion

In conclusion of the work, *Lactobacillus* strains isolated in this study from the different dairy samples have in vitro properties that make them potential candidates for probiotic applications. These results collectively suggest that isolates from curd samples have promising properties that are important for potential probiotics. Hence, more research is needed to exploit other potential probiotic properties of these strains. Further, in-vivo trials are needed to determine whether they function as probiotics in real-life situations for human health benefits. *Lactobacillus* isolates also confirmed some probiotic properties which suggest their possible use in the medical field and most of the food industry. Indeed, a process for the incorporation of these isolates is under some more investigation by our research group. However, more studies are needed to complete the isolation and characterization of novel strains of *Lactobacillus* spp. and other probiotic bacteria that could be beneficial for human health.

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