IN-VITRO EVALUATION FOR CHOLESTEROL REDUCTION POTENTIAL OF *LACTOCOCCUS SPECIES* ISOLATED FROM GARLIC AND GINGER

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خلاصہ

اسٹریپٹو کوکیسی خاندان کے اندر لیکٹک ایسڈ بیکٹیریا ہوتا ہے۔ یہ بیکٹیریا مائیکروایروفیلک، ہوموفرینٹیٹو، گرام پازیٹو ہیں جو ہیکسوز کو لیکٹک ایسڈ پیدا کرنے کے لیے خمیر کرتے ہیں۔ وہ عام طور پر محفوظ (GRAS) کے طور پر پہچانے جاتے ہیں اور ڈیری خمیر شدہ مصنوعات جیسے دہی اور پنیر میں استعمال ہوتے ہیں۔ مطالعہ کا مقصد لییکٹوکوکس پرجاتیوں کی پروبائیوٹک صلاحیت کا اندازہ لگانا تھا جو کولیسٹرول کو کم کرنے میں مدد کرتے ہیں۔ لہسن اور ادرک مقامی بازار سے جمع کیے گئے۔ ڈی مین روگوسا ایگر کو لییکٹوکوکس لیکٹس کریم کو سفید کالونیوں سے الگ کرنے کے لیے استعمال کیا گیا تھا MRS آگر پر گرام مثبت کوکسی شکل والے بیکٹیریا خوردبینی تحقیقات کے بعد مائکروسکوپ کے نیچے پائے گئے۔ بیکٹیریا کی مزید تصدیق بائیوکیمیکل اور کاربوہائیڈریٹ ابال کے ٹیسٹوں سے ہوئی۔ لییکٹوکوکس کے لیے تمام بائیوکیمیکل ٹیسٹ منفی تھے سوائے میتھائل ریڈ کے جو مثبت تھا۔ پھر اینٹی بائیوٹک حساسیت کا تعین کربی باؤر ڈسک کے پھیلاؤ کے طریقہ کار کا استعمال کرتے ہوئے کیا گیا۔ اس نے Amoxicillin Clavulanic Acid، Piperacillin / Tazobactam، Clindamycin اور Kanamycin کی طرف ایک درمیانی رینج کی نمائش کی جبکہ Chloramphenicol، Ofloxacin، Ciprofloxacin، اور Levofloxacin کی طرف حساس ہے۔ Lactococcus lactis کی کولیسٹرول میں کمی کی صلاحیت کا تعین 620nm پر سپیکٹروفوٹومیٹر کے ذریعے کیا گیا۔ کولیسٹرول کے انحطاط کے نتائج کولیسٹرول کے اس فیصد کے حساب سے ریکارڈ کیے گئے جو ضم ہو گیا ہے۔ نتائج سے پتہ چلتا ہے کہ لہسن سے الگ تھلگ لیکٹوکوکس لیکٹس نے ادرک کے مقابلے میں 600µg/ml کے ارتکاز میں 12.97% زیادہ کولیسٹرول کو کم کیا ہے جو کہ اسی ارتکاز میں 7.62% ظاہر کرتا ہے۔ موجودہ مطالعہ سے پتہ چلتا ہے کہ لییکٹوکوکس لییکٹیس ایس پی کی کولیسٹرول کو کم کرنے کی صلاحیت۔ لہسن سے الگ تھلگ دل کی بیماریوں کے علاج میں مصنوعی ادویات کے میدان میں استعمال کیا جا سکتا ہے۔

Abstract

The genus *Lactococcus* contains Lactic acid bacteria within the family *Streptococcaceae*. These bacteria are micro aerophilic, homo fermentative, Gram-positive that ferment hexoses to produce lactic acid. They are generally recognized as safe (GRAS) and used in dairy fermented products like yogurt and cheese. The objective of the study was to assess the probiotic ability of *Lactococcus* species that help reduce cholesterol. Garlic and Ginger were collected from the local market. De Man Rogosa agar was used for isolation of *Lactococcus lactis* cream to white colonies were showed on MRS agar Gram positive cocci- shaped bacteria were found under the microscope after microscopic investigation. The bacteria were further confirmed by biochemical and carbohydrate fermentation tests. All the biochemical tests were negative for *Lactococcus* except Methyl Red which was positive. Then the antibiotic susceptibility was determined using Kirby-Bauer disc diffusion method. It exhibited an intermediate range towards Amoxicillin Clavulanic Acid, Piperacillin / Tazobactam, Clindamycin, and Kanamycin while sensitive toward Chloramphenicol, Ofloxacin, Ciprofloxacin, and Levofloxacin. The cholesterol reduction potential of *Lactococcus lactis* was determined by spectrophotometer at 620nm. The cholesterol degradation results were recorded by the percentage of cholesterol that has assimilated. The results showed that the *Lactococcus lactis* isolated from garlic has degraded more cholesterol 12.97% at concentration of 600µg/ml as compared to ginger which showed 7.62% at the same concentration. The present study showed that the cholesterol degrading potential of *Lactococcus lactis* sp. Isolated from garlic could be utilized in the field of synthetic medicines in treating cardiovascular diseases.

Introduction

Cholesterol is an essential substance that plays an essential role in cell connection in the brain, maintaining the cell membrane, producing hormones, and forming vitamin D in the skin. About 30% of cholesterol comes from food and 70% from liver activity. When the level of cholesterol increases in the blood, it can cause many problems that have dangerous consequences, such as cardiovascular diseases (Dunn-Emke *et al.*, 2001).Cardiovascular diseases (CVDs) are considered the leading causes of mortality in the world. As reported by the World Health Organization (WHO), 17.9 million people died due to CVDs in 2019, which was 32% of global deaths. The most common type of CVD is coronary heart disease (CHD), which has caused the largest number of deaths since 2000. The epidemiological studies demonstrated a positive correlation between raised serum cholesterol levels and CHD risk (Keleszade *et al*., 2022). The increasing levels of low-density lipoprotein cholesterol (LDL-C) and systolic blood pressure play an important role in the development of cardiovascular disease (Groenland *et al*., 2022). To reduce the serum cholesterol level, probiotic bacteria have attracted the vast attention of researchers, physicians, and consumers. Therefore, the demand for probiotics for lowering blood cholesterol has increased over the past few decades. It was assumed that the reduction of cholesterol was increasing with increasing bile salt concentration in the medium. Several in vitro studies reported that the degradation of cholesterol was significant in the presence of bile salts (Anila *et al.*, 2016). Increasing levels of cholesterol may be decreased by consumption of yogurt containing specific probiotic bacteria (LAB), dietary fiber, and low-fat and low-cholesterol diets (Dunn-emke *et al.*, 2001). Health benefits of probiotics include reducing cholesterol in serum, suppressing cancer, acting as an antidiabetic agent, enhancing natural resistance in the gastrointestinal tract against infectious disease, and using them as food supplements for Lactose intolerance (Kumar *et al*., 2010).Characteristics of a probiotic include viability without pathogenicity or toxicity, tolerance of the acidic pH of the gut to metabolize there, adherence to the mucosal walls of the gastrointestinal tract, and ability to maintain the balance of the inhabitant microflora (Venkatesan *et al*., 2012).Most common genera belonging to probiotics comprise the species of *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Propionibacterium, Leuconostoc, Corynebacterium*, *Lactococcus, Bacillus, Saccharomyces,* and *Aspergillus* (Hossain *et al.*, 2017). They are Gram-positive anaerobic, non-spore-forming bacteria that produce lactic acid in the presence of carbohydrates. Lactic acid-producing bacteria belong to different genera, such as *Lactobacillus, Lactococcus, Enterococcus, Bifidobacterium, Pediococcus, Streptococcus*, and *Leuconostoc*. They play an important role in the human body by colonizing different parts of the body, such as the nasopharyngeal mucosa, gut, intestine, and mucosa of the vagina (Teuber, 2008). There are 19 species of *Lactococcus with* validly published names. They are widely present in carbohydrate-rich environments and isolated from various sources such as plants, milk, fish, and animals. The psychrophilic LABs, *Corynebacterium, Lactococcus,* and *Leuconostoc,* play a beneficial role in spoilage meat and have bioprotective characteristics (Hilgarth *et al*., 2020).The Lactococcus species present in plant materials have the ability to inhibit the growth of pathogenic bacteria in the intestine as well as reduce the serum cholesterol level in the human body(Lecerf and De Lorgeril, 2011).Various strains of *Lactococcus* have cholesterol-reduction potential. The mechanism of *Lactococcus* that removed cholesterol from the media was the adhesion of cholesterol to the cell surface and the assimilation of cholesterol by the cells (Groenland *et al*., 2022).Medicines are not advised for decreasing cholesterol levels due to their side effects. A substance that does not have any side effects is required to reduce cholesterol levels. Soybean-fermented food with lactic acid bacteria greatly decreased hyperlipidemia. *Lactococcus* in fermented foods decreased the risk of atherosclerosis and cardiovascular disease (Avci *et al*., 2018).

Materials and Method

Sample collection

A total of 10 garlic and ginger samples were collected from the local market. The samples were grinded by grinder machine and inoculated on MRS agar plates by spread plates method then incubated for 24h at 37°C under anaerobic condition. Mixed growth was streak on MRS agar for pure isolation(Lee *et al.*, 2020).

Identification and characterization of *Lactococcus Lactis*

For phenotypic characterization of *Lactococcus lactis* Gram staining and various biochemical test such as Methyl red test, Voges-Proskauer test, Indole test, catalase test, oxidase test, Citrate utilization test, and carbohydrate fermentation test were performed according to protocol mentioned by (Vendrell *et al.*, 2006).

Antibiotics susceptibility test

The isolates were examined for antibiotic susceptibility by using Kirby-Bauer disc diffusion method. The antibiotics included Amoxicillin Clavulanic acid, Gentamycin, Piperacillin / Tazobactam, Clindamycin, and Kanamycin, Chloramphenicol, Ofloxacin, Ciprofloxacin, and Levofloxacin were used (Sadeghi, 2016)

Cholesterol reduction test: Cholesterol reduction test was performed to check the degrading ability of *Lactococcus* lactis. The first5g of commercially available water-soluble cholesterol was added to 95ml distil water. Then cholesterol was made soluble by combining it with warm ethanol and heating it with a few drops of chloro form. After evaporation of chloroform and ethanol the crystals of cholesterol were formed. A total of 12 test tubes divided into 3 groups 5-garlic 5-gingerand 2 utilized as control were filled with MRS broth and supplement with cholesterol that solubal in water with different concentration such as 600µg/ml, 400µg/ml,200µg/ml. The garlic and ginger tubes were inoculated with 1% *Lactococcus lactis* culture. The 2 test tubes were utilized as negative control. All test tubes were incubated for 24h at 37℃ in anaerobic jar. After incubation the cells of *Lactococcus lactis* were harvested by centrifugation at 10000 rpm for 10 mints and washed with distilled water twice. The isolated cell was again added in MRS broth containing various concentration of water-soluble cholesterol (200µg/ml, 400µg/ml, 600 µg/ml). Again, incubated at 37℃ for 24h. The cell degradation ability of *Lactococcus lactis* was determined spectrophotometer at 620nm and compared with negative control. The following equation was used to calculate cholesterol degradation. Cholesterol Assimilation (A) = 100-(B\C) *100 (Bashir *et al.*, 2020)

- A= % of cholesterol degraded
- B = Absorbance of sample containing cells
- C = Absorbance of the sample without cells

Results and Discussion

Round, opaque, convex, medium size, cream to white in color colonies with entire edges were examined on MRS agar plates. Gram-positive cocci shape bacteria were examined that showed the phenotypic characteristics of *Lactococcus lactis*. The results of biochemical and carbohydrate fermentation tests are shown in tables 1 and 2.

Table 1. Biochemical characterization of isolated bacteria.

Biochemical Test	Observations	Results
Indole	Absence of red colored ring in test tube	Negative
Catalase	No bubble formation on slide	Negative
Oxidase	Absence of purple colour on filter paper	Negative
Methyl Red	Transformation of yellow to red colour	Positive
Voges-Proskauer	No transformation of yellow to pink	Negative
Citrate Utilization	No transformation of green to blue colour	Negative

Table 2. Carbohydrate fermentation test for *Lactococcus lactis*

Carbohydrate	Observations	Results
Glucose	Transformation of red to yellow colour	Positive
Lactose	Transformation of red to yellow colour	Positive
Sucrose	Transformation of red to yellow colour	Positive
Maltose	Transformation of red to yellow colour	Positive

Antibiotic Susceptibility Test

Antibiotic sensitivity of isolated *Lactococcus lactis* was confirmed against commonly used antibiotics by Kirby-Bauer disc diffusion method. The zone of inhibition was measured. *Lactococcus lactis*exhibited an intermediate range towards Amoxicillin Clavulanic Acid Gentamycin, Piperacillin / Tazobactam, Clindamycin, and Kanamycin while susceptible to Chloramphenicol, Ofloxacin, Ciprofloxacin, and Levofloxacin.The antibiotic susceptibility of isolated *Lactococcus lactis* against commonly used antibiotics are summarized in table 3.

Table 3. Antibiotic discs used against *Lactococcus lactis* isolated from garlic and ginger.

Antimicrobial Agent	**Disc Content (µg)**	**Zone of Inhibition (mm)**
Kanamycin \ k	14µg	intermediate
Clindamycin \ CD	2µg	intermediate
Ofloxacin \ OFX	5µg	susceptible
Ciprofloxacin \ CIP	5µg	susceptible
Levofloxacin \ LEV	5 v	susceptible
Piperacillin / tazobactam \ TZP	100\10µg	intermediate
Amoxicillin clavulanic acid \ AUG	30µg	intermediate
Chloramphenicol \ C	30µg	intermediate
Gentamycin / CN	10µg	intermediate

Cholesterol reduction test results

The cholesterol degrading ability of *Lactococcus lactis* was determined by spectrophotometer at 620nm and compared with negative controls after 24h incubation. The *Lactococcus lactis* isolated from garlic has degraded more cholesterol 12.97% at concentration of 600µg/ml while ginger isolates showed 7.62%. The results are shown in Table 4 and5.

Table 4: The Effect of *Lactococcus lactis* isolated from garlic on cholesterol degradation.

Sample	**Concentration of water-soluble cholesterol (µg\ml)**	**Optical Density**	**Percentage of cholesterol degradation (%)**
Garlic	200µg\ml	0.563	4.83%
	400µg\ml	0.572	9.35%
	600µg\ml	0.584	12.97%

Table 5: The Effect of *Lactococcus lactis* isolated from ginger on cholesterol degradation.

Sample	**Concentration of water-soluble cholesterol (µg\ml)**	**Optical Density**	**Percentage of cholesterol degradation (%)**
Ginger	200µg\ml	0.572	3.07%
	400µg\ml	0.629	4.81%
	600µg\ml	0.637	7.62%

Probiotics are live microorganisms that play an important role in human and animal health as proved by many studies. However, the clinical use of probiotics for cholesterol reduction required more research and justification before its implication. Previous*in-vivo* research indicates that probiotics help to maintain a healthy lipid profile by lowering serum cholesterol (Ooi and Liong, 2010). Total cholesterol and LDL-C reduction in hyper cholesterolemic men lowers the risk of cardiovascular disease(Huang *et al.*, 2013).Modifying diets through probiotic interventions is one of the promising and cost-effective strategies for lowering serum cholesterol levels. This paper describes the study conducted based on previous studies that were successfully completed. The objective of the study was to assist the *in-vitro* evaluation for cholesterol reduction potential of Lactococcus species isolated from garlic and ginger. *Lactococcus lactis* were isolated by using De Man Rogosa and Sharp agar. It showed cream to white colonies and microscopic examination revealed Gram positive Purple and cocci shape under microscope. Further identification was done based on various biochemicals test (indole test, oxidase test, catalase test, Voges Proskauer test, citrate utilization, methyl red) and carbohydrate fermentation test. All showed negative except Methyl red was positive that affirmed the identification of *Lactococcus lactis*. The isolated bacteria showed intermediate range toward Amoxicillin Clavulanic Acid, Piperacillin/ Tazobactam, Clindamycin, and Kanamycin while susceptible toward Chloramphenicol, Ofloxacin, Ciprofloxacin, and Levofloxacin. The cholesterol reduction potential of *Lactococcus lactis* was determined by spectrophotometer at 620nmcompared with un-inoculated controls. The *Lactococcus lactis* which has isolated from garlic revealed the highest cholesterol assimilation 12.97% at concentration of 600µg\ml while ginger isolates showed 7.62%. According to (Mariga *et al.*, 2011). The fermented leaves of Amaranthus are the potential source of probiotics, especially *Lactococcus* strains. It is suggested that the regular consumption of Amaranthus fermented leaves can help to maintain serum cholesterol level. Daliri *et al.*, suggested the consumption of Korean fermented soybean paste could reduce the cardiovascular disease by reducing cholesterol level (Daliri *et al.*, 2022).

Conclusion

It is concluded from the present study that garlic and ginger are the potential source of probiotics bacteria, specially *Lactococcus lactis*. For the first time *Lactococcus lactis* isolated from raw garlic and ginger could be used for the treatment of cardiovascular disease due to its cholesterol reduction potential. The main difference between garlic and ginger isolates from other sources is that they are non-pathogenic and do not contain virulence factors. Moreover, *in-vivo* studies are needed to prove the hypercholesterolemia effect of *Lactococcus lactic*. Furthermore, *in-vitro* studies are necessary to identify the mechanism which has involved in the cholesterol assimilation by such a promising isolation.

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