HARNESSING THE POWER OF LACTIC ACID BACTERIA FROM LOCAL YOGURT: A PROMISING APPROACH FOR ENHANCING SOURDOUGH FERMENTATION

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خلاصہ

سیلیاک بیماری ایک آٹومیمون عارضہ ہے جو گلوٹین مالیکیولز سے پیدا ہوتا ہے ۔ اناج میں موجود پرولین سے بھرپور باقیات سیلیاک بیماری کے مریضوں میں منفی مدافعتی ردعمل میں ثالثی کرتے ہیں ۔ پاکستان میں بالغوں کے مقابلے میں بچوں میں سیلیاک بیماری کا زیادہ پھیلاؤ ہے ۔ نفسیاتی عوارض اور سیلیاک بیماری کے درمیان تعلق طویل عرصے سے جانا جاتا ہے ۔ سیلیاک بیماری کے مریضوں میں افسردگی اور دیگر نفسیاتی علامات کی نشوونما کا ایک اہم خطرہ ہے ۔ سیلیاک بیماری گٹ ڈیسبیوسس کا باعث بھی بنتی ہے ۔ لیکٹک ایسڈ بیکٹیریا گرام مثبت ، ایسڈ روادار ، چھڑی یا کوکی کی شکل کے بیکٹیریا ہوتے ہیں ۔ ایل اے بی اینٹی آکسیڈینٹ انزائم تیار کرتا ہے اور پروٹولائٹک انزائم تیار کرکے گلوٹین کے مالیکیولز کو خراب کرتا ہے ۔ وہ انزائموں کو اپ ریگولیٹ کرکے ڈپریشن کی علامات کو بھی کم کرتے ہیں ، جو ڈپریشن میں کم ہوتے ہیں ۔ مقاصد: اس موجودہ مطالعے کا مقصد مقامی دہی کے نمونوں سے لیکٹک ایسڈ بیکٹیریا کو الگ کرنا اور ایل اے بی کے ساتھ خمیر خمیر کے ذریعے گلوٹین کے مالیکیولز کو خراب کرنا ہے ۔ طریقہ کار: دہی کے نمونے کراچی کے مختلف مقامات سے اکٹھے کیے گئے ۔ ایم آر ایس میڈیا ، میکروسکوپک اور مائکروسکوپک تشخیص میں لیکٹک ایسڈ بیکٹیریا کو الگ کیا گیا ، اور ایل اے بی کی تصدیق کے لیے کیٹلیز ٹیسٹ سمیت بائیو کیمیکل ٹیسٹ کیے گئے ۔ ایل اے بی آئسولیٹس کے ساتھ خوراکی خمیر کیا گیا ۔ ایک ریپڈ ٹیسٹ کٹ نے گلوٹین کے مالیکیول کے انحطاط کا جائزہ لیا ۔ نتائج: گندم کے ڈیگریڈ نمونوں کے تابع ہونے پر تمام آئسولیٹ گیس اور بلبلے پیدا کرتے ہیں ۔ تاہم ، آئسولیٹ 9 نے مثبت کنٹرول کے مقابلے میں بلبلے کی تشکیل ، گیس کی پیداوار ، اور کھٹی کی پروفنگ کے لحاظ سے بہترین نتائج دیے ۔ اس مطالعہ سے پتہ چلا کہ ایل اے بی خمیر کے دوران گندم میں موجود گلوٹین مالیکیولز کی تعداد کو نمایاں طور پر کم کرتا ہے ۔ نتیجہ: اس مطالعے کا مقصد دہی سے لیکٹک ایسڈ بیکٹیریا کو الگ کرنا ، اور سیلیاک بیماری میں اس کا اطلاق کرنا تھا ۔ ایل اے بی مصنوعات کو بہتر بحالی کے لیے آپریشن کے بعد کے مریض کی خوراک میں شامل کیا جا سکتا ہے کیونکہ ایل اے بی اینٹی آکسیڈینٹ انزائمز کی پیداوار کے ذریعے مدافعتی نظام کو مضبوط کرے گا ۔ ایل اے بی کو مقامی طور پر گلوٹین فری مصنوعات کی تیاری کے لیے استعمال کیا جا سکتا ہے

Abstract

Celiac disease is an autoimmune disorder triggered by gluten molecules. The proline-rich residues present in the grains mediate the adverse immune response in patients with celiac disease. There is a high prevalence of celiac disease in children as compared to adults in Pakistan. The association between psychiatric disorders and Celiac disease has long been known. There is a significant risk of the development of depression and other psychiatric symptoms in patients with celiac disease. Celiac disease also leads to gut dysbiosis. Lactic acid bacteria are gram-positive, acid-tolerant, rod or cocci-shaped bacteria. LAB produce anti-oxidant enzymes and degrades gluten molecules by producing proteolytic enzymes. They also mitigate the symptoms of depression by up-regulating the enzymes, which are down regulated in depression. This present study aims to isolate lactic acid bacteria from local yogurt samples and to degrade the gluten molecules by sourdough fermentation with LAB. Yogurt samples were collected from various sites in Karachi. Lactic acid bacteria were isolated in MRS media, macroscopic and microscopic evaluation, and biochemical tests including catalase test was performed for confirmation of LAB. Sourdough fermentation with LAB isolates was carried out. A rapid test kit evaluated gluten molecule degradation. All isolates produced gas and bubbling when subjected to degrade wheat samples. However, isolate 9 gave the best results in terms of bubble formation, production of gas, and proofing of the sourdough when compared to the positive control. This study revealed that LAB significantly degrades the number of gluten molecules present in wheat during fermentation. ***Conclusion:*** This study aimed to isolate the lactic acid bacteria from yogurt, and its application in celiac disease. LAB products can be added to the diet of post-operative patient's diets for better recovery as LAB will strengthen the immune system by the production of antioxidant enzymes. LAB can be used for the production of gluten-free products locally.

Keywords: Gluten; Lactic acid bacteria (LAB); Sourdough fermentation; Yogurt

Introduction

Celiac disease is an autoimmune disorder triggered by gluten molecules. This condition serves as a paradigm for immune based disorders with genetic and environmental risk factors, and the complex interplay between genes, diet, and the microbiome is significant for the development of celiac disease (Lebwohl, 2018). A cross-sectional study conducted in Islamabad reported a higher prevalence of celiac disease in childhood than in adults in Pakistan (Jamila *et al.,* 2018). Gluten is an environmental trigger for Celiac disease. It is a storage protein found in wheat, rye, and barley grains. The gluten matrix and its activities are significant in influencing the dough quality of bread and other baked products including spaghetti, cakes, pastries, and cookies. This proline-rich residue creates tight and compact structures that can mediate adverse immune reactions in Celiac disease (Biesiekierski, 2017). An imbalance in oxidative defense system, due to disrupted to enzymatic and non-enzymatic antioxidant activities has been observed in Celiac disease (KhalKhal *et al.,* 2019).

Lactic acid bacteria (LAB) are a group of gram-positive, acid-tolerant microorganisms that are non-motile, non-spore-forming, and typically exhibit a rod-shaped or cocci-shaped morphology. These bacteria have a relatively low guanine + cytosine (G + C) content (Bintsis, 2018). In their metabolic processes, LAB primarily produce lactic acid as the main end-product under anaerobic conditions. Additionally, they synthesize a variety of metabolites crucial for the nutritional, sensory, and technological characteristics of fermented food products (Rodríguez *et al.,* 2019). The utilization of LAB in food fermentation represents one of the oldest methods of food preservation known to humanity (Bintsis, 2018). As fermentative organisms, LAB possess key metabolic traits that contribute to their significance in food processing. These include their capacity to generate sourness and unique flavors, their ability to break down proteins, their production of viscous exopolysaccharides, and their capability to inhibit undesirable bacteria when necessary (Wang et al., 2021). Furthermore, the presence of LAB in fermented foods and dietary supplements has been associated with various health benefits for both humans and animals (Feng & Wang, 2020).

The sourdough fermentation process with LAB creates an ideal environment for the degradation of cereal prolamin, a semi-liquid dynamic pH system that optimally activates and stimulates cereal proteases within their pH range (Graça, 2021). Under controlled conditions, LAB can effectively break down the major gluten protein, gliadin (Angelis *et al.,* 2006). Lactobacilli, a type of LAB, utilize their proteolytic system to release amino acids necessary for their growth and development. The sourdough fermentation process also yields high-quality gluten-free products (Scherf, 2018). Furthermore, the use of specifically selected sourdough LAB cultures as fermentation starters is recommended as a means to eliminate the risk of gluten contamination in gluten-free products (Cristofori *et al.,* 2020). This approach ensures the production of safe and gluten-free food products, which are particularly important for individuals with gluten intolerance or sensitivity. As patients diagnosed with celiac disease are recommended to follow a strict lifelong gluten-free diet (Canbanillas, 2020). Current study aims to isolate different strains of LAB from yogurt samples and to eliminate gluten molecules in flour by application of sourdough fermentation with the isolated strains. The study provides a solution for the production of gluten-free wheat products using LAB strains. The inclusion of fermented wheat products with LAB may reduce gluten immunogenicity in patients with celiac disease.

Materials and Methods

***Collection of Local Yogurt Samples*:** Ten samples of yogurt were collected at various sites in Karachi. All samples were labeled and stored right away at low temperatures (Saavedra *et al.,* 2003).

***Isolation of Lactic Acid Bacteria*:** Isolation of LAB was performed by preparing 10 times serial dilutions followed by inoculation in 10 ml of MRS broth. The broth tubes were incubated at 37°C for 48-72 hours. Pure cultures of LAB isolates were obtained by the spread plate method (Saavedra *et al.,* 2003).

***Morphological Characterization*:** After obtaining the pure culture, the morphological characteristics such as shape, size, colony color, elevation, texture, and arrangement were observed. Gram staining was performed according to the standard procedure for each LAB isolate to confirm the morphology and Gram reaction. The culture slides were observed microscopically at 100 x magnification (Saavedra *et al.,* 2003).

Biochemical Characteristics

***Catalase Test*:** The catalase test is used to identify the organisms that contain catalase enzymes. Catalase is an oxidoreductase enzyme that converts hydrogen peroxide which is a powerful oxidizing agent into water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \text{ (gas bubbles)}$$

Fresh cultures of isolates were obtained by overnight 24hrs incubation. Catalase test was performed by taking a loopful of fresh culture onto the glass slide; two drops of 3% hydrogen peroxide solution were added to the cultures and were observed for bubble formation (Salaj *et al.*, 2013).

***Sourdough Fermentation*:** Whole wheat was purchased from a local store. 100 g of wheat was transferred to each sterilized 250 ml pyrex jar. The jars were placed under UV light in a laminar flow hood for approximately 10 minutes to remove any type of impurities that would interfere with the fermentation process. Suspensions of LAB isolates were prepared in PBS and compared with 1 McFarland standard. 3 ml of the suspension was added to a 100 g wheat sample and sterile distilled water was added until a sourdough mixture of thick consistency was obtained. A negative control was prepared by adding distilled water and wheat, and a positive control was prepared by adding 3 grams of yeast to 100g of wheat. All jars were covered with food-grade cling wrap, placed at room temperature for about 24-48 hours. Samples fermented with the best LAB strain were baked at 24 and 48 hours respectively to record textures, aromas, and rising of sourdough. The best strains were also selected for further analysis.

***Rapid Kit Test for Detection of Gluten Molecules*:** The bread samples for 24 and 48 hours were homogenized. 2 grams of sample were added into the extraction bottle containing extraction buffer. The bottle was closed and gently mixed with the help of a disposable pipette, the two drops of the sample were added to a dilution bottle containing the dilution buffer. Then it was mixed gently by inverted repeats. With the help of the disposable pipette, 4 drops of the sample were added to the S zone of the kit. After 10 minutes, the results were observed.

Results and Discussion

The outcomes of this study provided useful insights into the application of LAB in celiac disease. Celiac disease affects 0.14-4.4% of people in different parts of the world and is more common in children and adolescents (Jamila *et al.*, 2018). It demonstrated that gluten free products can be produced through sourdough fermentation and relief could be provided to the individual with stress-like conditions just by adding a dairy product like yogurt which is a rich source of LAB. Local yogurt samples were collected from various sites in Karachi. **Table** 1 represents the location from where the yogurt samples were collected. Some of the isolates were identified as slow-growing LAB strains because they grew more slowly and required longer incubation times as depicted in Figure 1. Microscopic characterization was done by performing gram staining. Gram reaction along with the morphology was observed for each isolate at 100 x magnification under a microscope. All the isolates were gram-positive and have either bacilli or cocci morphology as mentioned in Table-2 and Figure-2.

Table-1: Locations of the sample collection.

Samples	Localities
1	University Road, Karachi
2	Mehmoodabad, Karachi
3	North Nazimabad, Karachi
4	Safoora, Karachi
5	Gulzar e Hijri, Karachi
6	Gulistan-e-Jauhar, Karachi
7	DHA, Karachi
8	Gulshan, Karachi
9	Nazimabad, Karachi
10	Malir Cantt, Karachi

Table-2: Colonial and Micoscopic characterisation of isolated Lactic acid bacteria.

Samples	Colonial characteristics				Microscopy	
	Forms	Elevation	Margin	Color	Gram reaction	Shape
1	Large circular	Flat	Entire	Yellowish white	Gram positive	Bacilli
2	Small circular	Raised	Entire	White	Gram positive	Cocci
3	Small circular	Convex	Entire	White	Gram positive	Bacilli
4	Small circular	Convex	Entire	Creamy white	Gram positive	Cocci

4	Medium circular	Convex	Entire	Yellowish white	Gram positive	Bacilli
6	Punctiform	Umbonate	Entire	White	Gram positive	Bacilli
7	Small circular	Convex	Entire	White	Gram positive	Cocci
8	Irregular	Convex	Entire	White	Gram positive	Cocci
9	Small circular	Convex	Entire	White	Gram positive	Cocci
10	Small circular	Convex	Entire	White	Gram positive	Cocci

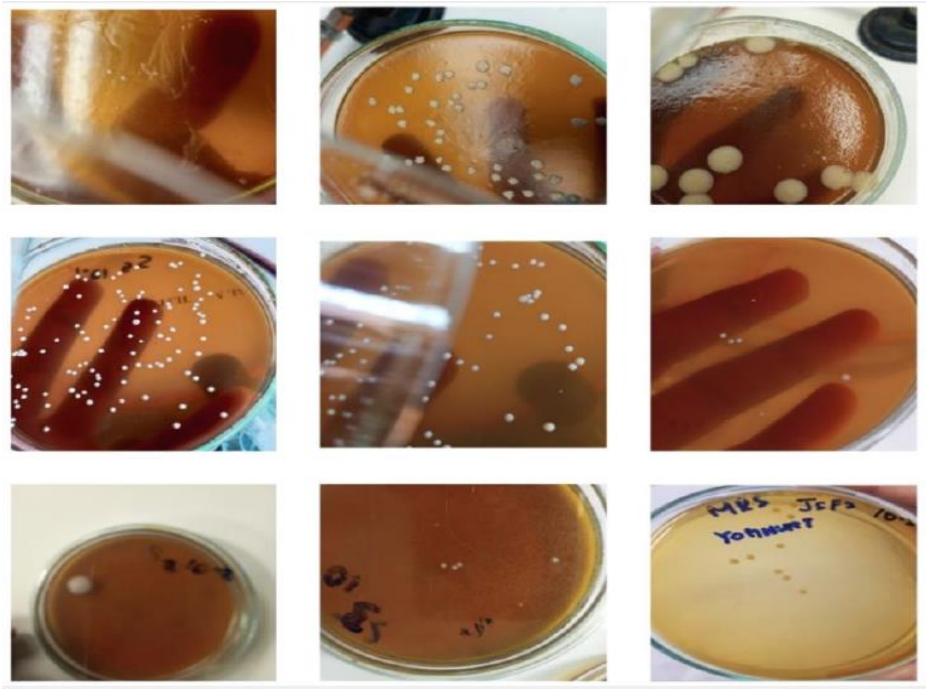

Fig. 1: Isolated lactic acid bacteria from yogurt samples exhibiting macroscopic characteristics.

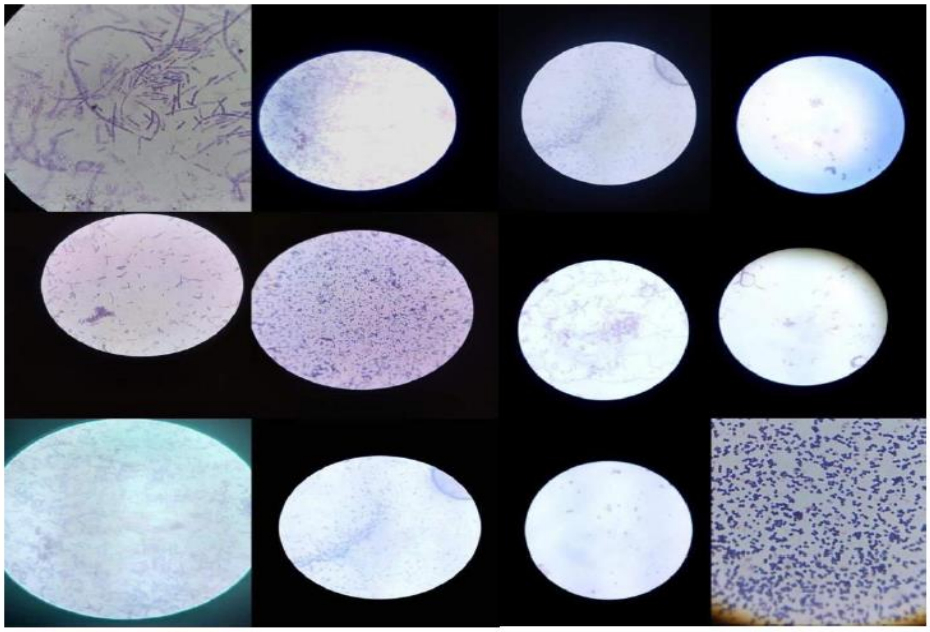

Fig. 2: Gram reaction of Lactic acid bacteria isolated from yogurt samples.

Catalase test was performed to ensure that all the isolates are LAB. No bubbling was observed upon exposure of colonies with hydrogen peroxides. All isolates were recorded as catalase-negative, which is the characteristic of LAB. Ibrahim *et al.* (2015) reported the catalase negative activity of lactic acid bacterial species isolated from mangoes. During the sourdough fermentation process, all isolates produced gas and bubbles when subjected to degrade wheat samples which is indicative of active fermentation. However, isolate 9 gave the best results in terms of bubble formation, production of gas, and proofing of the sourdough when compared to the positive control. No bubble formation, gas production, and proofing of dough was observed in negative controls. Results of 24 hours' fermentation of wheat samples were considered best as compared to the 48 hours' results. As over proofing occurred after 48 hours and sourdoughs were flattened along with excessive gas production. After proofing, the sourdoughs fermented with LAB isolate 9 were baked at 180^0C for about 12-15 minutes. After baking, the aroma and texture of the sourdough bread were recorded. Various microbes are involved in wheat fermentation. However, genera Lactobacilli is dominant in sour dough fermentation (Corsetti *et al.*, 2004). Figure-3 exhibits the LAB-fermented wheat samples. There was no proofing observed in the dough of negative control. The highest proofing and bubble formation was observed in the sample fermented with isolated 9 as compared to the rest of the wheat sample fermented with the remaining isolates.

Fig. 3: LAB fermented wheat along with positive and negative control.

Figure-4 shows the characteristics of dough after fermentation. The volume of dough has been increased. Bubble formation can be observed along the sides of the pyrex jar beneath the surface. Figure-5 shows the baked sourdough bread. The negative control slices of bread were flat and had no aroma. Whereas the rest of the fermented bread was proofed, tiny air pockets were observed in all the sourdough pieces of bread. 24 hours sourdough breadcrumb was less hardened and has high improvement as compared to 48 hours. Bartkiene *et al.* (2017) reported the sour dough fermentation by LAB and the dough was tested for the production of bread.

Fig. 4: Characteristics of dough after fermentation with LAB isolates

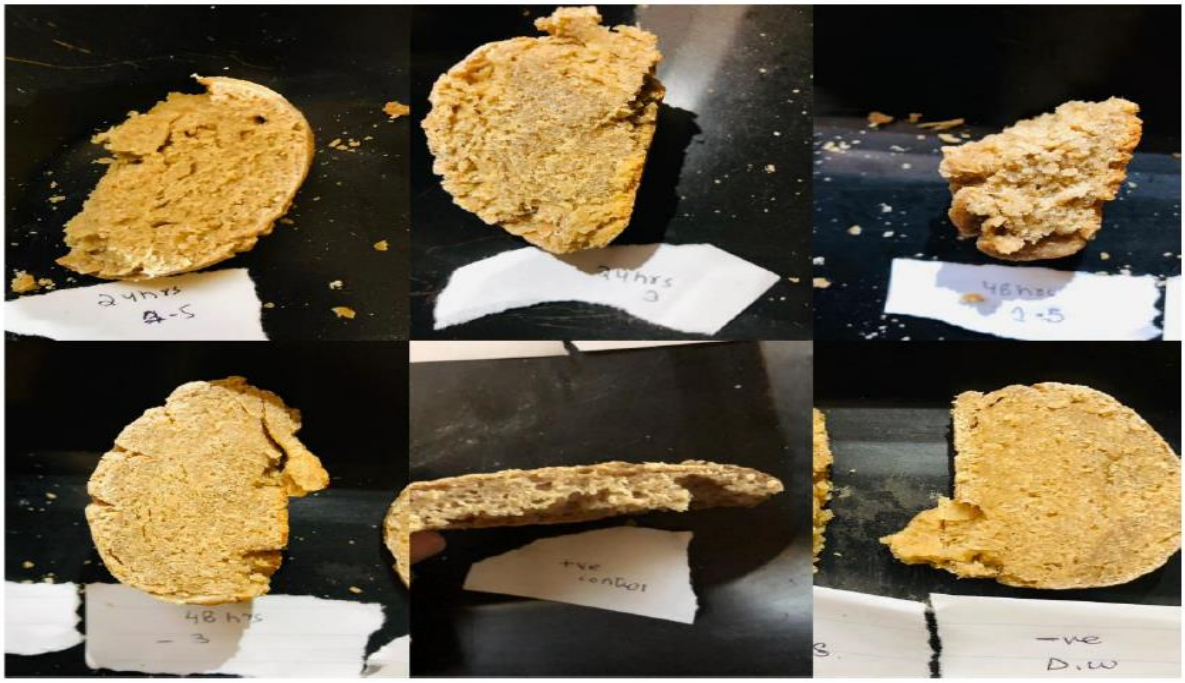

Fig. 5: Baked sourdough bread with the LAB isolate 9 along with positive and negative control.

A pink line was observed in the kit, which indicated the presence of some gluten molecules greater than 20 ppm in sourdough bread. Many studies have reported that LAB degrade the gluten molecules by cleaving the proline-rich residues responsible for immunogenicity. The gluten is degraded by the production of proteolytic enzymes produced by LAB during the process of fermentation. The results of our sourdough fermentation confirm the degradation of the gluten molecule by LAB but the gluten molecules were not degraded up to the safe consumption level which is 20 ppm. A possible factor could be the high ratio of wheat, or the use of only a single strain, not a combination of strains. The production of gluten free products in Pakistan can be done simply by the application of LAB at the industrial level. Utilizing specific sourdough lactic acid bacteria cultures as fermentation starters is recommended as a method to mitigate the potential for gluten contamination (Cristofori *et al.*, 2020). The best strain of LAB degrading the gluten molecules can be isolated from natural sources including dairy products. Since most of the gluten-free products are imported and are very costly to be afforded by the middle and lower class socioeconomic statuses. Local production makes gluten-free products available to everyone. Further, this research paves the way for the improving the quality of life for individuals with celiac disease when applied at industrial scale.

Conclusion

This study aimed to isolate the LAB from yogurt, and its application in celiac disease. LAB products can be added to the diet of post-operative patient's diets for better recovery as LAB will strengthen the immune system by the production of antioxidant enzymes. LAB can be used for the production of gluten-free products locally.

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