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Production of Reuterin by *Lactobacillus reuteri* and Its Antimicrobial Activity in Fermented Milk Products

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Abstract

This study investigates the production of reuterin by *Lactobacillus reuteri* ATCC 53608, both in vitro and in situ in fermented milk products, and evaluates its antimicrobial activity against various microorganisms. Reuterin was synthesized in aqueous glycerol solution and within fermented milk, then tested against starter cultures (*Lactobacillus delbrueckii subsp. bulgaricus*, *Streptococcus thermophilus*), spoilage organisms (*Penicillium expansum*), pathogenic bacteria (*Staphylococcus aureus*, *Salmonella enterica subsp. enterica*, *Listeria monocytogenes*), and a surrogate pathogen (*Escherichia coli* DH5α). The results demonstrate that reuterin remains stable during refrigerated storage. Gram-positive bacteria were more resistant to reuterin than Gram-negative bacteria, with *E. coli* DH5α being the most sensitive (MIC 0.9 mM), while *P. expansum* and *L. reuteri* ATCC 53608 tolerated concentrations up to 10 mM and 8.5 mM, respectively. The presence of reuterin did not significantly affect the quality parameters of the fermented milk product, including pH, acidity, soluble solids, and color.

Subject Areas

Traditional Biotechnology, Food Technology

Keywords

Fermented Milk Products, *Lactobacillus reuteri*, Reuterin

1. Introduction

The shelf life of yogurt and fermented milk is most commonly extended by refrigeration; however, its duration varies depending on storage temperature, presence

of spoilage and pathogenic microorganisms, and other factors. Foodborne illnesses caused by *Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp.*, and *Listeria spp.* have led to the use of chemical preservatives such as benzoic and sorbic acids [1] [2]. Growing consumer demand for natural alternatives has driven research into biopreservation, particularly using lactic acid bacteria (LAB) capable of synthesizing antimicrobial compounds [3]-[5]. Among these, *Lactobacillus reuteri* can produce reuterin—a broad-spectrum antimicrobial agent—during anaerobic glycerol metabolism, offering a promising natural strategy for food preservation.

Recent peer-reviewed literature underscores the expanding role of reuterin as an efficacious natural biopreservative in dairy products. A 2022 review delineates the multifunctional antimicrobial mechanisms of the reuterin system and outlines challenges related to scale-up production for industrial use [6]. Concurrently, studies conducted in 2023 demonstrate that the synergistic application of reuterin with bacteriocins such as nisin and pediocin significantly inhibits microbial proliferation in raw milk, thereby prolonging shelf life without adversely affecting fundamental quality parameters [7]. Additionally, emerging evidence highlights the cooperative action of reuterin alongside other microbial metabolites, including organic acids and hydrogen peroxide, in enhancing antifungal and antibacterial effects within dairy preservation strategies.

2. Fermented Milk Products

2.1. The Nutritional and Health Benefits of Fermented Dairy Products

Fermented dairy products represent highly valuable and health-promoting foods widely utilized in human nutrition. In addition to their well-established nutritional and biological value, these products may exert beneficial effects on human health. According to several studies, fermented dairy products may contribute to immune system enhancement and the prevention of cardiovascular diseases [8]. Fermented dairy products are obtained through lactic acid fermentation (as in yogurt) or through a combination of lactic acid fermentation and yeast fermentation (as in kefir). These processes not only improve the digestibility and shelf life of dairy products but also enhance their nutritional profile. Fermented dairy products serve as an excellent source of proteins, calcium, phosphorus, magnesium, and vitamins B2 (riboflavin) and B12 (cobalamin). Common examples of fermented dairy products include sour milk, yogurt, acidophilus milk, kefir, kumis, cream, and a variety of cheeses. The regular consumption of fermented dairy products is associated with multiple health benefits, supporting their inclusion as functional foods in the human diet.

2.2. Microbial Cultures of Fermented Dairy Products

In addition to their own microflora, fermented dairy products contain cultures of various bacterial species, which are significant in both nutritional and biological

aspects. The starter cultures used in the dairy industry include: *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Bifidobacterium* [2] [3].

3. *Lactobacillus reuteri*

Lactobacillus reuteri (See Figure 1) is a heterofermentative LAB found in food and the gastrointestinal tracts of humans and animals [9]. It plays a protective role against pathogens and helps stabilize gut microflora. Its ability to synthesize reuterin depends on factors such as temperature, pH, oxygen concentration, biomass, and cell age, which must be optimized for effective food applications.

Figure 1. *Lactobacillus reuteri.*

3.1. Strain Selection and Cultivation

The selection of appropriate *L. reuteri* strains is critical for effective reuterin production. Strains are typically isolated from fermented foods or gastrointestinal samples and screened for their ability to produce reuterin under laboratory conditions. Cultivation is performed in MRS broth under anaerobic conditions, with optimization of growth parameters to maximize biomass and metabolic activity.

3.2. Genetic and Metabolic Characteristics

L. reuteri possesses the pdu (propanediol-utilization) operon, which is essential for the conversion of glycerol to reuterin [10] [11]. The genetic regulation of this pathway, as well as the presence of accessory genes, influences the efficiency of reuterin synthesis. Metabolic profiling has shown that reuterin production is associated with specific growth phases and is enhanced by the availability of glycerol as a substrate.

3.3. Factors Affecting Reuterin Synthesis

Reuterin synthesis is influenced by environmental and physiological factors, including temperature, pH, substrate concentration, and incubation time [10] [12]. Optimal production is typically achieved at 37˚C, pH 6.5, and with excess glycerol. Oxygen presence can inhibit reuterin formation, necessitating strict anaerobic

conditions during the synthesis phase.

3.4. Application in Food Systems

The integration of *L. reuteri* into fermented milk products requires careful consideration of its compatibility with starter cultures, its impact on sensory properties, and its ability to produce reuterin *in situ*. Studies have demonstrated that co-cultivation with traditional starter cultures can be achieved without significant negative effects on fermentation kinetics or product quality [9] [12].

4. Reuterin

Reuterin (3-hydroxypropionaldehyde) is a low-molecular-weight, neutral compound with broad-spectrum antimicrobial activity [10] [11]. It inhibits the growth of bacteria (including *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, *Clostridium*, and *Staphylococcus*), yeasts, fungi, and protozoa. Reuterin is synthesized by *L. reuteri* during anaerobic metabolism of glycerol and can be produced both *in vitro* and *in situ* in food matrices (see **Figure 2**).

Figure 2. 3-hydroxypropionaldehyde.

4.1. Synthesis of Reuterin

4.1.1. Biomass and Reuterin Production *In Vitro*

L. reuteri ATCC 53608 was cultured in MRS broth, followed by incubation with glycerol under anaerobic conditions. After cell harvesting and washing, the biomass was incubated with glycerol to induce reuterin synthesis. The supernatant was collected, filtered, and used as the reuterin extract.

4.1.2. Quantification of Reuterin

Reuterin concentration was determined using a colorimetric method based on the reaction of reuterin-derived acrolein with tryptophan in acidic conditions, measuring absorbance at 560 nm. Calibration was performed with acrolein standards.

4.1.3. *In Vitro* Inhibitory Testing

The antimicrobial activity of reuterin was assessed against various microorganisms. Bacterial cultures were exposed to different concentrations of reuterin, and minimal inhibitory concentrations (MICs) were determined by the absence of growth after incubation.

4.1.4. *In Situ* Production in Fermented Milk

Fermented milk was prepared with pasteurized skim milk, inoculated with starter cultures and *L. reuteri* ATCC 53608. Glycerol was added during fermentation to stimulate reuterin production [12]. After fermentation, pathogenic and spoilage organisms were introduced to simulate post-process contamination. Products

were stored at 4˚C for shelf-life analysis.

4.2. Packaging and Storage

Fermented milk products containing reuterin were stored in refrigerated conditions for up to 28 days. Microbial stability and product quality were monitored throughout storage.

4.3. Antimicrobial Activity of Reuterin

4.3.1. Effect on Starter Cultures

Reuterin exhibited inhibitory effects on starter cultures, but these were less pronounced than on pathogenic and spoilage organisms [10] [12].

4.3.2. Survival of Microorganisms in Fermented Milk

Gram-positive bacteria (e.g., *L. delbrueckii*, *S. thermophilus*) showed higher resistance to reuterin, with MICs above those for Gram-negative bacteria. *E. coli* DH5*α* was the most sensitive (MIC 0.9 mM) [13]. *P. expansum* and *L. reuteri* ATCC 53608 tolerated up to 10 mM and 8.5 mM, respectively.

Escherichia coli DH5*α* is widely employed as a surrogate pathogen in antimicrobial efficacy studies due to its well-characterized genetic background and non-pathogenic status. Although it lacks the virulence factors characteristic of pathogenic *E. coli* strains, DH5*α* exhibits similar susceptibility profiles, rendering it a suitable model organism for evaluating antimicrobial agents in food matrices. This strain's widespread use in laboratory investigations is supported by its safety and its ability to effectively mimic pathogen responses under controlled conditions.

4.4. Impact on Product Quality

Reuterin addition did not significantly alter key quality parameters (pH, acidity, soluble solids, color) of the fermented milk product during storage.

The incorporation of reuterin, produced by *Lactobacillus reuteri*, into fermented dairy products has been demonstrated to exert minimal impact on key sensory attributes such as pH, acidity, color, and texture, thereby preserving the organoleptic quality of the product. From a regulatory perspective, reuterin and reuterin-producing *L. reuteri* strains benefit from Generally Recognized As Safe (GRAS) status in food applications. Nonetheless, sensory outcomes may be concentration-dependent, necessitating optimized dosing strategies to mitigate any potential off-flavors or modifications in fermentation dynamics. Regulatory frameworks currently emphasize the safety of residual antimicrobial compounds, although explicit limits specific to reuterin continue to evolve in alignment with advancing research.

5. Conclusions

Reuterin produced by *L. reuteri* ATCC 53608 demonstrates strong antimicrobial activity against a range of spoilage and pathogenic microorganisms in both *in*

vitro and *in situ* conditions. Its stability during refrigerated storage and lack of negative impact on product quality support its potential as a natural biopreservative in fermented milk products. Further research should explore its application in other food matrices and assess consumer acceptance.

This investigation utilized a single *Lactobacillus reuteri* strain (ATCC 53608), which may restrict the broader applicability of the findings, considering the strain-dependent variability in reuterin synthesis and antimicrobial potency. Moreover, the absence of consumer sensory evaluation represents a critical gap in assessing commercial acceptability. Future studies should incorporate multiple *L. reuteri* strains to account for phenotypic diversity and conduct comprehensive sensory trials to evaluate consumer preferences and acceptance. Furthermore, detailed exploration of different dairy matrices, long-term storage effects, and synergistic interactions between reuterin and other biopreservatives is warranted to advance practical applications.

Conflicts of Interest

The author declares no conflicts of interest.

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