Biooptimization of Bacteriocin Production from a Local Isolate of *Lactiplantibacillus plantarum* and Evaluation of its Inhibitory Efficacy Against Some Foodborne Pathogens.

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Abstract

This work was designed to determine the effect of different fermentation parameters on *bacteriocin* production by a local strain of *Lactiplantibacillus plantarum*. The *bacteriocin* produced under the most suitable fermentation parameters was purified to homogeneity to assess its inhibitory effect against some common foodborne pathogens. The present study was conducted to optimize the production of *bacteriocin* from a local strain of *Lactiplantibacillus plantarum* in order to find its inhibitory potential and use it as a natural biopreservative to ensure food safety. The bacterial strain MKN, isolated from corn silage, was molecularly identified and deposited in the GenBank of the National Center for Biotechnology Information under the accession number LC939791.1. The VITEK 2 compact system gave the identification of the strain as that of Staphylococcus aureus and Escherichia coli with an accuracy of 99%.

The isolate was further tested for its antimicrobial activity against some foodborne pathogens. It was noted that the MKN isolate demonstrated the best inhibition compared to the other isolates tested; zones of inhibition measuring 24 and 28 mm were recorded against *S. aureus* and *E. coli*, respectively. Optimize *bacteriocin* production by studying the influence of culture conditions on *bacteriocin* production. The best medium for *bacteriocin* production proved to be skim milk. The maximum level (20 mg/mL) was achieved at 37°C after 72 h of incubation. Results confirmed that the indigenous *L. plantarum* MKN strain has great potential as a good *bacteriocin* producer against major foodborne pathogens. The optimized production parameters achieved in this study would therefore serve as a good baseline for increasing *bacteriocin* output and making it easier to use as a substitute for natural preservatives in the food industry. This strain can, therefore, be considered a valid candidate for enhancing food quality, shelf life, and consumer safety through sustainable biopreservation means.

Keywords: *Bacteriocin*, Biopreservation, VITEK 2, Corn Silage, *Plantaricin*.

Introduction

Foodborne diseases constitute major health problems worldwide, with millions of cases and deaths recorded annually and a substantial economic impact due to reduced food quality and withdrawal of contaminated food products from the market [1]. *Staphylococcus aureus* and *Escherichia coli* are some of the most important foodborne pathogens since they can produce toxins that cause food poisoning and intestinal diseases in humans [2, 3]. While the use of chemical preservatives has largely been successful at inhibiting the growth of these microorganisms, there is increasing concern about their safety and possible effects on consumers. This has renewed the interest of researchers and the food industry in safe and sustainable alternatives, particularly because

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consumers are now demanding natural foods without artificial additives [4].

In this context, biopreservation using lactic acid bacteria has been recently suggested as a means to enhance food safety and shelf life by applying beneficial microorganisms or their metabolites of antimicrobial compounds [5]. Among the microorganisms, lactic acid bacteria (LAB) are the most frequently used because they are generally regarded as safe organisms and can produce a broad spectrum of antimicrobial compounds, including organic acids, hydrogen peroxide, and bacteriocins [6].

Bacteriocins are proteins or peptides that are highly inhibitory against many foodborne pathogens and spoilage bacteria [7]. They can be used as preservatives in place of chemicals since they are relatively stable and biosafe. Among bacteriocin-producing species, strains of *Lactiplantibacillus plantarum* have recently become very popular because they can produce more than one type of bacteriocin, which has been proven very effective in inhibiting most foodborne pathogens; these bacteriocins are referred to as plantaricins [8].

Recent studies have indicated that the source of isolation plays a significant role in the biological efficacy of bacterial strains. Promising reservoirs for isolating strains with high capacities for the production of antimicrobial compounds include natural environments that have microbial competition, such as silage [9]. This would also be useful in the optimization of production conditions and the selection of suitable nutrient media to increase bacteriocin yield and reduce production cost, thus enhancing the potential for industrial-scale application [10].

This study was therefore carried out to isolate and characterize a local strain of *Lactiplantibacillus plantarum* from corn silage, assess its inhibitory activity against some foodborne pathogens, and optimize the production of bacteriocin under low-cost nutrient media. The work's novelty can be credited to the fact that it used a local isolate registered in the GenBank database and tested its applicability for biopreservation in food, thereby contributing to the development of natural and safe alternatives to the conventional chemical preservatives.

Although plantaricin production by *Lactiplantibacillus plantarum* has been studied extensively, little is known about bacteriocin-producing strains isolated from Iraqi corn silage. The effects of activation strategies and production media on plantaricin yield from locally isolated strains have also received limited attention. This study therefore focused on optimizing plantaricin production by a newly isolated *L. plantarum* strain, MKN (NCBI accession LC939791.1), and assessing its antimicrobial activity against selected foodborne pathogens. The findings offer new insight into the possible use of indigenous bacteriocin-producing strains for food biopreservation.

Material and Methods

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Bacterial Isolation and Identificati

The food samples were corn silage, meat, local yogurt, and commercial yogurt. These were collected to ensure a wide diversity of bacterial isolates that could be capable of producing bacteriocins. The primary isolations were carried out on appropriate culture media, ensuring all aseptic conditions. Later, the purified isolates were identified using the VITEK® 2 automated diagnostic system (BioMérieux, France). This system is based on the most accurate and rapid technique of species identification through

the biochemical characteristics of bacteria. In this system, the GP ID card is meant for Gram-positive bacteria and the GN ID card for Gram-negative bacteria. Each card has a series of 43-47 biochemical tests which are automatically interpreted to give a very reliable final diagnosis [11].

Evaluation of the Inhibitory Activity of Bacterial Isolates

The well diffusion method was used to assess the antibacterial activity of selected isolates. Test bacteria used were isolates of Staphylococcus aureus and Escherichia coli to represent Gram-positive and Gram-negative bacteria, respectively. The diameters of the zones of inhibition formed around the wells were measured after the appropriate incubation period. In this study, the diameter of the inhibition zone was used as an indicator in measuring the efficiency of the isolates in producing inhibitory compounds, including bacteriocins [12].

Optimizing Bacteriocin Production Conditions

To optimize bacteriocin yield, various factors related to the production process were investigated. The first phase was to compare triple activations on MRS medium, quadruple activations based on exchange between culture media, and triple activation enhanced with filtered skim milk. Other factors considered included the efficacy of three culture media - MRS Broth, skim milk, and Nutrient Broth - in the support of bacterial growth and bacteriocin production. Temperature (30, 35, and 37°C) and incubation periods (24, 48, and 72 hours) were also studied in an integrated factorial design to ascertain the best production conditions that would lead to the highest concentration of the bioactive compound. Other factors considered were temperature and time of incubation [13,14].

Measurements and Statistical Analysis

Bacteriocin production was estimated by the measurement of its concentration, expressed in mg/mL. Its biological efficiency was determined by measuring the diameter of inhibition zones expressed in millimeters. All measurements were taken as the average of three determinations performed to ensure accuracy and minimize experimental error.

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The results are expressed as the Mean ± Standard Deviation (Mean ± SD). The data were analyzed using suitable statistical methods to determine the significance of the differences among the treatments at $P \leq 0.05$ by one-way analysis of variance (ANOVA). Means were compared using Tukey's HSD test at a significance level of $P \leq 0.05$. Results are reported as mean ± standard deviation [15].

Results and Discussion

The VITEK 2 system was used for final identification of the taxonomical characteristics of the pathogenic bacterial isolates. This method of diagnosis is rapid, accurate, and highly efficient in diagnosing microbial infections. The GP ID Card, designed for use with Gram-positive bacteria like Staphylococcus aureus, has 43 tests in 64 wells with special media for different types of reactions. Diagnostically, there was a 99% concordance rate. The GN ID Card, designed for use with Gram-negative bacteria like *Escherichia coli*, has 47 tests in 64 wells with special media for different types of reactions. It also achieved a 99% diagnostic accuracy rate.

These results confirm the efficiency of the VITEK 2 system in the rapid and accurate diagnosis of bacteria of medical and nutritional importance. They are consistent with the results obtained by [16]. who stated that this system gives a high level of reliability in identifying different bacterial species on the basis of their biochemical characteristics as isolates.

Evaluation of the Inhibitory Activity of Local Isolates Against Pathogenic Bacteria

The ability of the bacterial isolates to produce antimicrobial compounds can be evaluated through their inhibitory activity, which may reflect their capacity to synthesize broad-spectrum antimicrobial compounds. According to [17], this has been adopted as the prime selection criterion for identifying strains that can be used in biotechnological processes and for food preservation. In this study, four indigenous isolates of Lactiplantibacillus plantarum obtained from corn silage, traditional fermented milk, commercial fermented milk, and meat were evaluated for their inhibitory activity against pathogenic strains of *Staphylococcus aureus* and *Escherichia coli* using the Well Diffusion Method.

Activity among the four isolates. The isolate from corn silage had the highest antimicrobial activity, with inhibition zones of 24 and 28 mm against S. aureus and E. coli, respectively. The isolates from traditional fermented milk and commercial fermented milk came next with inhibition zones of 22 and 20 mm, respectively. The meat isolate had the least inhibitory activity, with zones of 18 and 20 mm, respectively. Similarly, the isolates displayed higher inhibition zones against E. coli compared to *S. aureus*, which may be associated with better permeability of the compounds that cause inhibition through the outer membrane of Gram-negative bacteria [18]. Also found similar results in their research on bacteriocins produced by strains of *L. plantarum*.

The better performance of the isolate obtained from corn silage could be due to the acidic and anaerobic conditions in the natural fermentation process of silage. Such an environment would promote the natural selection of strains capable of producing higher levels of antimicrobial compounds as a competitive survival mechanism against other microorganisms. According to [19], the *Lactiplantibacillus plantarum* strains isolated from silage environments express enhanced genetic systems for bacteriocin production compared with strains from other ecological niches; they thus increase their

antimicrobial efficiency and competitive advantage.

Table 1. Inhibition diameters of the four local isolates against pathogenic bacteria.

Isolates	Corn silage	meat	local milk	commercial milk
S. aureus	24	18	22	20
E. coli	28	20	25	22

Selection of the Most Efficient Isolate

The corn silage-derived Lactiplantibacillus plantarum isolate was chosen as the best isolate due to its stronger antagonistic activity against the pathogenic bacteria tested. It had significantly larger zones of inhibition against both Staphylococcus aureus and Escherichia coli compared to the other isolates. This meant that it had a higher capacity to produce antimicrobial compounds, particularly the bacteriocin Plantaricin. Therefore, it was chosen to be labeled as the producer strain for subsequent experiments on Plantaricin production, purification, and biological applications. The NCBI GenBank database received it under the name *Lactiplantibacillus plantarum* strain MKN with the accession number LC939791.1. This registration validated and scientifically justified the valuable contribution made through the registration of a new local Plantaricin-producing strain. Similar reports exist on highly productive bacteriocin-producing strains selected through similar methods as those used in the present study by Todorov and Franco [20].

Determination of the Optimal Conditions for Plantaricin Production by *Lactiplantibacillus plantarum*

Some factors that affect Plantaricin production by the chosen *Lactiplantibacillus plantarum* isolate were studied, such as the method of inoculum activation, culture medium composition, incubation period, temperature, and nutritional supplements. The study aimed to find out the conditions that result in maximum bacteriocin yield. The indicator used to measure the efficiency of production of Plantaricin was the inhibitory activity of the cell-free supernatant against pathogenic bacteria. Similar optimization strategies have been used successfully in other reports for enhancing bacteriocin productivity in lactic acid bacteria [21].

Activation of the Selected Lactiplantibacillus plantarum Inoculum

A. Activation in MRS Broth

This technique involved three successive activation cycles in MRS broth with an incubation period of 24 h for each cycle. The results showed weak bacterial growth with small colony formation on MRS agar. This was probably due to the short incubation period; 24 h was not enough for the bacterial cells to reach the late logarithmic growth phase. It can also be said that repeated cultivation in a single medium without nutritional diversity can limit cellular adaptation to subsequent changes in the environment. Results obtained were similar to those of Shaaban et al. [22], who stated that short-term activation in a single medium lowers cell density and reduces bacteriocin productivity.

B. Four-Step Activation Using MRS Broth and Skim Milk

This protocol entailed four sequential activation steps in MRS broth and skim

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milk, with an incubation time of 24 h for each step. The results obtained indicated relatively weak growth but with larger colony size. The response observed may be due to nutritional shock from the transfer of cells, previously adapted to MRS broth, into skim milk. Abrupt changes in nutrient composition can lengthen the lag phase of the cells' growth, and even though there is casein and minerals for cell enlargement, it does not directly support rapid division [23] made a similar observation when they stated that an abrupt switch of culture medium could have negative impacts on the growth performance of bacteria.

C. Improved Three-Step Activation Using Filtered Skim Milk

The protocol involved two activation stages in MRS broth followed by a third stage in skim milk, with an incubation period of 48 h for each step. This method outperformed the previous protocols by producing dense bacterial growth and large colonies. The enhanced performance can be ascribed to several factors. A 48-h incubation period allowed the bacterial cells to reach the late logarithmic phase, where metabolic activity is at its maximum. This gradual transition helped reduce cellular stress and facilitated physiological adaptation. The final product, synthesized by healthy cells that were not subjected to stress, was contamination-free because the cells had enough time to adapt metabolically; they were inoculated into sterile filtered skim milk (0.22 μm membrane filter) [21] stated that gradual multi-stage activation, combined with prolonged incubation and medium diversification, can increase bacteriocin production by 30–50%. The latter activation protocol was used for the subsequent stages of the study.

Effect of Culture Medium on Plantaricin Production

The culture medium and its nutritional components rank among the most influential parameters regarding bacteriocin production by Lactiplantibacillus plantarum. Carbon sources, nitrogen sources, and growth factors can bring about significant changes in the efficiency of production. Therefore, the ability to support Plantaricin production by the selected isolate was tested in three different culture media, namely MRS broth, skim milk, and nutrient broth (Table 2).

The results showed that skim milk was the most effective medium for Plantaricin production, with a yield of 19 mg/mL. This was followed by MRS broth with 18 mg/mL. No detectable activity was observed in nutrient broth. From these results, it can be said that environments rich in nutrients and that contain easily available proteins and milk sugars are better placed for bacteriocin biosynthesis than general media. [22] also made similar conclusions and pointed out that nutrient composition is a key factor in the optimization of bacteriocin production by lactic acid bacteria.

The high performance of skim milk in Plantaricin production can be related to its high content of casein protein. This provides the essential amino acids required for biosynthesis of bacteriocins, since bacteriocins are proteinaceous peptides. It also contains lactose as a good carbon and energy source, and vitamins and minerals that occur naturally to help bacterial metabolism and enhance metabolite production. [23] found that dairy-based substrates, particularly milk and cheese whey, offer an inexpensive and highly effective medium for bacteriocin production by *Lactiplantibacillus plantarum*; productivity can be matched with that in commercial MRS medium.

Inability of Nutrient Broth to support Plantaricin production can be related to the

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absence of some special nutritional factors needed for lactic acid bacteria growth and secondary metabolite synthesis. Important components that would stimulate the pathways for biosynthesis of bacteriocins are also not available in this medium—Tween 80, yeast extract, and manganese salts [17] demonstrated that in the absence of factors that would stimulate the pathway, production could be significantly reduced or totally arrested even when the cells continue to grow.

Table 2. Effect of culture medium type on Plantaricin production.

Culture medium	Plantaricin concentration mg/ml
Skim Milk	19.00 ± 0.40 a
MRS Broth	18.00 ± 0.40 b
Nutrient Broth	0.00 ± 0.00 c

Effect of Incubation Period on Plantaricin Production Efficiency

The influence of incubation time on Plantaricin production efficiency was further investigated by studying three incubation periods: 24, 48, and 72 h using MRS broth at 37°C (Table 3). Results indicated a significant increase in Plantaricin concentration with increased incubation time from 24 to 72 h. The highest level of production was recorded after 72 h of incubation; it was 20 mg/mL. Lower concentrations of 10 and 16 mg/mL were observed after 24 and 48 h, respectively.

This also means that increased accumulation of Plantaricin in the cell-free supernatant is closely related to the dynamics of growth of the bacteria and the stage of their development through physiological stages for maximum metabolite production. A 24-h incubation period might be too short to get the cells maximally active for substantial bacteriocin accumulation; 72 h would be more appropriate, as genes associated with Plantaricin would have enough time for expression and the subsequent secretion of the bacteriocin to the extracellular environment [17,23]. share this view and report that after 72 h of incubation, the maximum level for Plantaricin was reached during the stationary phase of bacterial growth. They further note that after the logarithmic growth phase, bacterial cells would direct a good part of their metabolic activity to secondary metabolites, which include antimicrobial compounds. Yang et al. The author stated that incubation beyond the logarithmic growth phase allows the bacterial cells to redirect a significant portion of their metabolic activity toward secondary metabolites, including antimicrobial compounds.

An incubation period of 72 h was taken as the most suitable for the subsequent stages of the study which will follow.

Table 3. Effect of incubation period on Plantaricin production efficiency.

Incubation duration	Plantaricin concentration mg/ml
24 hours	10.00 ± 0.40 c
48 hours	16.00 ± 0.40 b
72 hours	20.00 ± 0.40 a

Effect of Temperature on Plantaricin Production Efficiency

To evaluate the effect of temperature on Plantaricin production by the selected Lactiplantibacillus plantarum isolate, three incubation temperatures (30, 35, and 37°C)

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were investigated using MRS broth for 48 h following inoculum activation according to the selected third activation method, as shown in Table (4). These results indicated 37°C as the most favored temperature for Plantaricin production, where the highest concentration of 20 mg/mL was obtained. Lower concentrations of 16 and 8 mg/mL were obtained at 35 and 30°C, respectively.

The better performance at 37°C can be explained by the fact that this temperature is an optimal growth condition for *Lactiplantibacillus plantarum*. At this temperature, metabolic enzymes undertake bacteriocin biosynthesis at maximum efficiency, and therefore, bacterial cells realize the highest physiological and metabolic activity. In contrast, lower temperatures such as 30 and 35°C reduce enzymatic activity and retarded bacterial growth; hence, biosynthesis and secretion of Plantaricin are limited. Similar results were also reported by [22]. who stated that the optimal temperature range for bacteriocin production by *L. plantarum* strains is between 35 and 37°C [26]. demonstrated also that deviations above or below this optimal thermal range can significantly suppress the expression of bacteriocin-related genes, resulting in reduced production efficiency.

The results showed that 37°C is the best incubation temperature for further assays of Plantaricin production.

The higher plantaricin production at 37°C may result from increased metabolic activity and greater enzyme efficiency, since this is the optimum growth temperature for *L. plantarum*. At that temperature, nutrient use and expression of bacteriocin-related genes are likely at their highest, leading to increased plantaricin synthesis.

The highest plantaricin production at 72 h may coincide with the shift from late exponential growth into early stationary phase, when bacteriocin biosynthesis is often stimulated as part of the bacteria's strategy for competing and surviving.

Table 4. Effect of temperature on the production efficiency of Plantaricin.

temperature	protein concentration mg/ml
30°C	8.00 ± 0.40 c
35°C	16.00 ± 0.40 b
37°C	20.00 ± 0.40 a

Conclusion

Results of the study revealed that the local isolate *Lactiplantibacillus plantarum* strain MKN from corn silage had very good inhibitory activity against both *Staphylococcus aureus* and *Escherichia coli*, thereby indicating high potential for the production of effective antimicrobial compounds. Bacteriocin productivity was significantly influenced by the source of isolation; the silage-derived isolate outperformed isolates from other food sources. The three-stage activation method in MRS broth and filtered skim milk improved bacterial growth and Plantaricin production under the most favorable conditions. Skim milk was economically viable and efficient as a culture medium; it gave higher yields of bacteriocin compared to the other media tested. The period of incubation was also increased up to 72 h to increase Plantaricin production; 37°C was found to be the best temperature for maximum yield. Generally, the results indicate the potential use of the local MKN isolate as a promising source of Plantaricin

for biopreservation and food safety applications, hence reducing the dependence on chemical preservatives to enhance the safety and shelf life of foods.

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