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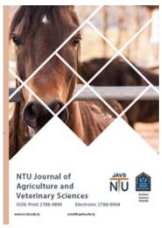

Effect of certain physical and chemical factors on the development of biofilms in *Lactobacillus plantarum*

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A B S T R A C T

Lactobacillus plantarum is known to produce biofilms and planktonic cells. It has the ability to grow in MRS broth on inert surfaces. This study aimed to examine the ability of *L. plantarum* to generate biofilms and planktonic cells under diverse environmental conditions. The study also included the effect of temperature and pH changes on the growth of biofilms. In addition, the study investigated the effect of different sugars added to the MRS medium on biofilm formation. The results confirmed that the optimal temperature for biofilm growth is 37°C, and that the best pH 8. It was noted that the use of MacConkey's medium hinders bacterial growth.. Conversely, the addition of sugars to media environments significantly enhances biofilm formation, with biofilm production increasing alongside concentrations of glucose, sucrose, fructose, maltose and starch. The production of biofilms by *L. plantarum* holds importance for food processing and preservation, and presents diverse potential medical applications.

INTRODUCTION

Lactobacillus plantarum is a Gram-positive bacterium with rounded ends. It occurs either singly, in pairs, or in short chains. It can growth over a wide pH range from 3.4 -8.8 in diverse environmental settings, such as dairy and meat, as well as many plant fermentations. *L. plantarum* contributes to food spoilage, impacting items such as meats and juice. Notably, these bacteria are catalase-negative. It is facultative anaerobes with (5-10%) of CO_2. It can live and resist the acideic conditions (Giraud *et al*., 1991).

L. plantarum is used in the production of fermented foods due to its ability to produce multifaceted enzymes in the industrial sector, such as starch, beverages, food and textiles (Behera *et al*., 2018).

Extensive therapeutic researchers found that *L. plantarum* has an effect on a wide range of disease-causing bacteria (Zakarov and Lovett, 2012). *L. plantarum* is used to treat upper respiratory infections, as they stimulate immunomodulatory and anti-inflammatory effects. After the emergence of the COVID-19 pandemic, *L. plantarum* has shown promising results in its control (Wang *et al*., 2020).

L. plantarum is used in the production of dairy and meat, vegetable fermentations, a variety of fermented foods, pickled and salted vegetables, and sourdough. (Yue *et al*., 2013). This bacterium is characterized by the production of biofilms that limit the colonization of harmful bacteria. Biofilm formation begins by generating a polymeric layer of extracellular sugars on various surfaces. Biofilms can also form on tissues. For example, in the oral cavity specifically on tooth surfaces, which leads to modification of the physicochemical conditions that affects the adhesion of bacteria. (Guzma´n-Soto *et al*., 2021).

Biofilm formation is best on surfaces that are continuously immersed in water, including multiple stages. This process begins with adhesion of bacteria on a suitable surface and begins the formation of small colonies that eventually lead to the development of a three-dimensional structure of cells (Donlan *et al*., 2002). Several studies have revealed the ability of *L. plantarum* to form biofilms, in de Man, Rogosa, and Scharpe (MRS) broth on inert surfaces such as polystyrene and glass. Multiple factors influence biofilm development including temperature, type of nutrient medium, pH, growth stage, and resistance to surface water (Klimko *et al*., 2020).

The current study aimed to evaluate the ability of *L. plantarum* to produce biofilms and planktonic cells under environmental, chemical, and physical conditions such as temperature and pH. On the other hand, the type of medium and the addition of sugars to it may have an effect on the growth of biofilms.

MATERIALS AND METHODS

Bacterial isolation and activation

An isolate of *L. plantarum* was sourced from the Life Sciences Department at the University of Kirkuk, isolated from plant-derived samples using one colony from plate and were diagnosed by gram stain at the Food Sciences Department within the College of Agriculture and Forestry at the University of Mosul.

A single bacterial colony is transfered by needle to a test tube, containing 5 ml of MRS broth (UK) LAB, and incubated at 37ºC for 24 hours. Following incubation, a loopful of well-developed bacteria was taken and streaked onto MRS agar plates using the streak plate technique. The plates were further incubated at 37ºC for 48 hours (Akbas and Kokumer, 2015).

Production of biofilms from *L. plantarum* and staining:

20 µl of liquid bacterial cultures aged for 24 hours of incubation at 37°C were taken to evaluate their ability to produce biofilms.. For each well, 130 µl of MRS medium were added and placed in a well of a 96-well tissue culture plate (Cell Cult, Nunclon, U.S.A). The plates were incubated for 24 hours at 37°C, following the procedure outlined by Coffey and Anderson (2014).

The number of viable cells in each well was quantified using the drop count method by diluting on the agar medium. Subsequently, the biofilms were plated using the drop method as described by Herigstad et al. (2001).

A portion of the upper broth medium was carefully withdrawn from each hole. The biofilms were washed three times with saline to eliminate non-adherent cells. Excess liquid was then rinsed off and biofilms were collected using saline containing 0.2% EDTA, according to Lefebvre et al. (2016). Incubation was performed at 37°C for 48 hours. The number of cells was recorded using the falling counting method. Then, the membrane was prepared and stained by adding crystal violet according to Coffey and Anderson (2014).

Influencing the temperature of incubation on the production of biofilms by *L. plantarum*:

The bacteria were grown in MRS medium at three temperatures: 8, 25, and 37 °C for 24 hours. Six holes with two replicates were used for the isolation to perform the counting, and one hole was used for comparison for the isolation on the plate. The plates were incubated at 37°C.

Effect of pH on biofilm formation:

Isolates were cultured under varying initial pH values: 4, 5, 6, 7, and 8, and were incubated at 37°C for 48 hours. The pH of the MRS broth was adjusted

using (HCl) and (NaOH) solutions at 0.1 normality, measured with a pH meter from Pye Unicam (U.S.A.). The modified media were sterilized using autoclaving and left to cool. The modified media were then inoculated onto the agar plate by Coffey and Anderson (2014).

Effect of medium type on *L. plantarum* growth for biofilm production:
Three chemical media were utilized for bacterial growth: MRS broth, Tryptic Soy Broth (TSB) (Guangdong, China), and MacConkey broth (Guangdong, China). The plates were incubated for 48 hours at 37°C, following the method of Coffey and Anderson (2014).

Effect of sugar type in the medium on the formation of biofilms by *L. plantarum*:
The medium underwent modification to ascertain optimal conditions for bacterial growth by incorporating different percentages (0.5, 1, 1.5, and 2%) of various sugars (glucose, sucrose, galactose, fructose, maltose, arabinose, and starch). Stock solutions of the media were prepared, sterilized, and then inoculated onto pre-determined sites on the plate. The plate was incubated at 37°C for 48 hours Coffey and Anderson (2014).

Results

The findings from Figure 1 illustrate *L. plantarum* bacteria's capacity to generate biofilms following a 48-hour incubation period at 37°C. The logarithmic cell counts for biofilms amounted to 7.740, and the planktonic cells numbered 7 during the 48-hour incubation at 37°C.

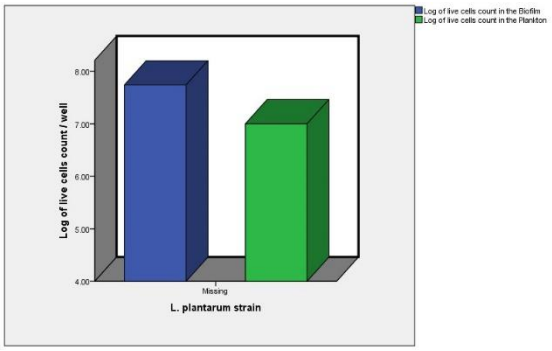

Figure 1: Production of biofilms by *L. plantarum* bacteria at 37°C for 48 hours.

The Figure 2 illustrates the result of *L. plantarum* biofilm formation test, stained with crystal violet, using a light microscope. It is apparent that cells are present in the adhesive substance surrounding the biofilm cells, which remained intact at the well's base despite the washing procedure.

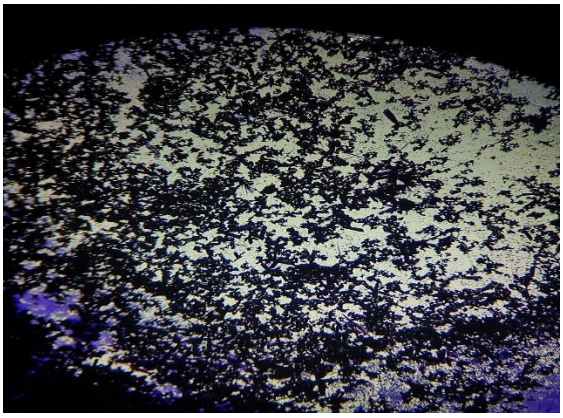

Figure 2: Biofilms of *L. plantarum* isolates at 24 hours old, observed under a light microscope with a magnification of 40X.

Biological membranes are characterized of microorganisms linked to either inert or living surfaces, encapsulated by a self-generated polymer matrix external to the cell. *L. plantarum* form is adopted to promote metabolic collaboration and provide defense against adversarial substances, with biofilm formation being influenced by environmental factors like immune responses and antimicrobial treatments (Corte *et al*., 2019; Song *et al*., 2019).

Effect of temperature on the production of biofilms by *L. plantarum*:

Biofilm formation was assessed at temperatures of 8, 25, and 37°C over a 48-hour period. At 25°C, the logarithm of live cells in biofilms was 8.74, and for attached cells, it was 8.698. Conversely, at 37°C, the logarithm for biofilms was 9.176, while for planktonic cells, it was 8.301. Interestingly, no biofilms or attached cells were observed at 8°C, as indicated in the figure's (3) results.

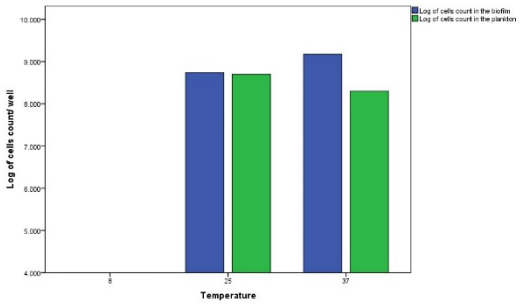

Figure 3: The influence of incubation temperature on the production of biofilms by *L. plantarum*.

Our results revealed that the growth and development of biofilms vary with temperature, and the optimal temperature for biofilm production is identified as 37°C. This underscores the heightened activity of *L. plantarum* in biofilm production at 25°C and 37°C compared to the incubation temperature of 8°C. In summary, an elevated

temperature correlates with an increased formation of biofilms, aligning with the discoveries of Jalilsood *et al*. (2015), who noted that *L. plantarum* biofilm formation occurs at 30°C and is further improved by raising the temperature to 35°C. These outcomes are also consistent with the findings reported by Matejčeková *et al*. (2016).

The impact of initial concentration (pH) on the production biofilms by *L. plantarum*:

Figure 4 illustrates the pH's impact on the logarithmic values of live cells in biofilms and attached cells at a temperature of 37°C over a 48-hour duration.

It was observed that the logarithmic production of biofilms is only minimally affected by pH values at 4, 5, 6, and 7 (7.371, 7.389, 7.243, and 7.380) respectively, with a notable increase noted at pH 8 (8.875). Similarly, the production of planktonic cells exhibited relatively consistent proportions between pH values 4-7 (6.602, 7.176, 7.176, 6.602), respectively. However, a significant increase in planktonic cell formation was observed at pH 8, with a logarithmic value of 8.

The production of biofilms and planktonic cells seems to be relatively unaffected by variations in neutral and acidic pH but increases when the pH becomes more alkaline for the bacteria. This phenomenon may be attributed to enzymatic and metabolic processes occurring within the cell (Pannella *et al*., 2020).

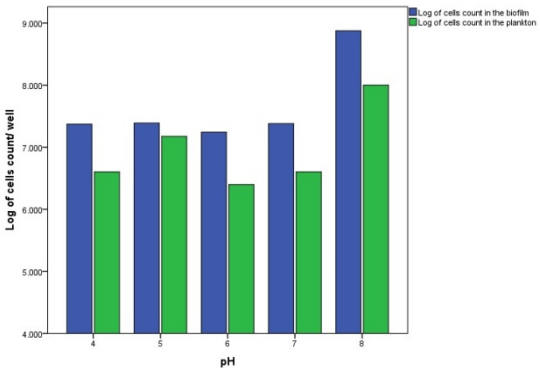

Figure 4: Impact of pH on the biofilm of *L. plantarum* bacteria at 37°C for 48 hours.

Our study findings diverged from those of Bonneville *et al*. (2021), who observed biofilm production by the *L. plantarum* strain on solid carbon sheets at 11°C or 25°C while our study found the best temperature is 37°C. They used a nutrient-rich Tryptic Soy Broth-Yeast Extract (TSB-YE) medium under various nutritional and low-temperature conditions. Our results align with a study that focused on biofilm growth detection at pH 3.2 and 3.5. This study evaluated the biofilm lifestyle patterns of *L. plantarum* strains in the laboratory as an innovative and suitable biotechnological strategy to ensure L-malic acid conversion under stress conditions during incubation times (24 and 72 hours), highlighting that the ability to produce biofilms depended on stress, regardless of the isolation source and standard growth conditions (Pannella *et al*., 2020; Salas-Jara *et al*., 2016).

The effect of the type of media on the production of biofilms by *L. plantarum*:

Illustrated in Figure 5 is the logarithmic count of viable bacterial cells and the production of biofilms and planktonic cells for the three types of media. The most favorable and highest growth occurred in MRS broth, exceeding that of TSB broth. Conversely, MacConkey broth showed no observable growth.

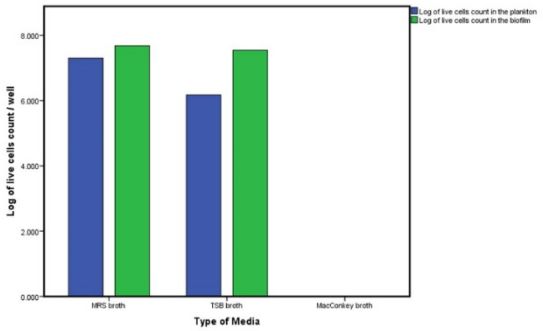

Figure 5: The impact of the type of medium on the production of biofilms by *L. plantarum* at an incubation of 37°C for 48 hours

During the test of the influence of adding MacConkey medium on the biofilms produced from MRS medium, and assessing the effect of this addition on the biofilms, Figure 6 depicts a substantial decrease after 24 hours of incubation. At the initial time point, the logarithmic count of live cells in the biofilms was 8, decreasing to 3.77 over the specified period.

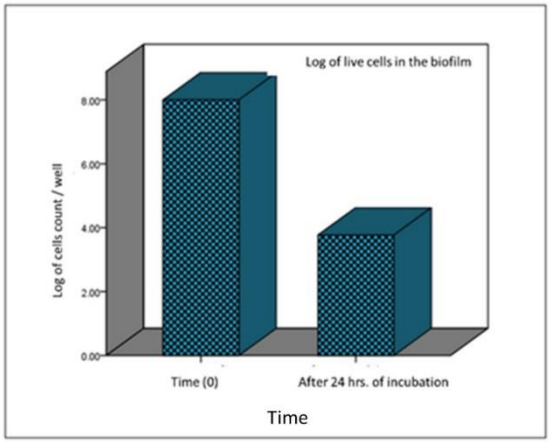

Figure 6: The impact of adding MacConkey on the logarithmic count of live cells in the biofilms produced by *L. plantarum* after incubation of 24 hours

In a previous study conducted to monitor *L. plantarum* growth in MacConkey medium for in vivo tests, 6-week-old mice were used and fed

cheese. The strains Lp790, Lp813, and Lp998, with surface permeability to water. Regarding the translocation assessment, *L. plantarum* couldn't grow on MacConkey agar, and there was no observed microbial translocation to extraintestinal sites such as the liver and spleen through the oral administration of the three strains at selected doses (Zago *et al*., 2011).

The impact of adding sugars on the production of biofilms and planktonic cells:

Figure 7 depicts the influence of adding sucrose to the medium on the logarithmic count of viable cells in both biofilms and planktonic cells. The logarithmic count increased as the concentration of sucrose (0.5%, 1%, 1.5%, and 2%), in comparison to the control sample. The 2% concentration exhibited the least growth among the four sugar concentrations when compared to the control sample. The figure illustrates a rise in the logarithmic count of live cells in biofilms and planktonic cells with an increasing sugar concentration until a certain point, beyond which a decline in growth is observed. This suggests that an excessive concentration adversely affects the metabolic activities of bacteria beyond their tolerance level.

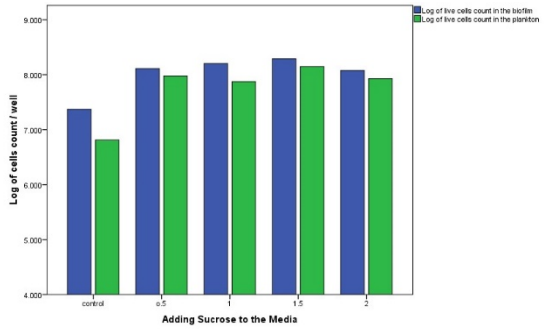

Figure 7: The impact of adding sucrose on the formation of biofilms by *L. plantarum*

Figure 8 demonstrates the influence of adding glucose on the logarithmic count of live cells in both biofilms and planktonic cells when varying concentrations (0.5%, 1%, 1.5%, and 2%) are introduced into the medium, in comparison to the control sample. Minor differences in growth enhancement are observed at different concentrations compared to the control sample.

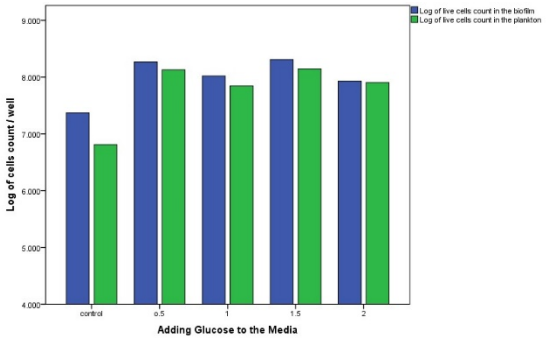

Figure 8: The impact of adding glucose on the production of biofilms by *L. plantarum*

When employing various concentrations of fructose (0.5%, 1%, 1.5%, and 2%) to highlight the impact of fructose addition on the growing biofilms and planktonic cells in bacteria, Figure 9 reveals that there are no discernible differences in the logarithmic count of live cells in both biofilms and planktonic cells at the fructose concentrations added to the medium compared to the control sample.

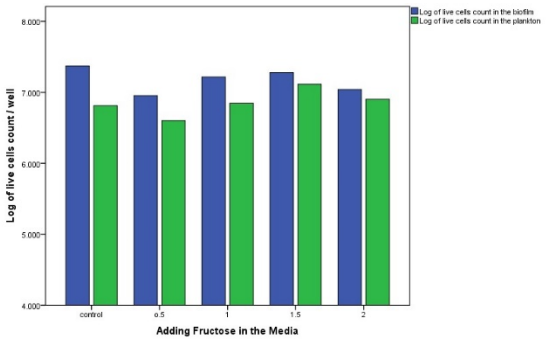

Figure 9: The impact of adding fructose on the formation of biofilms by *L. plantarum*.

Figure 10 indicates a fluctuation in the logarithmic count of live cells for the bacteria compared to the control sample. The growth of biofilms and planktonic cells initiates at a relatively high level with galactose concentrations of 0.5% and 1%, followed by a gradual decline for concentrations of 1.5% and 2%.

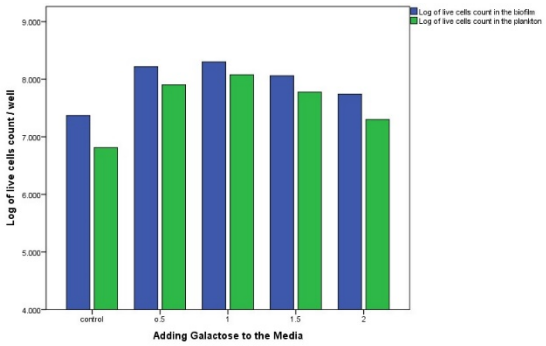

Figure 10: The impact of adding galactose on the production of biofilms by *L. plantarum*.

Figure 11 illustrates the logarithmic count of live cells with the addition of different concentrations of maltose. The results show noticeable variations compared to the control sample. Maltose has an observable effect on both growth and the production of biofilms across various concentrations. However, distinct differences are observed in the concentrations for planktonic cells, particularly at levels of 0.5%, 1.5%, and 2%, when compared to planktonic cells in the control sample. It is notable that there is a clear reduction in logarithmic growth for the 1% concentration.

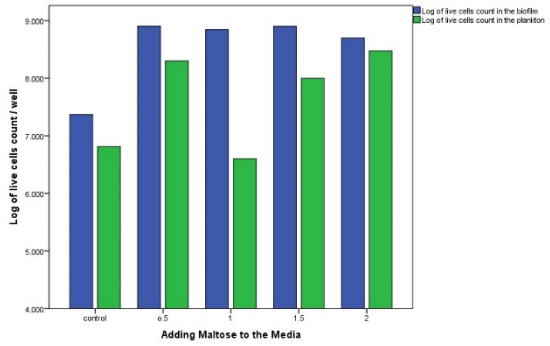

Figure 11: The impact of adding maltose on the production of biofilms by *L. plantarum*.

Figure 12 demonstrates the influence of adding arabinose sugar at equivalent concentrations to other sugars on the production of biofilms and planktonic cells. The obtained results closely resemble those of sucrose and glucose, indicating a notable increase in biofilm growth with an elevated arabinose concentration in the medium compared to the control sample.

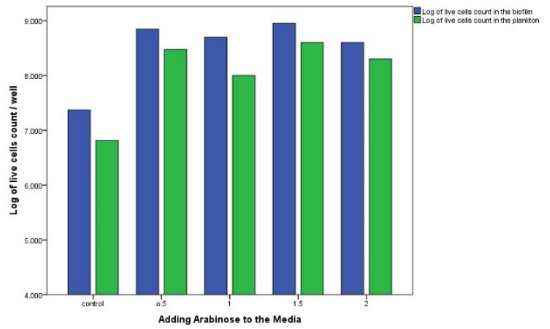

Figure 12: The impact of adding arabinose on the production of biofilms by *L. plantarum.*

In this study, we introduced varying concentrations of starch as a polysaccharide for comparison with other sugars added to the cultivation media, aiming to understand their influence on the development of biofilms and planktonic cells. Figure 13 depicts the composition of biofilms and planktonic cells at different starch concentrations added to the medium. The results reveal a notable increase in the logarithmic count of live cells in *L. plantarum* compared to the control sample as the starch concentration rises. This outcome contrasts with the effects of other sugars, where an increase in biofilm formation was observed at a 2% starch concentration, unlike the decrease seen with other sugars. This distinction may be attributed to starch being a complex sugar that does not exert the same pressure on the cells as observed with other sugars.

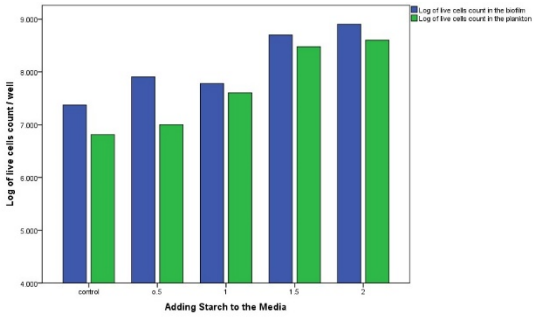

Figure 13: The impact of adding starch on the production of biofilms by *L. plantarum*.

Generally, the addition of sugars at specific concentrations to food environments can significantly support the exponential growth of biofilms and planktonic cells, as well as enhance membrane resistance against pathogenic bacteria. In general, adding sugars at the concentrations used to food media can support production Biomembranes and suspended cells, and these results are consistent with the previous study (Razmjooei *et al*., 2020). Our findings align with the results of Razmjooei et al. (2020). They generated linoleic acid using *L. plantarum* in biofilm reactors by introducing different sugars (glucose, lactose) or a combination of glucose and lactose to the MRS medium. Supplementary analyses revealed that, contingent on the carbon source used for *L. plantarum* growth in biofilm reactors, the biomass quantity fluctuated. The outcomes indicated that utilizing a compound of lactose and glucose yielded the highest biomass (3.66 g/L), and the most significant biofilm accumulation on the metal support was associated with the broth enriched with glucose-lactose. This investigation proposed a high capability of *L. plantarum* immobilized on the metal support to produce a significant amount of CLA isomers using the glucose-lactose blend. The researchers also pointed out that the sole essential sugar for biofilm formation is glucose. Its simulative effect was observed when incorporating glucose into the nutrient medium, leading to an increased cell count compared to the control membrane cultured on a sugar-free medium, in accordance with our study results.

Adding peptide mixtures and sugars can increase cell and cell membrane growth for *L. plantarum* strain Z7, and the controlled secretion of biofilms by the bacterial quorum-sensing system enhanced the

bacteria's ability to resist adverse environments, thereby promoting bacterial growth and proliferation (Ding and Li, 2020). In general, the impact was balanced when using sugar concentrations less than 2% for any of the studied sugars (glucose, sucrose, fructose, lactose, galactose, arabinose, starch).

CONCLUSIONS

The production of biofilms by *L. plantarum* is crucial for food production and preservation. The formation of these biofilms is influenced by important factors, such as the optimal growth temperature of 37°C. The suitable pH for the growth of biofilms is 8. Additionally, the addition of sugars to the nutritional environments is considered important for enhancing the growth of biofilms and suspended cells. The use of MRS medium is observed to impede bacterial growth.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this work.

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