Effect of *Hibiscus Sabdariffa* Extract on Growth and Survival of *Lactobacillus Acidophilus in vitro*

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ABSTRACT

Maintaining the growth and long-term survival of probiotic bacteria is a major challenge in the development of functional and probiotic based food products, thereby highlighting the need for natural growth supporting agents. The present *in vitro* study assessed the effect of *Hibiscus sabdariffa* extract on the growth and survival of the probiotic bacteria *Lactobacillus acidophilus* under controlled laboratory conditions. A 60 day experimental study was conducted using De Man, Rogosa and Sharpe (MRS) broth and agar media supplemented with and without supplementation of hibiscus extract. Bacterial growth and survival were assessed on days 0, 15, 30, 45 and 60 using the serial dilution and spread plate method and the results were expressed as colony forming units per milliliter (CFU/mL). The study design allowed the assessment of both short-term growth patterns and long-term survival trends of the probiotic under supplemented and control conditions, this helped to understand how the plant extract affected the probiotic growth and survival over time. The hibiscus-supplemented samples showed higher CFU counts compared to the control, with maximum bacterial growth seen on day 15. The highest growth was observed in sample B at 10^{-3} dilution medium (150 CFU/mL) followed by sample A at 10^{-3} dilution medium (134 CFU/mL) and control at 10^{-3} dilution medium (120 CFU/mL). After day 15, a gradual decline was observed in CFU values across all samples and better survival of bacteria was seen in hibiscus-supplemented media at day 60. Statistical analysis indicated significant differences between supplemented and control groups. The findings of this study indicated that *Hibiscus sabdariffa extract* enhanced the growth and prolonged the survival of *Lactobacillus acidophilus* highlighting its potential application as a natural functional ingredient for improving probiotic viability in food and nutraceutical formulations.

Keywords: Herbal extract, Probiotic, Functional Food, Bioactive compounds

INTRODUCTION

In recent years, there has been increasing interest in functional foods due to their potential to provide health benefits beyond basic nutrition. Functional foods are designed to support physiological functions and reduce the risk of chronic diseases, while being consumed as part of a regular diet. Consumer acceptance of functional foods has grown steadily, especially for products that promote gut health, immunity, and metabolic regulation[1].

The development of functional foods has shifted toward a holistic approach that considers bioactive components, safety, sustainability, and long-term health benefits in addition to nutrient composition[2]. Functional food ingredients that support gut health have gained attention due to the key role of the gut microbiome in overall health. As a result, probiotics, paraprobiotics, and plant-derived bioactive compounds are widely explored for their potential to improve gastrointestinal and immune function[3].

Probiotics are defined as live microorganisms that offer health benefits when consumed in adequate amounts and play a vital role in maintaining gut microbiome balance, digestion and immune function[4]. A healthy gut microbiome is important for metabolic regulation, immune function and protection against harmful microorganisms[5]. Among various commonly used probiotic strains *Lactobacillus acidophilus* has been well studied due to its safety, its ability to survive harsh conditions of gastrointestinal tract and its positive effect on intestinal health[6],[7].

Lactobacillus acidophilus supports gut health by producing lactic acid, which lowers intestinal pH and inhibits the growth of harmful bacteria[6]. It also enhances gut barrier integrity and supports immune responses[5]. However the effectiveness of probiotic bacteria depends largely on their ability to grow, survive and remain viable during storage and consumption[4]. Environmental stress, oxidative damage, nutrient limitation and varying pH can reduce probiotic viability, thereby decreasing their functional effectiveness[8].

In recent years, natural plant-derived compounds have gained attention for their ability to support probiotic growth and stability. Plant extracts rich in polyphenols, flavonoids and organic acids have been reported to enhance probiotic survival by reducing oxidative stress and supporting bacterial metabolism[9],[10]. These bioactive compounds may act as protective agents by maintaining bacterial membrane integrity and improving stress tolerance[11]. Since they come from natural sources, plant-based components are considered safer than synthetic additives and they are increasingly being used in functional foods[12].

Hibiscus sabdariffa is a medicinal plant commonly consumed as herbal tea and traditional drinks and it is well known for its rich phytochemical composition. It contains phenolic acids, flavonoids, anthocyanins and organic acids which contribute to its antioxidant, antimicrobial and anti-inflammatory properties[13],[14]. The strong antioxidant potential of hibiscus has been associated with its ability to destroy free radicals and reduce oxidative stress[15]. These properties suggest that hibiscus may also provide a favourable environment for beneficial microorganisms.

Several studies have demonstrated that plant polyphenols can positively influence probiotic growth by protecting bacterial cells from oxidative damage and enhancing metabolic activity[10],[16]. Polyphenols have also been shown to interact with bacterial membranes, which help improve cell stability and survival[11]. Organic acids present in plant extracts help maintain a mildly acidic environment that favours the growth of *Lactobacillus spp.* while inhibiting pathogenic bacteria[14].

Recent evidence suggests that *Hibiscus sabdariffa* extract may exhibit prebiotic-like properties by acting as a natural growth supporting substrate for probiotic bacteria[17]. Flavonoids present in hibiscus have also been shown to stimulate carbohydrate utilization pathways in *Lactobacillus* species, leading to improved growth rates[10].

Despite growing evidence on the antimicrobial and antioxidant properties of *Hibiscus sabdariffa*, limited studies have evaluated its direct effect on the long-term growth and survival of probiotic bacteria, particularly *Lactobacillus acidophilus*. Most studies have focused on short-term growth responses or different probiotic strains under different experimental conditions[6],[18]. Long-term survival studies are important, as probiotic viability during storage determines their effectiveness in functional food products[12].

In-vitro studies using controlled laboratory conditions provide a reliable approach to study interactions between plant extracts and probiotic bacteria. De Man, Rogosa and Sharpe (MRS) media is commonly used for culturing *Lactobacillus* species due to its nutrient rich composition[6]. Supplementation of MRS media with plant extracts allows evaluation of growth-promoting effects under standardized conditions[8]. The spread plate method with serial dilution is a widely accepted technique for assessing bacterial viability and growth patterns over time[18].

Considering the bioactive composition of *Hibiscus sabdariffa* and the increasing interest in natural prebiotic sources, it is important to evaluate its effect on probiotic bacteria such as *Lactobacillus acidophilus*. Understanding how hibiscus extracts influences probiotic growth and survival may contribute to the development of novel functional food ingredients and probiotic formulations with improved stability and efficacy[7], [9].

Therefore, the present study was undertaken to assess the effect of *Hibiscus sabdariffa* extract on the growth and survival of *Lactobacillus acidophilus* cultured in De Man, Rogosa and Sharpe (MRS) media under *in-vitro* condition over a period of 60 days.

METHODOLOGY

Study Design

A 60 day *in-vitro* experimental study was conducted to evaluate the effect of *Hibiscus sabdariffa* extract on the growth and survival of *Lactobacillus acidophilus*. The study involved single time supplementation of hibiscus extract into De Man, Rogosa and Sharpe (MRS) broth, with bacterial growth monitored at day 0, 15, 30, 45, and 60 using the spread plate technique.

Variables

Independent variable: *Hibiscus sabdariffa* extract

Dependent variable: Growth and survival of *Lactobacillus acidophilus*

Sample selection and Study Location

Fresh *hibiscus sabdariffa* flowers were obtained from two local vendors to ensure consistency and variability in extract quality. A total of 2 kg of fresh flowers were collected.

All experimental procedures were carried out in the Microbiology Laboratory, Dr. BMN College of Home Science.

Preparation of *Hibiscus sabdariffa* extract

Fresh calyces of *Hibiscus sabdariffa* were washed thoroughly with distilled water, oven dried at 30°C for 4-6 hours, and ground into a fine powder. The powder was sieved to remove lumps and stored in airtight containers. For extract preparation, 10g of hibiscus powder was mixed with 200mL distilled water and gently boiled for 10 min in a water bath. The extract was cooled at room temperature and filtered through Whatman No.1 filter paper. The extract was stored at 4°C until use[17]. Extracts obtained from two different vendors were labelled as Sample A and B.

Selection of MRS Medium

De Man, Rogosa and Sharpe (MRS) medium is widely used for cultivating lactic acid bacteria as it provides essential nutrients and selectively supports *Lactobacillus* growth. Previous studies have shown that MRS medium can be optimized or supplemented with alternative nutrient sources without affecting probiotic viability or function[20-22]. Therefore, standard MRS medium was used as the base medium in the present study, and *Hibiscus sabdariffa* extract was added to evaluate its effect on the growth and survival of *Lactobacillus acidophilus*.

Preparation of culture media

De Man, Rogosa and Sharpe (MRS) broth and agar were prepared according to standard protocols for the cultivation of *Lactobacillus acidophilus*. The media were sterilized by autoclaving at 121°C for 15 minutes. Agar media were poured at 45-50°C into sterile petri plates. Broth media were prepared in 500 mL volumes for each experimental group and stored under refrigerated conditions until use.

Experimental grouping

Three experimental groups were prepared:

(i) Control - MRS media without hibiscus extract

(ii) Sample A - MRS media supplemented with 10% hibiscus extract (Vendor 1)

(iii) Sample B - MRS media supplemented with 10% hibiscus extract (Vendor 2)

Culture revival and inoculation

A commercially available probiotic capsule containing *Lactobacillus acidophilus* was used as the bacterial source. The capsule was aseptically opened, and the contents were suspended in sterile MRS broth and incubated at 37°C for 24 hours to activate the culture. 10 mL of the activated culture were aseptically transferred into 500 mL of sterile MRS broth for each experimental group. Hibiscus extract was added to the respective groups to achieve a final concentration of 10%. All flasks were incubated at 37°C for 24 hours (Day 0) and subsequently stored at 4°C.

Sampling time points and serial dilution

Samples were collected on Days 0,15,30,45 and 60. Serial dilutions were prepared using sterile normal saline up to 10^{-5} dilution following standard microbiological procedures.

Enumeration by spread plate method

Aliquots (0.1 mL) from 10^{-3}, 10^{-4} and 10^{-5} were spread onto MRS agar plates using a sterile L-shaped spreader. Plates were incubated at 37°C for 24-48 hours. Visible colonies were counted and results were expressed as colony forming units per milliliter (CFU/mL).

CFU/mL = (No. of colonies x Dilution factor) / Volume plated (mL)

Assessment of bacterial survival

The survival of *Lactobacillus acidophilus* was evaluated by comparing CFU counts obtained at different time points throughout the 60 day study period between control and hibiscus supplemented groups.

Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation. Statistical comparison between control and experimental groups were carried out using t-test with $p < 0.05$ considered statistically significant.

RESULTS

The present study assessed the effect of *Hibiscus sabdariffa* extract on the growth, viability, and survival of *Lactobacillus acidophilus* cultured in De Man, Rogosa and Sharpe (MRS) media over a period of 60 days. Growth was assessed at five fortnightly intervals (Day 0, 15, 30, 45, and 60) using the spread plate method. The findings showed that hibiscus supplementation had a positive growth-promoting and survival-enhancing effect on *Lactobacillus acidophilus* compared to unsupplemented control media.

Growth pattern at different time intervals

Across all groups, bacterial growth followed a characteristic pattern, with a rapid increase from day 0 to day 15, followed by a gradual decline until day 60. Both hibiscus supplemented samples (sample A and sample B) consistently exhibited higher colony forming units (CFU/mL) values than the control at all time points. The highest bacterial proliferation was observed on day 15.

At the dilution medium of 10^{-3} sample B showed the highest CFU count (150 CFU/mL), followed by sample A (134 CFU/mL) and then control (120 CFU/mL). Similar trends were observed at 10^{-4} and 10^{-5} dilutions, with hibiscus supplemented samples showing higher CFU counts compared to the control. CFU counts decreased with increasing dilution across all groups. The detailed CFU values across different dilution and time points are presented in Table 1.

Table 1: Growth of Lactobacillus acidophilus in Control and Hibiscus-Supplemented Media (CFU/mL)

Days	Control	SAMPLE A			SAMPLE B		
	Control 10^{-3} (CFU/mL)	Sample A 10^{-3} (CFU/mL)	Sample A 10^{-4} (CFU/mL)	Sample A 10^{-5} (CFU/mL)	Sample B 10^{-3} (CFU/mL)	Sample B 10^{-4} (CFU/mL)	Sample B 10^{-5} (CFU/mL)
0	0	0	0	0	0	0	0
15	**120**	**134**	**100**	**89**	**150**	**112**	**73**
30	91	102	80	65	100	99	65
45	80	93	69	50	89	75	58
60	42	69	38	32	70	45	28

Table 1 showed the colony forming unit (cfu/mL) counts of *Lactobacillus acidophilus* which was calculated using formula = Number of colonies x Dilution factor / Volume of sample plated (in mL). The assessment was done at an interval of five fortnightly. Data showed that control groups as well as for hibiscus supplemented groups at three dilution medium i.e. 10^{-3}, 10^{-4} and 10^{-5}. The data also showed the growth patterns and differences in bacterial proliferation under different media composition.

Effect of Hibiscus Extract on Bacterial Survival

The long-term survival of *Lactobacillus acidophilus* was improved in hibiscus-supplemented groups compared to control. At day 60 survival values at the 10^{-3} dilution medium were 70 CFU/mL for sample B, 69 CFU/mL for sample A and only 42 CFU/mL for the control.

Mean CFU analysis further supported these findings. Sample B (10^{-3}) recorded the highest mean CFU value (81.8 ± 54.46), followed by Sample A (79.6 ± 50.22), whereas the control showed a lower mean CFU (66.6 ± 46.54). A summary of mean CFU values, standard deviation and peak growth day is shown in Table 2.

Table 2: Summary of the growth of *Lactobacillus acidophilus* in Control and Hibiscus-Supplemented Media (CFU/mL)

Groups	Mean ± SD	Peak Day	Increase from Day 0
Control 10^{-3} (CFU/mL)	66.6 ±46.54	15	120
Sample A 10^{-3} (CFU/mL)	79.6 ±50.22	15	134
Sample A 10^{-4} (CFU/mL)	57.4 ±39.15	15	100
Sample A 10^{-5} (CFU/mL)	59 ±33.65	15	89
Sample B 10^{-3} (CFU/mL)	81.8 ±54.46	15	150
Sample B 10^{-4} (CFU/mL)	66.2 ±44.95	15	112
Sample B 10^{-5} (CFU/mL)	44.8 ±30.27	15	73

Table 2 summarizes the growth pattern of *Lactobacillus acidophilus* in control and hibiscus-supplemented media at different dilutions. The highest mean CFU values were observed in hibiscus-supplemented samples, particularly sample B at 10^{-3} dilution medium. Overall, hibiscus-supplemented groups showed greater increase in CFU from day 0 compared to the control, indicating improved growth performance.

Survival Trend over 60 Days

All groups peaked at Day 15 followed by a gradual decline in CFU values until day 60. The hibiscus-supplemented samples retained a greater proportion of viable cells throughout the study period. Sample B consistently showed high survival compared to Sample A and control. The survival trend of *Lactobacillus acidophilus* over time is summarized in Table 3.

Table 3 Survival trend of *Lactobacillus acidophilus* over 60 Days

Days	Control 10^{-3} (CFU/mL)	Sample A 10^{-3} (CFU/mL)	Sample B 10^{-3} (CFU/mL)
0	0	0	0
15 (Peak)	120	134	150
30	91	102	100
45	80	93	89
60 (Survival)	42	69	70

Table 3 showed the survival pattern of *Lactobacillus acidophilus* over the 60 day period. The colony forming unit values at each time point was compared among control, sample A and sample B at the 10^{-3} dilution. This suggested the long-term viability and decline trends under different media conditions. Hibiscus supplemented groups A and B showed significantly higher survival at Day 60 compared to control because the extract provided bioactive compounds which helped protect the bacteria.

Statistical analysis

Statistical comparison using t-test showed a significant difference ($p < 0.05$) between the control and Sample A at the 10^{-3} dilution, confirming that hibiscus supplementation significantly enhanced bacterial growth. A significant difference was also observed between Sample A and Sample B at the 10^{-4} dilution ($p = 0.05$), where Sample B showed slightly superior growth.

No statistically significant differences were observed between Sample A and Sample B at the 10^{-3} and 10^{-5} dilutions, suggesting that both hibiscus-supplemented media supported probiotic growth effectively, with Sample B showing marginally better performance. The results of statistical analysis are presented in Table 4.

Table 4 Analysis of colony forming unit values between the Control and Hibiscus supplemented samples using t-tests

Comparison	**t-test value**	**Interpretations**
Control 10^{-3} vs Sample A 10^{-3}	0.03	Statistically significant ($p < 0.05$)
Control 10^{-3} vs Sample B 10^{-3}	0.06	Not significant ($p >0.05$)
Sample A 10^{-3} vs Sample B 10^{-3}	0.56	Not significant ($p >0.05$)
Sample A 10^{-4} vs Sample B 10^{-4}	0.05	Statistically significant ($p = 0.05$)
Sample A 10^{-5} vs Sample B 10^{-5}	0.57	Not significant ($p >0.05$)

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Table 4 showed the statistical comparison of colony forming unit (CFU) values between control and hibiscus-supplemented groups using t-tests to assess the effect of hibiscus extract on probiotic growth. No statistically significant difference ($p<0.05$) was observed between control 10^{-3} and sample B 10^{-3}, between sample A 10^{-3} and sample B 10^{-3}, or between sample A 10^{-5} and sample B 10^{-5}. However, a statistically significant difference ($p<0.05$) was observed between sample A and sample B at dilution medium of 10^{-4} where sample B showed slightly higher growth. Significant difference was observed between control 10^{-3} and sample A 10^{-3}, which indicated improved bacterial growth in the hibiscus-supplemented sample.

Comparative growth analysis

Graphical comparison of growth curves showed that hibiscus-supplemented samples consistently showed higher peak growth and better long-term viability than the control. Sample B showed the strongest growth-promoting effect, achieving the highest peak CFU and maintaining superior survival until Day 60.

The control group displayed a normal growth-decline pattern typical of *L. acidophilus* cultured in MRS media without supplementation, confirming that the enhanced performance of experimental groups was due to hibiscus extract rather than media composition alone. These growth trends are illustrated in Figure 1.

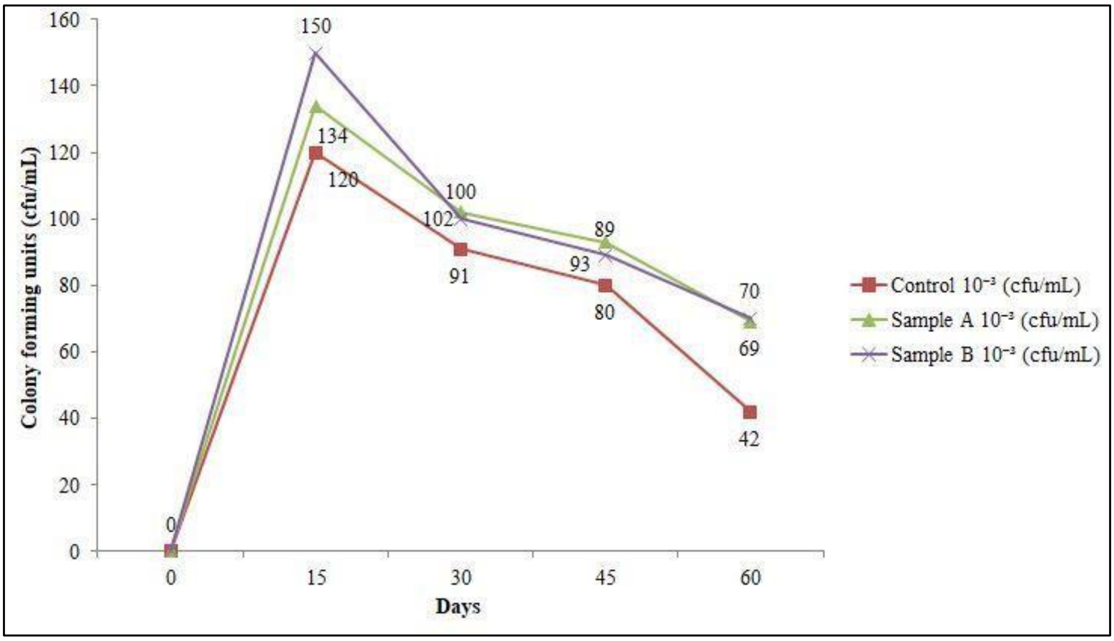

Figure 1 Combined comparison of Control, Sample A, and Sample B (10^{-3}) over 60 days

Figure 1 showed that in the combined comparative study of control, sample A, and sample B at 10^{-3} dilution medium over 60 days suggested that sample B showed the highest growth 150 CFU/mL on day 15 followed by sample A 134 CFU/mL while the control peaked at 120 CFU/mL. At day 60 sample B maintained a survival value of 70 CFU/mL followed by sample A at 69 CFU/mL and then the control declined to 42 CFU/mL. This indicated that Hibiscus supplementation significantly enhanced growth, with sample B showing the greatest effect.

DISCUSSION

The present *in-vitro* study showed that supplementation of *Hibiscus sabdariffa* extract significantly enhanced the growth and survival of *Lactobacillus acidophilus* compared to the control. The higher CFU counts observed in hibiscus-supplemented media indicate that hibiscus extract provided a favourable growth environment for the probiotic culture. Similar growth-promoting effects of hibiscus-based formulations on probiotic bacteria have been reported in recent functional food studies[23,24].

The enhanced growth observed up to day 15 may be due to the presence of bioactive compounds such as polyphenols, flavonoids and anthocyanins in *Hibiscus sabdariffa*. These compounds are known to support microbial metabolism and reduce oxidative stress, thereby improving probiotic growth during the active phase of incubation[25-27]. Anthocyanins and polyphenols have also been reported to exhibit prebiotic-like properties by selectively supporting beneficial microorganisms without exerting inhibitory effects[26,28]. Optimization studies on hibiscus calyces have further confirmed their high antioxidant potential, supporting their role in improving microbial performance[27].

The gradual decline in CFU counts after day 15 across all experimental groups is consistent with nutrient depletion and accumulation of metabolic by-products during prolonged storage, as commonly observed in probiotic survival studies[30-31]. However, the higher CFU values retained in hibiscus supplemented samples up to day 60 suggest a protective effect of hibiscus extract on probiotic cells. Previous studies have shown that hibiscus-derived antioxidants improve bacterial stress tolerance and long-term viability by preserving cell membrane integrity and reducing oxidative damage[27-28].

The enhanced survival of *Lactobacillus acidophilus* in hibiscus-supplemented media may also be associated with the organic acid and antioxidant profile of *Hibiscus sabdariffa*, which creates a mildly acidic environment favorable for lactic acid bacteria. Studies in animal models have demonstrated that hibiscus supplementation improves metabolic function and reduces oxidative stress, further supporting its role as a functional bioactive ingredient[34,35]. Additionally, hibiscus-enriched beverages and herbal tea have been reported to possess strong antioxidant and antidiabetic properties, highlighting their relevance in functional food systems[36].

Variations observed between sample A and sample B may be due to differences in the phytochemical composition of hibiscus calyces obtained from different sources. Several studies have reported that the phenolic content, antioxidant capacity and organic acid profile of *Hibiscus sabdariffa* vary depending on cultivation, geographical origin and extraction condition[27,36]. Such variability can influence probiotic growth-supporting potential and functional outcomes in fermented and nutraceutical products[32,33].

Recent literature highlights the growing interest in integrating probiotics with bioactive plant compounds to develop functional and synbiotic foods. Synbiotic formulations have been shown to enhance probiotic efficacy, particularly in metabolic and inflammatory conditions[37,38]. Probiotic strains exhibiting antioxidant, antibacterial and bacteriocin-producing properties further strengthen the functional value of such formulations. The findings of the present study are consistent with these observations and support the potential application of *Hibiscus sabdariffa* extract as a natural growth-supporting component in probiotic and functional food formulations[39,40].

Overall, the results of this study strengthen existing evidence that hibiscus-derived bioactive compounds can enhance probiotic growth and survival. The use of hibiscus sabdariffa extract may represent a natural and effective strategy to improve probiotic stability, thereby contributing to development of functional foods with enhanced microbial viability and health promoting potential.

CONCLUSION

The present *in-vitro* study clearly demonstrated that supplementation of *Hibiscus sabdariffa* extract enhanced both the growth and long-term survival of *Lactobacillus acidophilus* when compared to unsupplemented control media. Hibiscus-supplemented samples consistently exhibited higher viable counts, with peak bacterial growth observed on day 15, followed by better maintenance of probiotic activity and survival up to day 60. These findings indicate that hibiscus extract provided a favorable environment for probiotic growth and stability during the storage period.

The improved performance of *Lactobacillus acidophilus* in hibiscus-supplemented media may be attributed to the presence of bioactive compounds such as polyphenols, flavonoids and organic acids in *Hibiscus sabdariffa* which are known to support microbial metabolism and stress tolerance. Although a gradual decline in CFU values was observed after day 15 across all groups, the higher survival rates maintained in the supplemented samples highlight the protective role of hibiscus extract against environmental and storage related stress conditions. Variations observed between hibiscus samples obtained from different sources further emphasize the influence of phytochemical composition on probiotic support.

Overall, the findings of this study confirm that the objective of evaluating the effect of *Hibiscus sabdariffa* extract on the growth and survival of *Lactobacillus acidophilus* was successfully achieved. The results suggest that hibiscus extract has potential as a natural growth supporting component for probiotic cultures and may be explored further for incorporation into probiotic and functional food formulations. This study provides experimental evidence supporting the compatibility of hibiscus extract with probiotic bacteria.

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Conflict Of Interest

The authors declare no competing interest

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