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(Original Article)

Impact of Moringa Leaf Extract and Soybean Powder on the Growth rate of *Bifidobacterium animalis subsp. Lactis*

Fatma M. Ibrahim[1]; Asmaa M. Abdel-Rahman[2]; Aly I. Hassan[1] and Dalia G. Kamel[1*]

[1]Dairy Science Department, Faculty of Agriculture, Assiut University, Assiut 71515, Egypt
[2]Food Science and Technology Department, Faculty of Agriculture, Assiut University, Assiut 71515, Egypt

[*]Corresponding author e-mail: dalia.ali1@agr.aun.edu.eg
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Abstract

Natural prebiotic compounds could support the growth of probiotic bacteria and contribute to the balance of the intestinal microbiome effectively. The objectives of this study were to evaluate the moringa leaf extract (MLE) and soybean powder (SP) as natural prebiotic substances, added by different concentrations of each (0.5, 1.0, and 1.5% in MLE; 0.1, 0.3, and 0.5% in SP) on the viability of probiotic bacteria (*Bifidobacterium animalis* subsp. *lactis*) and acid production in sterilized milk. MLE and SP were sterilized and then were added to pasteurized buffalo skim milk (0.1% fat, 4.31% protein, 9.08% milk SNF,4.1% lactose, and 0.68% salt) after cooled to 40–42°C, *Bifidobacterium animalis* subsp. *lactis* (*Bif. lactis*) was inoculated (6.73 Log_{10} CFU/ml). Cell population and lactic acid production were determined during 24h. The results indicated that using 1.5% MLE as a prebiotic increased cell population to its maximum level (10.48 log CFU/ml) after 20 hours, while titratable acidity (TA%) recorded its maximum level (0.44± 0.01) after 24 hours of incubation. On the other hand, using 0.5% of SP after 24 hours of incubation recorded the maximum cell population (10.29 ± 0.02 log CFU/ml), and TA% recorded its highest level (0.44 ± 0.01). The results showed a direct relationship between the growth rate of *Bif. lactis* and rate of increase in the percentage of acidity. Moreover, the viability of *Bif. lactis* during incubation was enhanced significantly ($p < 0.05$) by the inclusion of MLE and SP.

Keywords: *Bifidobacterium animalis subsp. lactis; Growth curve; Moringa leaf extract; Soybean powder.*

Introduction

Bifidobacteria are microorganisms, which include several types such as *Bifidobacterium bifidum, Bifidobacterium longum*, *Bifidobacterium breve, Bifidobacterium adolescentis, Bifidobacterium animalis*, and *Bifidobacterium dentium*. These organisms are frequently present in the human digestive system and are widely known for their probiotic properties. It has multiple health benefits, including improving digestive and immune health, improving mental health via the gut-brain axis, and playing a significant part in preventing some chronic diseases, reducing obesity, and improving nutrient absorption (Sarita *et al*., 2024). Furthermore, *Bifidobacterium*

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animalis subsp. *Lactis* is a probiotic strain that is used in many food products and does not influence flavor, appearance, or mouthfeel, while it has a good ability to survive through the gastrointestinal tract. Thus, this probiotic has excellent benefits to human health (Haschke *et al*., 1998; Prasad *et al*., 1998; Möller and de Verse, 2004; Delgado *et al*., 2020). *Bifidobacteria* need special food sources, like prebiotics, to promote their growth, especially in environments that lack the complex sugars that they prefer. The International Scientific Association for Probiotics and Prebiotics (ISAPP) released a consensus statement in 2017 that described prebiotics as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al*., 2017). According to this definition, prebiotics are any substance that is specifically fermented by the helpful of gut bacteria, improving host health, and are not just dietary fibers. Moreover, recent studies revealed that a number of plant-derived extracts and natural powders that have prebiotic-like activity by boosting microbial diversity or encouraging the establishment of healthy gut microbiota (Kamel *et al*., 2019, 2022, 2023). Moringa leaves contain a variety of bioactive compounds, including phenols and flavonoids, as well as polysaccharides that exhibit prebiotic-like properties, promoting the growth of beneficial bacteria in the gut. These compounds stimulate the production of short-chain fatty acids (SCFAs) such as acetic, propionic, and butyric acid, which help lower the pH of the colon and promote the growth of beneficial bacteria such as *Bifidobacterium spp.* (Elabd *et al*., 2018). Previous studies showed that using moringa seed powder or extract enhanced texture, antioxidant capacity, consumer acceptability and probiotic viability in different dairy products (Zhang *et al*., 2019; El-Gammal *et al*., 2017; Ibrahim *et al*., 2024; Elkazaz *et al*., 2024). However, using concentrations higher than 3% resulted in relative inhibition in bacterial viability due to increased phenolics (Abidin *et al*., 2023), On the other hand, soybean gained increasing attention as a plant-based additive in dairy products due to its content of prebiotic components such as oligosaccharides (raffinose and stachyose) which promotes the growth of *Bifidobacteria,* as well as increase the production of short-chain fatty acids (SCFAs) such as acetic, propionic, and butyric acid, which improve colon health and reduce pH, inhibiting the growth of harmful bacteria. Also, soyabeans contain isoflavones (genistein and daidzein), and *Bifidobacterium* can convert isoflavones from their glycosidic form to their aglycone form, which increases their bioavailability and health benefits (Alegría *et al*., 2014; Gaya *et al*., 2017; Macedo *et al*., 2023). Furthermore, pervious studies used soyabeans and their derivates during making dairy products and illustrated the enhancement effect of those additives on the final product. (Kim *et al*., 2013; Lee *et al*., 2015; Olías *et al.*, 2023).

Indeed, the low viability of *Bifidobacterium* genus during storage, especially in yoghurt and cultured milk (Antunes *et al*., 2007) increased the need to use growth-promoting substances like moringa leaf extract and soybean powder to improve the growth rate of probiotics during processing, which led to positive health impacts. So, this study aimed to investigate different concentrations of moringa leaf extract and soybean powder on the growth rate of *Bifidobacterium animalis* subsp. *lactis*, as well as acid production in sterilized skim milk to choose the optimum concentration of each, which can stimulate the viability of *Bifidobacterium animalis* subsp. *lactis*.

Materials and Methods

Materials

Buffalo's milk used in this study was obtained from the herd of the Faculty of Agriculture, Assiut University. *Bifidobacterium animalis* subsp. *lactis* (*DSM 10140*) was obtained from the Egyptian Microbial Culture Collections (Cairo Microbiological Resources Center, Faculty of Agriculture, Ain Shams University MIRCEN, Cairo Egypt). Fresh Moringa leaves (*Moringa oleifera*) were obtained from the farm of the Faculty of Agriculture, Assiut University, Assiut, Egypt. Soybean was obtained from a local market (Assiut, Egypt). Furthermore, *lactobacillus* MRS broth was purchased from HIMEDIA, India, while L- Cysteine HCL and lithium chloride was obtained from Difco Laboratories, Detroit, MI, USA. Meanwhile, sodium hydroxide and phenolphthalein were obtained from EL-Gomhouria for Trading Chemicals and Drugs Co., Assiut city, Egypt.

Methods

1. Preparation of natural prebiotic

- Moringa leaf aqueous extract preparation.

Moringa leaves were soaked in cold water at 5 °C for three min, to remove impurities. Subsequently, the leaves were dehydrated in an oven at ambient temperature. The dried leaves were crushed into fine powder using a Grinder (Moulinex -LM2070, Egypt), sieved through a 60 mm mesh sieve, and stored at 7 °C until use. The aqueous Moringa extract was prepared by mixing 40 g of dried leaves powder in 100 mL of distilled water at 40°C for 24 h with shaking. Afterward, the extract was filtered twice with Whatman filter paper No1. Then filtrate was dried in an electric oven at 40°C for 24 h and store at 4°C until use (Arif *et al.*, 2022).

- Soybeans powder Preparation.

The seeds were soaked in cold water at 5 °C for three min. to remove impurities. The seeds were then dried in an electric oven at 40°C for four hours. The dried soybeans were then turned into powder using a high-speed grinder and stored at 4°C until use (Olaoye *et al*., 2022).

2. Microbial growth curve preparation

The Alfa-Laval separator, running at 16,000 rpm, was used to skim the milk as soon as it arrived at the lab, Skim milk was distributed into seven different conical flasks (200 ml into each sterilized flask), while moringa leaf extract (MLE) and soybean powder (SP) were each placed in separate flasks (250ml). All flasks were sterilized at 121°C for 10 min. After that the sterilized MLE was added to three flasks by concentrations (0.5, 1.0, and 1.5%), while SP was added to the next three flasks by 0.1, 0.3, and 0.5%. Meanwhile the last flask contained sterilized milk used as acontrol.

- Preparation of inoculum

Twenty-four hours before the start of each experiment, the culture *Bifidobacterium animalis* subsp. *Lactis* was revived in 10 ml of modified MRS broth (containing 0.05% L- Cysteine HCL and 0.3% lithium chloride) and incubated at 40°C for 24 h in an

anaerobic candle jar (Hughes and Hoover, 1995). The procedure of Hassan *et al*. (1989) was used to prepare the inoculum, as 10 ml of revived culture was added to 150 ml of sterilized skim milk, then incubated at 40°C overnight, and the first non-coagulated flask was used as inoculum (which contained about 6.73 Log CFU/ml).

- Bacteriological analysis

For bacteriological analysis, samples were withdrawn from all flasks under aseptic condition. Media used were modified MRS. of *Bifidobacterium animalis* subsp. *lactis* were enumerated in all skim milk flasks under investigation (without and with natural prebiotic addition) at 0, 4, 8, 12, 16, 20, and 24 h. by using modified MRS agar medium (m-MRS), supplemented with 0.3% lithium chloride and 0.05% L-Cysteine HCl, in accordance with Dave and Shah (1996). For 48 hours, the plates were incubated anaerobically at 40˚C. Colony Forming Unit (CFU)/ ml was used to calculate the tiny white colonies.

3. Chemical Analysis

Titratable acidity (TA) was determined according to AOAC (2020), and the results obtained were recorded as a percentage of lactic acid.

4. Statistical Analyses

The collected data were statistically analyzed using the CoStat statistical software (version 6.303). A completely randomized design (CRD) was applied. Differences among treatment means were tested using Duncan's multiple range test (DMRT) at a 5% probability level, as described by Gomez and Gomez (1984).

Results and Discussion

Impact of moringa leaf extract addition

1. Growth curve

Figure 1 presents the growth rate of *Bif. lactis* developed during 24 hours of propagation in sterilized skim milk without and with MLE addition. Data showed that the lag phase was observed between 0 and 4 hours of incubation in the control sample, while supplementation with MLE caused remarkable changes and the lag phase was not observed, which refers to the productivity improvement of cells and reflects the effect of MLE on enhancing and accelerate the viability and rate of growth of *Bif. animalis* subsp. *lactis*. A similar observation was found by Ali *et al*. (2019) when they studied the growth curve of *Bif. animalis* subsp. *lactis* HN019 in MRS broth. Furthermore, the data showed that at zero time,the *Bifidobacterium animalis* subsp. *lactis* trend in control (no MLE added) was noticeable when compared to other treatments. The statistical analysis showed a significant variation in the number of *Bifidobacterium animalis* subsp. *lactis* starter cultures throughout the incubation period. The growth and survival of *Bifidobacterium animalis* subsp. *lactis* during incubation improved by increasing the MLE addition percent in the sterilized milk, which could be attributed to the bioactive components content in MLE, of *Bifidobacterium animalis* subsp. *lactis* growth. Moreover, after 20 hours of incubation, the *Bif. lactis* count increased from 6.84 to 10.17 log CFU/ml with 0.5% MLE supplementation while increasing the addition level of

MLE to 1.5% raised the *Bifidobacterium animalis* subsp. *lactis* count to 10.48 log CFU/ml. On the other hand, of *Bifidobacterium animalis* subsp. *lactis* counts in the control sample recorded their highest value (9.93 log CFU/ml) at 24 h of incubation. The present data slightly differed from the data obtained by Ali *et al*. (2019). They investigated the growth curve developed by *Bif. animalis* subsp. *lactis* HN019 in MRS broth and found that the maximum count was achieved at 18 h (8.313 log CFU/mL), and this difference could be attributed to the variations in culture media, bacterial species, and enumeration techniques. Using *Bif. animalis* subsp. *lactis* in milk products should exceed 10^6 cells of viable probiotics per ml to achieve its improvement on the immune system, especially in elderly propels(Hill *et al.*, 2014). Moringa polysaccharides can serve as fermentation substrates unique to *Bifidobacteria* (Husien *et al*., 2024), which gave a potential explanation to the increase in the *Bifidobacterium* population at 1.5% MLE. Moringa's polysaccharides and phenols abundance improve the medium's reductive state and offer a low-molecular-weight energy source, hence lowering oxidative stress on cells (Elkazaz *et al*., 2024). Whereas Saila *et al*. (2025) showed that moringa addition slowed the growth of spoilage microbes and increased the survival of beneficial *Lactobacillus* during 5 days of refrigeration.

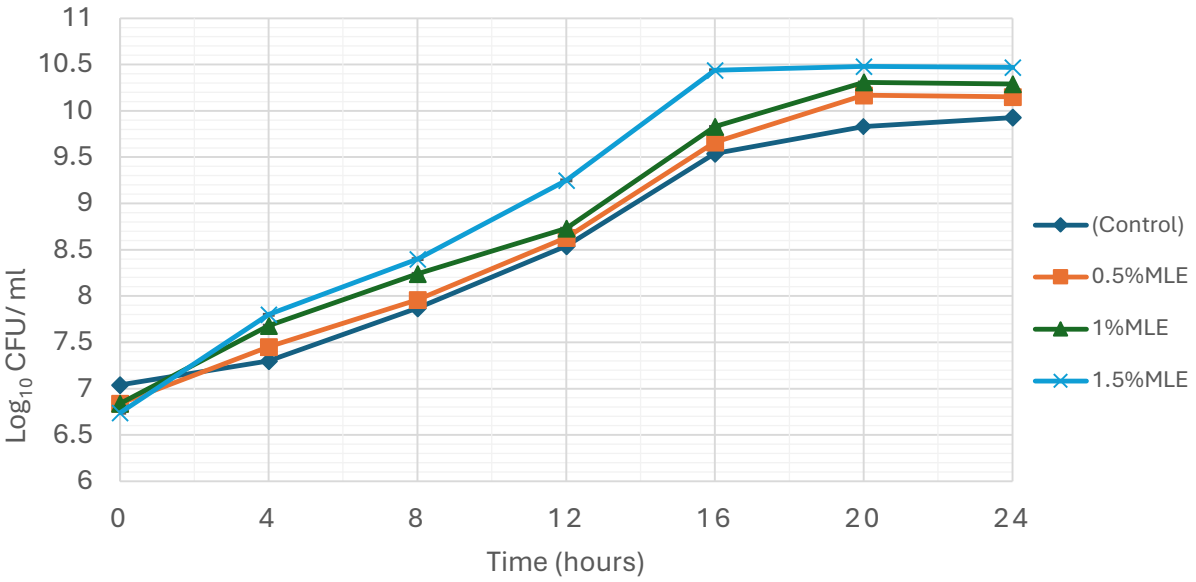

Fig.1. Effect of moringa leaf extract (MLE) supplementation on the growth rate (log_{10} CFU/mL) of *Bifidobacterium animalis subsp. lactis* in sterilized skim milk at 40°C.
Control: Skim milk contained *Bifidobacterium animalis subsp. lactis* (*Bif. lactis*) without any supplementation of moringa leaf extract (MLE); 0.5%MLE: Skim milk contained *Bif. lactis* and 0.5% MLE; 1%MLE: Skim milk contained *Bif. lactis* and 1% MLE; 1.5%MLE: Skim milk contained *Bif. lactis* and 1.5% M

2. Titratable acidity (TA)

Fig. 2 demonstrates that adding MLE affects TA (%) during 24 hours of incubation at 40°C. Data showed that the increase in MLE percentage was directly correlated with the increase in TA rate, and this increase persisted throughout 24 hours of incubation. At zero time, the acidity was 0.16 percent in control, and after 24 hours of incubation, this value increased to 0.36 %. The titratable acidity of the treated samples increased significantly ($p < 0.05$) during the incubation period when the MLE supplementation

increased from 0.5% to 1.5%, while 1.5% addition recorded the highest TA value (0.44%) after 24 hours of incubation. The growth of starter cultures throughout the incubation period caused the acidity to rise. The increase in MLE addition percentage caused metabolism activity improvement in the beginning cultures, which in turn raised the development of acidity (Elkazaz *et al*., 2024). Similar patterns were observed in other studies, which showed the impact of moringa addition on the sample acidity increase (Saeed *et al*., 2021).

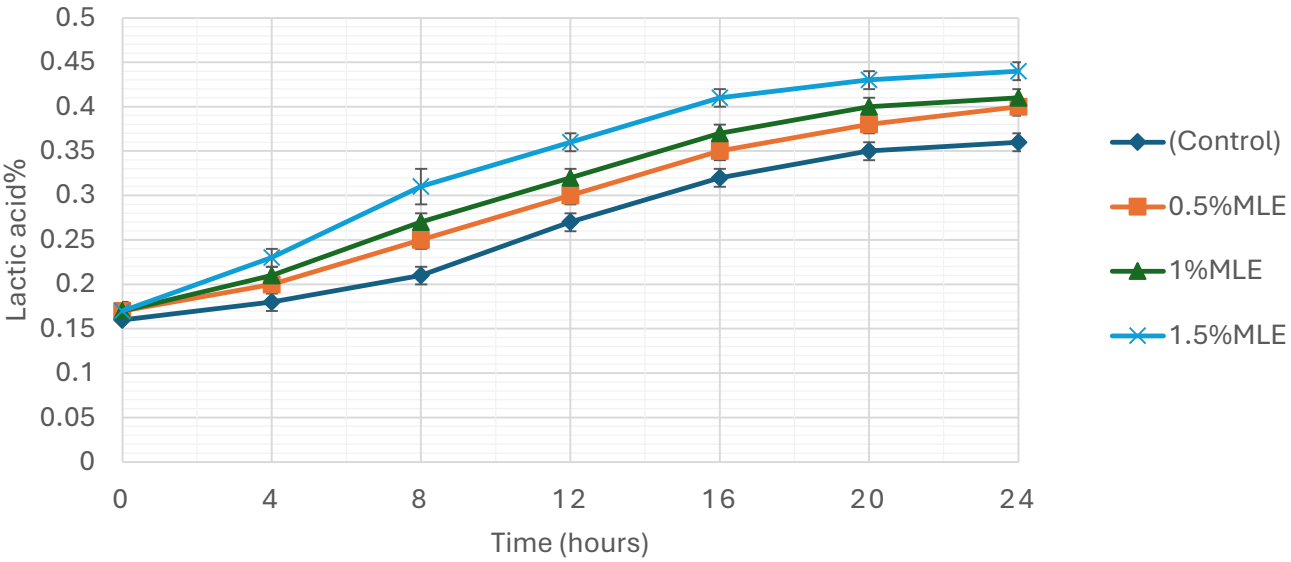

Fig.2. Effect of MLE addition on the rate of TA (%) during 24 h. of the growth of *Bif. lactis* in sterilized skim milk at 40°C. Control: Skim milk contained *Bifidobacterium animalis subsp. lactis* (*Bif. lactis*) without any supplementation of moringa leaf extract (MLE); 0.5%MLE: Skim milk contained *Bif. lactis* and 0.5% MLE; 1%MLE: Skim milk contained *Bif. lactis* and 1% MLE; 1.5%MLE: Skim milk contained *Bif. lactis* and 1.5% MLE

Furthermore, data in Table 1 illustrates mean square and p-values for *Bif. lactis* count and TA%. Table 1 shows that The *Bif. lactis* count is significantly ($p < 0.05$) affected by the addition of MLE, incubation duration, and their combination. Furthermore, the acidity of samples was significantly ($p < 0.05$) impacted by the additional percentage of MLE, incubation time, and their combination.

Table 1. Mean squares and P-values (in parentheses) for *Bif. lactis* count and TA values in sterilized skim milk without and with moringa leaf extract addition.

Factor	df	Mean Square (P-values)	
		***Bif. lactis* count**	**TA**
Treatment[1]	3	0.995 (<0.0001***)	0.0182(0.00***)
Time [2] **(hr.)**	6	21.705 (<0.0001***)	0.1006(0.00***)
Time (hr.) × Treatment	18	0.09 (<0.0001***)	0.000607(0.00***)

Bif. lactis: Bifidobacterium animalis subsp. lactis; TA: Titratable acidity.
[1] Treatments: Control, 0.5%MLE, 1%MLE, and 1.5%MLE; [2] Time: 0,4,8,12,16,20,24 hours; df: degrees of freedom; ***Statistically significant at *P<0.05*.

3. Impact of soybean addition

Growth curve

Figure 3 shows the *Bif. lactis* counts in sterilized skim milk without and with soybean addition (0.1, 0.3, and 0.5%) at 40°C. Data showed that fresh, non-significant differences were found in *Bif. lactis* counts between control and other treatments, whereas the lag phase continued until 4 hours in all samples under investigation. On the other hand, with the progress of incubation time, the *Bif. lactis* counts increased significantly ($p < 0.05$) in all samples, while *Bif. lactis* counts in control recorded the lowest value. Furthermore, after 24 hours of incubation at 40°C, *Bif. lactis* counts in sterilized skim milk containing 0.1% SP recorded 10.09 log CFU/ml, while 0.5% SP recorded the highest count (10.29 log CFU/ml).

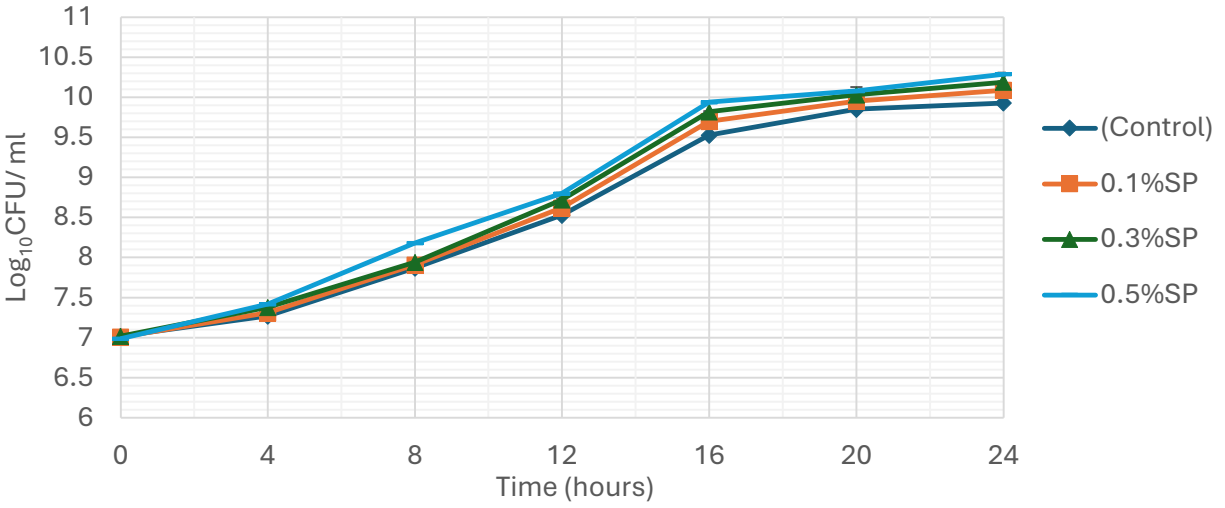

Fig.3. Effect of soybean powder supplementation on the growth rate (log_{10}CFU/mL) of *Bif. lactis* in sterilized skim milk at 40°C. Control: Skim milk contained *Bifidobacterium animalis subsp. lactis* (*Bif. lactis*) without any supplementation of soyabean powder (SP); 0.1%SP: Skim milk contained *Bif. lactis* and 0.1% SP; 0.3%SP: Skim milk contained *Bif. lactis* and 0.3% SP; 0.5%SP: Skim milk contained *Bif. lactis* and 0.5% SP

The oligosaccharides (such as raffinose and stachyose) content in soybeans powder, as well as nitrogen-rich peptides (from protein breakdown), had a role as prebiotic and could be fermented by *Bifidobacteria* (Dong *et al*., 2024). In this concept, s corresponding to the sizes of α<inf>2</inf>-, β-, and casein band. Yogurt-cheese made with added soy milk had similar elasticity (Ruiz de la Bastida *et al*., 2023) showed that the addition of soybean extract to soy drinks could preserve bacterial viability (they used probiotic *Bifidobacterium breve* INIA P734). Furthermore, (Tian *et al*.,2024) illustrated the role of short and low molecular weight peptides (≤ 1 kDa) in providing an osmotic barrier, maintaining the viability of *Bifidobacterium animalis* during drying and freezing processes, which explained the stability of bacterial counts in soybean- containing samples.

Titratable acidity (TA)

Figure 4 shows the influence of soybean powder on the TA (%) values of cultures during 24 hours of incubation at 40°C. Data showed that at zero-time, the control sample recorded the lowest value of TA (0.16%), while the addition of 0.5%SP recorded the highest value (0.19%). Whereas increasing incubation time affected TA significantly ($p < 0.05$) in all samples, and by the end of the incubation period, the sample supplemented with 0.5% SP recorded the highest TA% value (0.44%), while the control sample recorded the lowest value (0.36%). A similar trend was noticed by Park *et al*. (2012). On the other hand, increasing the SP addition percentage from 0.1% to 0.5% led to elevating the titratable acidity of samples during incubation time. Likewise, Baú *et al*. (2014) illustrated that increasing the percentage of soybean in probiotic kefir culture resulted in elevating the titratable acidity .This increase contributed to the high metabolism activity of starter cultures by increasing the soybean, which in turn elevates the acidity development (Lin *et al.*, 2024).

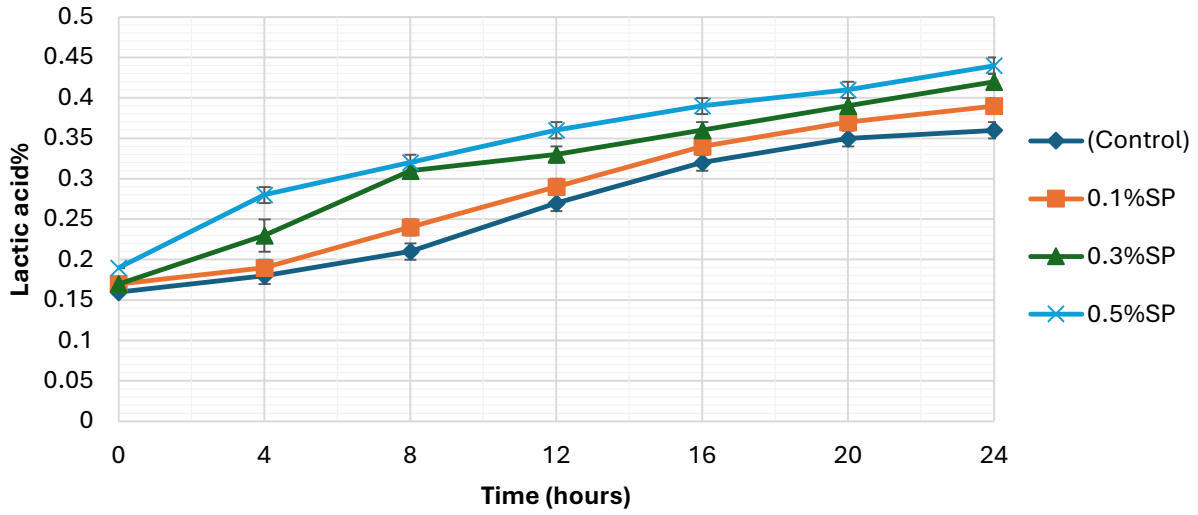

Fig.4: Effect of soybean powder addition on the rate TA (%) during the growth of *Bif. lactis* cultivated in sterilized skim milk at 40°C. control: Skim milk contained *Bifidobacterium animalis* subsp. *lactis* (*Bif. lactis*) without any supplementation of soyabean powder (SP); 0.1%SP: Skim milk contained *Bif. lactis* and 0.1% SP; 0.3%SP: Skim milk contained *Bif. lactis* and 0.3% SP; 0.5%SP: Skim milk contained *Bif. lactis* and 0.5% SP.

Furthermore, Table 2 shows the mean squares and P-values of *Bif. lactis* count and TA% values, which both have been significantly ($p < 0.05$) affected by adding soybean powder, incubation time as well as their interaction.

Table 2. Mean squares and P-values (in parentheses) for *Bif. lactis* count and acidity values in sterilized skim milk without and with soybean powder (SP) addition.

Factor	df	Mean Square (P-values)	
		***Bif. lactis* count**	**TA**
Treatment	3	0.230039 (0.00***)	0.02332 (< 0.0001***)
Time (hr.)	6	19.9627 (0.00***)	0.08516 (< 0.0001***)
Time (hr.) × Treatment	18	0.0135754 (0.00***)	0.000688 (< 0.0001***)

Bif. lactis: Bifidobacterium animalis subsp. *lactis*; DTA: Developed titratable acidity.
[1] Treatments: Control, 0.1% SP, 0.3%SP, and 0.5%SP; [2] Time: 0,4,8,12,16,20,24 hours; df: degrees of freedom; ***Statistically significant at *P<0.05*.

Conclusion

According to the current study's findings, the supplementation of 1.5% moringa leaf extract to skim milk before using inoculation by probiotic causes a significant increase in the growth rate of *Bifidobacterium animalis* subsp. *lactis*. However, this concentration seems to have a negative effect on *Bif. lactis* counts when the incubation time increased to 24 h. Meanwhile, using soybean powder (0.5g/ 100 ml) after sterilization could stimulate the growth rate of probiotic significantly, and higher percentages of soybean powder could be recommended for use in our future work due to the positive effect on growth rate as it caused a higher maximum cell population compared with the control sample. We could conclude that fortification of milk with moringa leaf extract or soybean powder (as prebiotics) during the production of probiotic dairy products would contribute effectively to maintaining human health.

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تأثير مستخلص اوراق المورينجا ومسحوق فول الصويا على معدل نمو *Bifidobacterium animalis* subsp. *Lactis*

فاطمة مصطفى إبراهيم[1]، أسماء محمد عبدالرحمن[2]، علي إسماعيل حسن[1]، داليا جمال كامل[1]

[1]قسم علوم الألبان، كلية الزراعة، جامعة أسيوط، أسيوط 71515، مصر.
[2]قسم علوم وتكنولوجيا الأغذية، كلية الزراعة، جامعة أسيوط، أسيوط 71515، مصر.

الملخص

تهدف هذه الدراسة لتقييم مستخلص أوراق المورينجا (MLE) ومسحوق فول الصويا (SP) كمواد بريبايوتيك طبيعية باستخدام تركيزات مختلفة من كلا منها (0.5، 1، 1.5% في أوراق المورينجا و0.1 و0.3 و0.5 %في مسحوق فول الصويا) على قابلية بكتريا Bifidobacterium *animalis subsp. Lactis (Bif. lactis)* للنمو وإنتاج حامض اللاكتيك في اللبن المعقم الخالي من الدسم. حيث تم تعقيم مستخلص أوراق المورينجا ومسحوق فول الصويا ثم أضيفت إلى اللبن الجاموسي المعقم الخالي من الدسم (0.1 % دهون، و4.31% بروتين، و9.08% جوامد صلبه لا دهنيه و4.1% لاكتوز، و0.68% ملح) بعد تبريده إلى 40-42 درجة مئوي، ثم تم تلقيح *Bifidobacterium animalis subsp. Lactis* (6.73Log_{10}CFU/ ml) فيه. تم تقدير عدد الخلايا وكذلك كمية حامض اللاكتيك الناتجة خلال 24 ساعة. أشارت النتائج إلى أن استخدام 1.5% من مستخلص أوراق المورينجا كمادة محفزة للنمو(بريبايوتيك) تسبب في زيادة اعداد الخلايا الي مستواها الأقصى (10.48Log_{10}CFU/ ml) بعد 20 ساعة، بينما سجلت الحموضة مستواها الأعلى (0.44%) بعد 24 ساعة من التحضين. ومن ناحية أخرى، سجل استخدام 0.5% من مسحوق فول الصويا بعد 24 ساعة من التحضين العدد الأقصى للخلايا البكتيرية محل الدراسة (10.29 Log_{10}CFU/ ml)، بينما سجلت الحموضة اعلي مستوي لها (0.44%). اظهرت النتائج التي تم الحصول عليها وجود علاقة طردية بين معدل نمو *Bif. lactis* وزيادة الحموضة. علاوة على ذلك فانه تم تعزيز قابلية *Bif. lactis* للنمو أثناء فترة التحضين بشكل معنوي ($p < 0.05$) عن طريق إدخال مستخلص أوراق المورينجا ومسحوق فول الصويا.

الكلمات المفتاحية: *مستخلص أوراق المورينجا، مسحوق فول الصويا، منحنى النمو.*