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Development and evaluation of a functional drink (probiotic supplement) fermented from local date juice by selected strains of *Lactobacillus bacteria*

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ABSTRACT

The objective of this research is to produce and test an innovative plant-based fermented functional drink (probiotic) using local date juice as alternative, safe and economical to conventional fermented dairy drinks and to consumers who are lactose intolerant or plant based drinkers. The date juice was then diluted to 18 °Brix soluble solids content without adding any external carbon source after which selected pure strains of lactic acid bacteria (LAB), such as *Lactobacillus acidophilus* and *Lactobacillus plantarum* were added to the medium. Fermentation was performed under constant condition at 37 ± 1 °C for 24 hours and the fermented product was kept on refrigeration at 4 °C for 21 days to observe the biological and physicochemical stability of the product. The data showed that date juice had an excellent biological efficacy in promoting the metabolic activity and fast growth of the two isolates, and the number of live bacteria reached more than the limit (10⁹ CFU/mL) at the end of fermentation. The beverage had a high and stable bacterial load even during cold storage, and was found to be stable above the internationally recommended therapeutic level (10⁷ cfu/mL) on the 21st day. The beverage had a consistent high level of bacteria and as the cold-storage period progressed, it remained consistently above 10⁷ CFU/mL, the internationally recommended therapeutic limit, until day 21. Because of the buildup of organic acids (lactic and acetic) during this microbial activity, there was a significant decrease in pH to 4.05 which resulted in the phenomenon of post-acidification which allowed the product to provide self-stabilization and natural protection against spoilage microbes. At the same time, sensory evaluation showed that there was a high level of consumer acceptance with an average score of 8.06, the natural sweetness of the reduced date sugars was able to compensate for the acidity generated by fermentation. It was found that the added value of local date crops could be maximized and they could be used industrially, producing 'sybiotic' drinks containing a high level of nutritional and medicinal values at low production cost.

Keywords: *Lactobacillus bacteria*, Juice, Functional Foods, Post-Fermentation, Plant-Based Probiotic Drinks

1. Introduction

The date palm (*Phoenix dactylifera* L.) is considered one of the most important strategic crops, it is one of the most substantial pillars of agriculture and the economy of the arid regions, particularly in the Republic of Iraq, where it is considered the homeland of the world's best date palms. To play a role not only as a consumer fruit, but to also serve as a pillar in sustaining GDP and improving global food security, dates are now being produced on a larger scale, with an average of 9 million tons per year (FAOSTAT, 2021) [1]. Due to its high reducing sugar content, essential minerals and phenolic compounds (Julai *et al.*, 2023) [2], this crop is an ideal nutrient medium for biological applications, and Date syrup is the pioneer product in this industry which is natural and healthy alternatives to refine sugar (Madi *et al.*, 2023) [3].

In the last ten years, there has been a dramatic transformation towards "functional foods" due to the increasing awareness of the relationship between food and public health. In this context, probiotics, especially the strains of lactic acid bacteria (LAB), have been identified as a promising field for scientific research because of their potential to enhance the immune system function and strengthen the balance of microorganisms in the gut (FAO *et al.*, 2001) [4]. The use of dairy products as the vector for these bacteria is, however, hindered by several factors, namely the prevalence of lactose intolerance in the world (up to 70% of people, Storhaug *et al.*, 2017) [5], the high cholesterol content of animal fats and the trend towards veganism. It shows the significance of this research in searching for new solutions that can synthesize the synergism between Iraq's agricultural wealth (Date Syrup) and biotechnology to produce a "sybiotic" environment, Dates can be used as a source of carbohydrates and energy (prebiotic), which supports the survival and activity of beneficial bacteria (probiotic) (Maqsood *et al.*, 2020) [6]. The use of Date Syrup as a growth medium for the strains

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of *Lactobacillus acidophilus* and *Lactobacillus plantarum* offers an alternative safe plant-based medium, and can produce products with high levels of potassium, iron, and natural antioxidants which are lacking in traditional dairy products (Karbası *et al.*, 2015; Mostafa *et al.*, 2021) [7, 8].

The study problem is the control of the biochemical parameters, in order to achieve the internationally accepted “therapeutic threshold” (at least 10^7 CFU/mL) and the retention of the sensory parameters attractive to the consumers.

In this regard, the purpose of this study is to create and assess an innovative functional beverage based on a plant-derived fermentation solution in the form of date juice, along with the study of the viability of the strains *L. acidophilus* and *L. plantarum* under refrigerated storage conditions and the modelling of their growth behavior. The overall goal of this research is to systematically develop and investigate a novel symbiotic drink using a local date juice (*Phoenix dactylifera* L.) as a bioactive fermentation medium. In the present study, the kinetic growth pattern and metabolic adaptability of *Lactobacillus acidophilus* and *Lactobacillus plantarum* have been studied in this high-sugar environment. Moreover, it seeks to determine the longevity of these probiotic strains in long-term storage in the refrigerator, which must remain at least 10^7 CFU/mL above the international regulatory limit. This study offers a scientific basis for a sustainable transition from dairy-based to plant-based functional alternatives in the global health-food sector by combining the three aspects physicochemical stability, sensory profiling, and economic feasibility analysis.

2. Methodology

Phoenix dactylifera L. date juice was collected as a main raw material for fermentation from local markets of Diwaniya Governorate in Republic of Iraq. The bacterial starter culture is made from two strain *L. acidophilus* and *L. plantarum*, both of which are pure and freeze dried supplied by Chr. The genetic and physiological stability was ensured using Hansen (Denmark). The culture media and chemicals used were MRS Broth and MRS Agar media supplied by Oxoid (United Kingdom), and 70% ethanol (BDH, United Kingdom), A 0.1 N sodium hydroxide (NaOH) solution, (Merck, Germany).

All the measurements and laboratory work were done with highly accurate analytical instruments and equipment, such as an analytical balance (Sartorius, Germany), an A pH meter (Knick, Germany), a digital refractometer (A, Germany) and a pH/conductivity meter (Instruments, Germany). A water bath (LAUDA, Germany), a laboratory incubator (Mettler, Germany) and a KRESS Optronic, Germany water bath.

2.1 Preparation of the fermentation substrate

The initial dates juice concentration of 70 °Brix was diluted with distilled water to decrease its high viscosity and to prevent the osmotic stress on the bacterial strains. The juice was continuously stirred with distilled water until the concentration of dissolved solids (TSS) was attained, which corresponds to a refractometer reading of (Nancib *et al.*, 2005) [9].

2.2. Heat treatment and cooling

Date juice was heat-pasteurized on water bath for 10 minutes at 85 °C to destroy microorganisms and inactivate spoilage enzymes. The heat treatment was then followed by rapid direct cooling under the temperature range (37 °C–40 °C) to prevent heat induced degradation of phenolic compounds (Abbès *et al.*, 2011) [10] and to condition the medium to the optimum temperature before microbial inoculation (Kchaou *et al.*, 2013) [11].

2.3 Inoculum preparation and activation

Both strains, *Lactobacillus acidophilus* and *Lactobacillus plantarum*, were cultured in MRS broth under anaerobic conditions at 37 °C for 24 hours to ensure optimal physiological activity and to reach the desired cell density before being added to the culture medium (Champagne *et al.*, 2008) [12].

2.4. Fermentation process and monitoring

Juice prepared with modified date (18 °Brix) was seeded with a 5% (v/v) bacterial starter culture. Fermentation was carried out in airtight glass flasks to provide anaerobic conditions at a constant temperature (37 °C) for 24 hours. It was periodically monitored for changes in pH, sugar consumption / total dissolved solids (TSS), accumulation of correctable acidity (TA, expressed as percentage of lactic acid). (Champagne *et al.*, 2018) [13]

2.5. Storage and stability assessment

After completing the fermentation process (24 hours), the fermented functional drink was kept at a refrigerated condition (4 °C) for 21 days. Samples were collected at 0, 7, 14 and 21 days for physicochemical and microbiological analysis, and to evaluate the bacterial stability and the product stability (Pimentel *et al.*, 2015) [14].

2.6. Physicochemical analyses

Immediately after calibration with standard Buffer solutions (pH 4.0 and 7.0), the pH values were measured directly by using a digital pH meter. Total soluble solid concentration was measured in °Brix with digital refractometer. The amount of titratable acidity (TA) was determined by titration with sodium hydroxide solution (0.1 N NaOH) to the equivalence point (pH 8.1), as described in the AOAC Standard Method No. 942.15 and reported as percentage of lactic acid equivalent (% lactic acid equivalent). (AOAC, 2016) [15].

2.7. Microbiological analyses and confirmation

The bacterial counts of viable strains were carried out by pour plate method with 10 fold serial dilutions in MRS agar incubated at 30 °C for 72 hours as per the international standard (ISO 15214:1998) [16]. The data were reported in units (Log CFU/mL). Morphological and enzymatic confirmatory tests were also performed such as Gram staining and catalase test to ensure purity of the growing strains and to determine that they were lactic acid bacteria (Gram positive and catalase negative).

2.8. Sensory evaluation

A preliminary sensory evaluation of the fermented functional beverage was done to find out the acceptability of its sensory attributes (color, aroma, taste, texture and overall product acceptability) from 30 (untrained) consumer participants. Using the 9-point hedonic scale, a score of (1) indicates “I dislike it very much” while a score of (9) indicates “I like it very much” (Meilgaard *et al.*, 2016) [17].

2.9 Cost and economic analysis

The direct cost calculation was used to estimate the cost of the production of the fermented functional drink at laboratory scale. The total direct operating costs have been determined (Cost of Goods Sold - COGS) by totaling the costs of the raw materials (date juice and bacterial starter culture) and the energy used in operation and processing (sterilisation, fermentation and cooling), and packaging materials, based on prevailing local market prices during the study period. The calculation of the production cost per unit (250 ml) was therefore based on the total production revenue per litre.

3 Results and discussion

3.1. Physicochemical changes in date juice during refrigerated storage

According to the findings, the pH gradually decreased from 4.48 ± 0.13 (mean $\pm$ SD, $p < 0.05$) on the first day to 4.05 ± 0.16 (mean $\pm$ SD, $p < 0.05$) at the end of the storage period. This was coupled with a concomitant elevation in total titratable acidity (TA%) from $0.23\% \pm 0.01\%$ to $0.36\% \pm 0.02\%$ (mean $\pm$ SD, $p < 0.05$). This physicochemical phenomenon is attributed to the continuous low-level metabolic activity of *Lactobacillus* bacteria during cooling. This is known as the “post-acidification” phenomenon, whereby bacteria continue to create organic acids, mostly lactic acid, and break down the remaining monosaccharides. These results are consistent with the

results of Nancib *et al.*, (2005) [9]. The high content of reducing sugars in date juice ensures the maintenance of metabolic activity of bacteria even at low temperatures. Date juice maintains metabolic activity of the bacteria even at low temperatures because of its nutrient rich environment, rich in reducing sugars. This will cause the pH to drop and create a self preserving environment to inhibit growth of pathogenic or harmful microorganisms. Concurrently, there is a decline in total soluble solids (°Brix) from 18.00 ± 0.56 to 16.20 ± 0.50 (mean $\pm$ SD, $p < 0.05$). It is evident evidence that some of the sugars in the solution have been consumed by the bacteria and used in the bacteria's fermentation process.

Table 1. The changes in pH, sugar level (Brix) and total acidity (%TA) of date juice during storage.

Storage Time (Days)	pH	Total Soluble Solids (°Brix)	Titrateable Acidity (TA %)
Day 0	4.48 ± 0.13^a	18.00 ± 0.56^a	0.23 ± 0.01^d
Day 7	4.35 ± 0.14^{ab}	17.50 ± 0.46^{ab}	0.27 ± 0.02^c
Day 14	4.20 ± 0.15^{bc}	16.80 ± 0.40^{bc}	0.31 ± 0.01^b
Day 21	4.05 ± 0.16^c	16.20 ± 0.50^c	0.36 ± 0.02^a

Values are Mean $\pm$ SD ($n = 3$). Superscript letters (a-d) in the same column indicate significant differences ($p < 0.05$, Tukey's test)

3.2. Microbial growth efficiency at the end of fermentation

The results of the used starters in terms of microbial growth efficiency are presented in this part at the end of the fermentation process. This indicator is one of the most important ones which shows the activity of lactic acid bacteria and their reproduction in the laboratory medium.

Table 2. The microbial growth efficiencies at the end of fermentation ($T = 0$).

Parameter	Unit	Mean $\pm$ SD ($n = 3$)
Microbial count	$\times 10^9$ CFU/mL	2.80 ± 0.10^a
pH	-	4.48 ± 0.13^a
Total acidity	% TA	0.230 ± 0.010^a

Values are Mean $\pm$ SD ($n = 3$). Superscript letters (a-d) in the same column indicate significant differences ($p < 0.05$, Tukey's test)

The results of microbial growth efficiency of the starters used at the end of the fermentation process showed that the medium (date juice) is a good probiotic carrier as the bacterial count (2.80 ± 0.10) $\times 10^9$ CFU/mL (mean $\pm$ SD, $p < 0.05$) at the end of fermentation, while the pH value of 4.48 ± 0.13 (mean $\pm$ SD, $p < 0.05$) indicates the ecological equilibrium of the culture with the bacterial isolation and nutritional component of the medium. This is in line with the findings of Naghmouchi *et al.*, (2020) [18] who indicated that dates are rich in minerals (e.g., magnesium, potassium) and amino acids, which are cofactors essential for promoting fast cell divisions and accumulation of biomass at a very high rate.

3.3. Stability of microbial counts and pH values during refrigerated storage

The study monitored microbial population and pH changes for 21 days at 4 °C during the estimated shelf life to assess the retention of the biological properties of a fermented beverage (Table 3).

Table 3. The time course of changes in pH values and microbial counts at refrigerated storage conditions.

Days	pH	Microbial count (CFU/mL)
zero	4.48 ± 0.14^a	$(2.80 \pm 0.10) \times 10^9^a$
7	4.35 ± 0.14^{ab}	$(1.50 \pm 0.05) \times 10^9^b$

Days	pH	Microbial count (CFU/mL)
14	4.20 ± 0.14^{ab}	$(6.20 \pm 0.20) \times 10^8^c$
21	4.05 ± 0.14^b	$(1.20 \pm 0.04) \times 10^8^d$

Values are Mean $\pm$ SD ($n = 3$). Superscript letters (a-d) in the same column indicate significant differences ($p < 0.05$, Tukey's test)

The results showed that although there was a natural gradual reduction in the number of live cells due to accumulation of direct acidity and heat stress, the bacterial count still remained above 10^8 CFU/mL till Day 21. The bacterial count on day 21 was $(1.20 \pm 0.10) \times 10^8$ CFU/ml (mean $\pm$ SD, $p < 0.05$).

This result exceeds the minimum acceptable limit which is set internationally and established in the recommendations of the Food and Agriculture Organization and the World Health Organization FAO/WHO, (2006) [19] and the International Society for Bioactive Compounds Hill *et al.*, (2014) [20], which needs to be at least in a range of 10^6 – 10^7 CFU/mL of live bacteria in functional foods and beverages at the time of consumption to ensure that enough live cells reach the intestines to provide the desired health benefits.

But, when the temperature dropped to 4 °C, the effect of it was to slow down the rate of cellular metabolic activity and hence to reduce the degradation rate of the cell, which prevented the degradation rate from becoming fast due to accumulation of low acidity 4.05 ± 0.16 (mean $\pm$ SD, $p < 0.05$). The results are in line with the ones obtained by Mauro *et al.* (2016) [21] in their study on the stability of *Lactobacillus* bacteria in non-dairy plant-based juices, who revealed that the natural compounds and soluble fibres present in the juice matrix have a cryoprotecting effect towards the bacterial cell membrane integrity and its protection from the environmental stress induced by the cooling and subsequent acidification of the juices.

3.4. Organoleptic evaluation and consumer acceptance

The fermented functional drink was evaluated organoleptically in an effort to determine the consumer acceptance of its organoleptic properties. Responses were collected from a laboratory sample of 30 participants (untrained consumers) using a 9-point hedonic scale, according to the methodology adopted by Meilgaard *et al.* (2016) [17]; the results are summarized in Table (4).

Table 4. Organoleptic Evaluation and Consumer Acceptance.

Sensory Attribute	Hedonic Score (Mean $\pm$ SD)	Descriptive Rating
Appearance & Color	$8.20 \pm \text{SD}$	Like Extremely
Aroma	$8.03 \pm \text{SD}$	Like Very Much
Flavor	$8.00 \pm \text{SD}$	Like Very Much
Texture	$8.00 \pm \text{SD}$	Like Very Much
Overall Acceptability	$8.06 \pm \text{SD}$	Like Very Much

Values are expressed as Mean $\pm$ Standard Deviation (SD). On a 9-point hedonic scale (1 = Dislike extremely, 9 = Like extremely) according to Meilgaard *et al.* (2016) [17]

The results presented in Table (4) showed high demand and acceptance of the fermented functional drink by the consumers and commercial feasibility of this novel formulation as a preliminary study; The average score for 'Overall Acceptability' was 8.06, which falls

within the 'Like Very Much' category of the hedonic scale developed by Meilgaard *et al.* (2016) [17]. Appearance/Colour had the highest sensory rating with an average of 8.20 (Like Extremely), this was due to the thermal and chemical stability of the natural pigment compounds in date juice not being affected by the heat treatment and fermentation processes, and not being subject to thermal degradation or drastic changes in colour.

In terms of taste and flavor (8.00) and texture (8.00), this high score is due to the sensory balance created by the sweetness of the date sugars, and the lively acidity that has been developed. This chemical-sensory balance has helped to diminish the strong tartness often found as a shortcomings of high-acid fermented beverages and makes this product a functional plant-based alternative functional superior to the dairy-based drinks in terms of sensory acceptability.

3.5. Confirmatory tests for isolate

The following simple diagnostic tests were performed: (1) to check purity of the bacterial culture used, and to eliminate any microbial contamination of the preparation and fermentation process.

1. Gram stain: On microscopic examination, gram-positive bacterial cells (purple in color) of regular rod-like shape were seen, which is typical of the genus *Lactobacillus*.
2. Catalase Test: Result was completely negative (no oxygen bubbles were produced after hydrogen peroxide was added) therefore this test excluded the presence of catalase positive aerobic bacteria as staphylococcus and Bacillus, and classified the bacteria as facultatively anaerobic lactic acid bacteria.

3.6. Cost and economic analysis

A direct cost analysis was performed to determine the production cost of the fermented functional drink during laboratory trial stage. The preparation of 1000 mL product of 18.0 °Brix is 244 mL of raw date juice plus 756 mL of water.

The price of the raw material was calculated at the local price of date juice (5,000 IQD/L) and the result was IQD (1,220/L). The total cost of producing 1 litre of the microbial starter was 2,500 IQD per litre, taking into consideration the direct operating cost (microbial starter, energy and packaging) (Table 5).

Table 5. Cost Estimation of Direct Costs of manufacturing one Liter of the Functional beverage (bottles).

Cost Item	Value (IQD/L)	Percentage of Total Cost (%)
Raw date date juice (244 mL)	1220	48.8%
Bacterial starter culture and processed water	280	11.2%
Energy and operations (sterilization, fermentation, refrigeration)	200	8.0%
Packaging (4×250 mL bottles with labels)	800	32.0%
Total Cost of Goods Sold (COGS)	2500	100.0%
Unit cost per bottle (250 mL)	625	—

The direct production cost of the product is 625 IQD per bottle of 250 mL (600 IQD per bottle of 200 mL), which highlights the high economic viability and significant competitive advantage of the product, when compared to the prices of functional and probiotic enriched drinks imported to the domestic market (1,250-2,000 IQD per bottle).

These data are an affirmation that local dates are viable for producing useful and value added products at minimal manufacturing cost.

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