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Original Article

Culture of Lactic Acid Bacteria in Natural Environments Based on Dates

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ABSTRACT

Introduction: Lactic acid bacteria are used in the food industry and have restrictive criteria for probiotic potential. The most common growth media used for lactic acid bacteria is de Man, Rogosa, and Sharpe culture medium (MRS). **Methods:** In this study, three culture media were developed (date powder DP, date core DC and date core associated with lentils CL) based on locally available plant materials with a low market value to obtain a less expensive culture medium compared to the reference medium MRS for the growth of lactic acid bacteria. Four lactic strains were used (*Lactobacillus acidophilus* LbA-CECT4529, *Lactobacillus plantarum* LbP-CECT 748, *Bifidobacterium animalis subsp lactis* Bb12 and *Bifidobacterium bifidum* Bb 443). The growth and acidification kinetics of the tested strains were evaluated. The content sugar was determinate with HPLC. **Results:** All lactic bacteria were able to grow on all culture media, but the best results were obtained with MRS and DP media. No significant difference ($p < 0.05$) was observed between DP and MRS medium. The consumption of sugars and proteins in the DP medium was good with all tested bacteria (70.87 to 81.96% and 71.42 to 80.90%, respectively). After the analysis of sugar content of DP medium before and after fermentation with high-performance liquid chromatography (HPLC), fructose was the only sugar detected (45.28µg/ml ± 0.24). After fermentation, 83% of fructose was consumed by Bb12. **Conclusion:** The present data allow us to conclude that date medium promotes the growth of lactic bacteria and can be considered as MRS standard medium substitute.

Key words: Dates, MRS, Lactic acid bacteria, Medium, Fermentation.

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INTRODUCTION

Lactic acid bacteria (LAB) are known as facultatively anaerobic, gram-positive bacteria, which primarily metabolize sugar to lactate. LAB mainly get ATP via the glycolytic pathway. The accumulation of too much lactate results in a large decrease in the pH of the culture media.[1]

LAB constitute a heterogeneous group of bacteria that are naturally found in many types of food, they are also commensals and are present as natural flora of human and animals. These species are predominant in natural microbial vaginal flora and human intestinal microbiota.[2]

LAB are an important part of the diet and are responsible for the fermentation of food products such as dairy, bread, meat, or vegetable origin[3]. The most common growth media used for lactic acid bacteria is de Man, Rogosa, and Sharpe culture medium.[4]

Date palm (*Phoenix dactylifera L.*) is the most important crop in arid and semi-arid areas. It plays an important role in the economic and social life of the people of these regions. Several biotechnological processes allow the production of biomass and various metabolites from date. This biomass production forms the basis of many industrial activities: yeast production, yeast production, etc.

Date palm by-products (leaves, trunk, stones, pedicels, etc.) have various uses in the Saharan regions. Date cores, in particular, are used for feeding livestock when they are not simply thrown away. Extensive research has been conducted on the valorization of date cores into activated carbon[5], feed supplement[6], citric acid and protein production[7], and as a traditional drug for its antimicrobial and antiviral properties.[8]

Dates are known for their nutritional value. Although they are rich in certain mineral salts, vitamins, and organic acids, their sugar content makes them premium food. Due to their high carbohydrate content and relatively long storage, dates and date core offer many technological possibilities depending on the treatment to which they are subjected.[9]

The aim of this study is the biovalorization of a low market value product (Mech Dagla variety dates), as well as a food industry waste (dates nuclei) to develop a new and less expensive culture media replacing the conventional MRS for the growth of lactic bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Four lactic bacteria strains were used in this study: *Lactobacillus acidophilus* LbA-CECT4529, *Lactobacillus plantarum* LbP-CECT 748 (Complutense University, Madrid, Spanish); *Bifidobacterium animalis subsp lactis* Bb12 and *Bifidobacterium bifidum* Bb 443 (Chr-Hansen,

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laboratory, Danmark). Bacteria cultures were stored at -80°C in MRS broth (Difco laboratories sparks, MD, USA) containing 15% of glycerol (w/v). The inoculums were prepared by transferring glycerol stock crop (100 µL) of each strain in 10 mL MRS broth and subsequently incubated under anaerobic conditions at 37 °C for 24 hours.

Preparation of broth culture media

Three natural growing media were developed, (DP) based on date powder, (DC) core of date powder, and (CL) lentils and date core powder. Date pulp is washed and is cut into small pieces and dried at 60°C for 72 H. After that, dates are ground with a crusher, and sieved with a 250 µm diameter sieve. The powder is kept away from moisture. The cores of dates and lentils were dried at 104°C for 5 hours, then cooled, grinded, and sieved. The liquid medium DP and DC are obtained by diluting the powder in distilled water 5% (w/v). The CL medium is an equal mixture of the two powders, dates cores and lentils at 2.5% (w/v) each one. After agitation for 30 min, the solution is centrifuged at 6000 rpm for 10 min, pH is adjusted to 6.5 ± 0.1 and the solution is autoclaved at 120 °C for 20 min.

Fermentation procedure of the different cultivated media

The inoculum was prepared by transferring 10 µL of each bacterium culture to 10 mL of MRS broth and incubated at 37°C for 24 h. The cells were collected in a sterile tube (15 mL) and centrifuged for 10 min at 6000 rpm. The resulting cap was suspended in sterile distilled water to adjust the optical density from 0.08 to 0.10 at A_{625} which corresponds to 10^8cfu/mL.[9] The different culture media (DP, DC, and CL) are inoculated with a 1% (v/v) of preculture of each strain and incubated at 37°C for 72 hours.

Chemical characterization of the culture media before and after fermentation

Determination of dry matter

The dry content is determined on an aliquot portion of 5 g of sample spread in a porcelain capsule and then dried at 103±2°C.[10]

Determination of total sugars

Total sugars were evaluated according to the method of Dubois. The absorbance was measured at 490 nm using Jenway-6715 Spectrophotometer (England).[11]

Determination of protein content

Proteins in the liquid medium were evaluated by the Bradford spectrophotometric method at a *wavelength of* 595 nm.[12]

Determination of growth and acidification kinetics

A sample is collected at the start of the fermentation (0 hours), and after 6h, 12h, 24h, 48h, and 72h to determine the produced biomass and measure the pH.

Bacterial enumeration

The growth kinetics of beneficial strains of the genera *Lactobacilli* sp and *Bifidobacterium* sp were determined by direct enumeration on MRS agar medium. A sample of 50 µL of each culture was diluted with a 450 µL of MRS both, then a volume of 10 µL was taken and cultured In a petri dish containing solid MRS. After incubation at 37°C For 48 hours, colonies numbers are calculated using the following formula: N= Σ n colonies. 1000 µL / 30 µL.[13]

pH measurements

The acidity developed in the culture media from the collected samples was assessed by measuring pH using a potentiometer (Jenway-6715 Spectrophotometer, England).

Sample preparation for high-performance liquid chromatography analysis

Derivatization of sugars with PMP

The phenyl-3-methyl-5-pyrazole (PMP) derivatization of monosaccharides was carried out according to the method described by Goubet *et al.*[14] Briefly, a sample of 20 µL of monosaccharide standards (glucose, fructose, sucrose) or broth samples was mixed with 400 µL of 0.3 M aqueous NaOH and 400 µL of 0.5 M PMP–methanol solution. The mixtures were allowed to react at 70 °C for 30 minutes in a water bath, and then cooled to room temperature and neutralized with 400 µL 0.3 M HCl. The resulting solution was separated by liquid-liquid extraction using a volume of chloroform (three times, 2 mL). After being shaken vigorously and centrifuged, the organic phase was carefully discarded to remove the excess reagents. Then the aqueous layer was analyzed by HPLC analysis.

HPLC analysis

The chromatographic analysis was performed on a Thermo Finnigan Surveyor Plus HPLC apparatus equipped with a thermo stated auto sampler (Auto sampler Surveyor Plus), a quaternary pump (Surveyor LC Pump Plus), and a diode-array detector (Surveyor PDA Plus) with a 5 cm Light Pipe flow cell.

Separations were performed on an Agilent Zorbax carbohydrate column (250 × 4.6 mm I.D., 5 µm). The mobile phase was composed of acetonitrile-water (80:20) and a flow rate of 1 mL/min. The injection volume was 5 µL. The UV spectra were recorded in the range of 200–400 nm and the DAD was set at 245 nm.

Statistical analysis

All measurements were repeated 3 times and expressed as means ± standard deviation. Data were analyzed using statistica® (6.0) software. Differences were considered significant when $p < 0.05$.

RESULTS

Physico-chemical characteristics of liquid culture media before fermentation

The conventional MRS medium was used as a reference medium for the growth of lactic acid bacteria. The results of the physico-chemical characteristics of each culture media are presented in Table 1.

All culture media pH was adjusted to 6.50 before sterilization, but after autoclaving at 120 °C for 20 min, it drops by a few units (1.12, 0.87, and 0.44 for DP, DC, and CL respectively). MRS pH stayed stable as it contains a buffer solution.

The dry matter content of our media ranges from 8% to 12%, which is less important than that found in the MRS (13.09%). For the protein content, DP media has slightly less proteins (1.12 mg⁄mL) than MRS does (1.27mg⁄mL). On the other hand, both CL and DC media contain higher levels in proteins (2.48 and 2.06 respectively).

In which concern total sugar, all developed culture medium have a higher content of sugars than the conventional MRS. The amounts of sugars were more important in DP media followed with DC, LC, and finally MRS with levels of 72.1, 64.5, 40.2, and 20.4 mg⁄mL respectively.

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Bacterial growth

The results for the growth kinetics of the four lactic strains used in this experiment are illustrated in Figure 1. According to the results, all developed culture medium were appropriate for the growth of all tested LAB strains.

Although DC and CL culture media had good amounts of sugars and proteins, lactic acid bacteria did not develop very well in this medium compared with the date powder medium (DP).

Bifidobacterium animalis subsplactis Bb12 had better assimilate the substrates of the four culture media than the rest of lactic bacteria. After 72h of incubation, the most important biomass was obtained with the standard medium (MRS) and date powder medium (DP) recording 17.35 and 14.39 log cfu /mL respectively. *Bifidobacterium bifidum* Bb443 was also able to well developed in the three natural culture media with maximum biomass of 11.89, 11.03, and 9.26 log cfu /mL with DP, CL, and DC. The growth on MRS remains better with 13.03 log cfu /mL after 72 h of incubation.

At the end of fermentation, the growth of both *Lactobacillus plantarum* LbP-CECT 748 and *acidophilus* LbA-CECT4529 was similar on the two culture media MRS and DP(12.58 and 11.85 log cfu /mL respectively, P=0.106).

Determination of acidification kinetics

In general, the synthesis of organic acids by lactic acid bacteria goes hand in hand with their growth kinetics. The most important organic acid production was obtained with the standard medium (MRS) containing glucose as a source of carbohydrates, followed by DP medium (Figure 2). These results are correlated with the biomass production in both media.

The production of organic acids in date core medium (DC) and the one combined with lentils (CL) were very low compared to MRS and DP media. All tested strains had a slow acidification power in both CL and DC media; therefore, pH did not drop more than 1.5 units.

After 72 h, no significant difference in pH was noticed ($p > 0.05$) in DP and MRS media at the end of fermentation.

Estimated consumption of total sugars and proteins after fermentation

The quantities of total sugars and proteins consumed by each strain and in the different culture media at the end of the fermentation are

Table 1: Physico-chemical characteristics of culture media.

Media	pH	Dry matter(%)	Proteins (mg/mL)	Total sugars(mg/mL)
MRS	6.50 ± 0.02	13.09	1.27 ± 0.06	20.4 ± 0.04
DP	5.38 ± 0.1	9.40	1.12 ± 0.04	72.1 ± 0.06
DC	5.63 ± 0.05	8	2.06 ± 0.03	64.5 ± 0.06
CL	6.06 ± 0.03	12	2.48 ± 0.02	40.2 ± 0.08

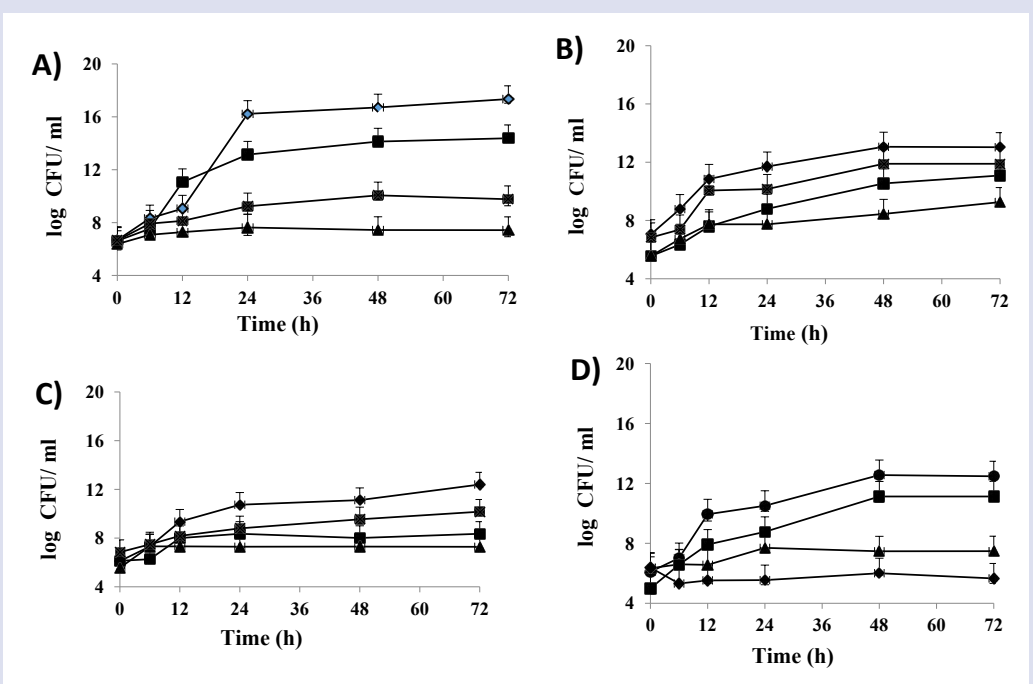

Figure 1: Growth kinetics of LAB strains **(A)** *Bifidobacterium animalis* subsp *lactis* Bb12**, (B)** *Bifidobacterium bifidum* Bb443**, (C)** *Lactobacillus acidophilus* LbA-CECT4529**, (D)** *Lactobacillus plantarum* LbP-CECT 748 monocultivated on complex media based on different substrates (date powder ■ **DP**, date core ♦**DC** with or without lentils ▲**CL**) and in standard medium●**MRS**.

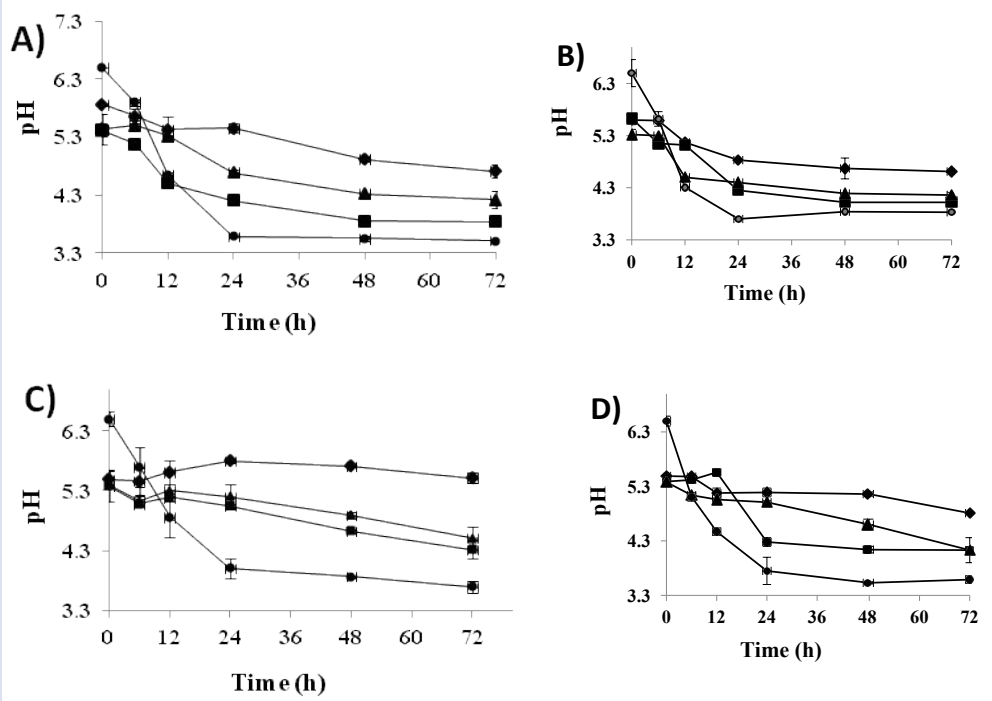

Figure 2: Acidification kinetics of LAB strains **(A)** *Bifidobacterium animalis* subsp *lactis*Bb12**, (B)** *Bifidobacterium bifidum* Bb443**, (C)** *Lactobacillus acidophilus* LbA-CECT4529**, (D)** *Lactobacillus plantarum* LbP-CECT 748 monocultivated on complex media based on different substrates (date powder ■ **DP**, date core ♦**DC** with or without lentils ▲**CL**) and in standard medium●**MRS**.

Table 2: Total sugars and proteins consumption in fermented culture media.

Medium		Quantity in Total sugar Consumed mg/mL	% of total sugar consumption	Quantity in total protein consumed mg/mL	%of total protein consumption
LbP-CECT 748	MRS	18.5 ± 0.1	91	1.01 ± 0.01	79.52
	DP	51.1 ± 0.06^{NS}	70.87	0.80 ± 0.02 ^{NS}	71.42
	CL	20.2 ± 0.02*	50.24	1.22 ± 0.01*	49.53
	DC	16.5 ± 0.08**	25.58	0.16 ± 0.01**	07.76
LbA-CECT4529	MRS	18 ± 0.05	90	0.98 ± 0.02	77.95
	DP	56.1 ± 0.09^{NS}	77.80	0.82 ± 0.005 ^{NS}	73.79
	CL	18.2 ± 0.01*	45.27	1.36 ± 0.1*	55.12
	DC	8.5 ± 0.08**	13.17	0.19 ± 0.02**	9.22
Bb12	MRS	19.00 ± 0.05	95	1.09 ± 0.05	85.80
	DP	59.1 ± 0.1^{NS}	81.96	0.90 ± 0.01 ^{NS}	80.90
	CL	20.20 ± 0.09*	50.24	1.46 ± 0.07*	58.8
	DC	13.50 ± 0.02**	20.9	0.18 ± 0.04**	8.54
Bb443	MRS	19.00 ± 0.01	95	1.06 ± 0.10	83.48
	DP	58.10 ± 0.04^{NS}	80.58	0.89 ± 0.06 ^{NS}	79.46
	CL	22.20 ± 0.02*	55.22	1.42 ± 0.09*	57.25
	DC	12.50 ± 0.03**	19.47	0.19 ± 0.03**	9.22

reported in Table 2. In general, Bb12 remains the strain that consumed the most important quantities of sugars and proteins in all media at the end of fermentation, followed by Bb 443.

At the end of fermentation, both *Bifidobacterium* strains consumed almost all the sugars (95%) and more than 83% of proteins in the MRS medium. In which concerns *Lactobacillus*, (91 and 90%) of sugars, and (79 and 78%) of proteins were consumed by LbP-CECT 748 and LbA-CECT4529.

Among all developed media, all lactic bacteria had a better consumption of sugars and proteins in DP medium. Values of 81.96 and 80.58 % of sugars were consumed by Bb12 and Bb443, respectively. Good protein intake was also noticed with all lactic bacteria in DP medium (71.42 to 80.90%). DP is a suitable culture media for the growth of LAB. The weakest consumption of both sugars and proteins were observed in the date core medium for all tested strains.

HPLC analysis

As the date powder culture media was the most interesting among all developed culture media, its monosaccharides profile before and

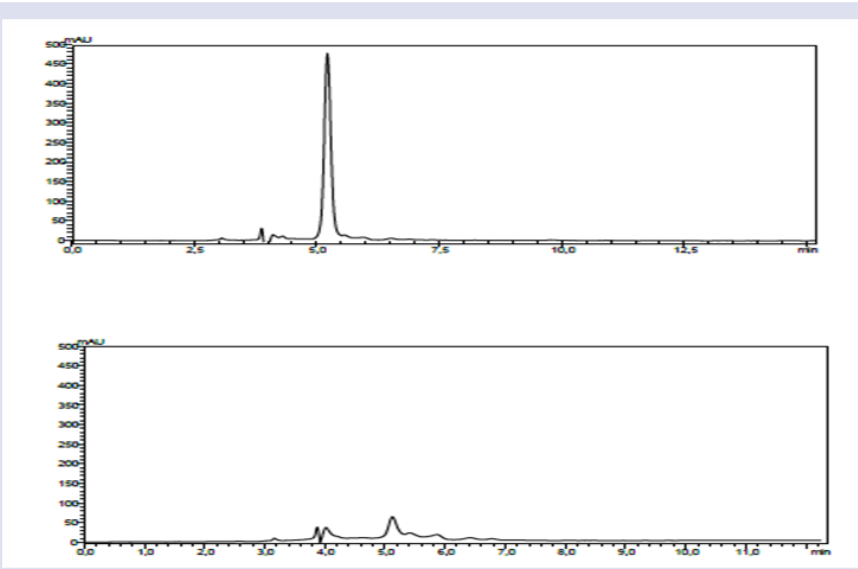

Figure 3: Chromatograms of sugar content in date powder culture medium (DP) broth before **(A)** and after **(B)** fermentation (72h) by *Bifidobacterium animalis subsp lactis* Bb12.

after fermentation was analyzed b HPLC (Figure 3). According to the results only one compound was detected, and by comparison to the retention time of different standard (glucose, fructose, sucrose), this compound corresponds to fructose. After quantification, the amount of fructose was 45.28µg/ml ± 0.24, but after 72 hours of fermentation, this quantity dropped to 7.68 µg /ml± 0.13. This means 83% of fructose was consumed by Bb-12 in date powder medium DP.

DISCUSSION

The changes in medium pH during a sterilization process can be explained by changes in medium components.[16-17] The results of this study are in agreement with the work of Schenk *et al.* 16 and Vuksanović *et al.*[18], the temperature of sterilization can affect media pH through protein denaturation, carbohydrate hydrolysis, and salt dissolution. This phenomenon was already reported by several studies.[19-20-21] Natural developed culture media pH is slightly acidic compared to the MRS medium. As the literature indicates, the optimal pH for lactic acid bacteria growth is ranged from 4.5 to 6.4.[22] It is important to highlight that all culture developed media pH are suitable for the growth of the four tested strains.

According to the literature, dates cores are richer in proteins than the fruit flesh, but despite this, Dates contain essential amino acids that can not be produced by the body and must be supplied in the diet.[23] Lentils are a rich source of high-quality proteins; it contains approximately 26% of crude protein.[24] This can explain how the addition of lentils to date cores has improved the protein content of the newly developed culture media (17%).

Carbohydrates are predominant components in dates, mainly reducing sugars (glucose and fructose), as well as non-reducing sugars such as sucrose, and small levels of polysaccharides (cellulose and starch).[25] According to literature, date flesh has a higher content of sugars.[26-27]

Herchi *et al.*[28], reported that the flesh of dates contains 83.46% of total sugars whereas the seeds have 78.69%. This can explain the difference in the total sugar obtained in DP and DC culture medium in present study.

Low growth of LAB in media based in cores (DC and CL) in this study is probably related to antimicrobial activity of cores and not the poverty of the media.[28] Lactobacilli are able to efficiently use both of the glycolytic pathways facilitates the degradation of a wider range of carbohydrates.[29-30] According to Huebner *et al.*[31], the ability of LAB to metabolize several carbohydrates is related to the intrinsic characteristics of strains and specific substrates, whose degree of polymerization determines the extent of their degradation. Cereals (wheat and oat bran), and their constituents have been reported to stimulate the growth of probiotic lactic strains.[32]

The change of culture media pH is considered as an indicator of carbohydrates fermentation. This is also the result of the production of organic acids by microorganisms.

These results are consistent with those obtained by several studies. In the presence of complex prebiotic substrates, the pH of the fermented medium generally decreases by only one pH unit.[33-34] This demonstrates the effectiveness of the culture media DP for the growth of lactic bacteria and could be a good replacement for much expensive MRS. The results of the consumption of sugars and proteins reflect the growth of the tested strains in the developed media, where there is a proportional relationship between the assimilation of the substrate and the kinetics of generated growth. Our results are in agreement with those obtained by Thorpe *et al.* [19], who found that at kimri and khalal stages the only sugar was fructose. At the rutab stage, the amount of fructose increased sharply; and at the tamer stage fructose was the only sugar detected.

CONCLUSION

Based on this study, the culture media prepared from date powder (DP) can be considered as an appropriate medium for the growth of lactic acid bacteria and especially *Bifidobacterium* species. All the tested bacteria demonstrated significantly high growth rate in DP and a good consumption of sugars and proteins during fermentation.

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DECLARATION OF COMPETING INTERESTS

The authors declare that there are no known conflicts of interest associated with this publication.

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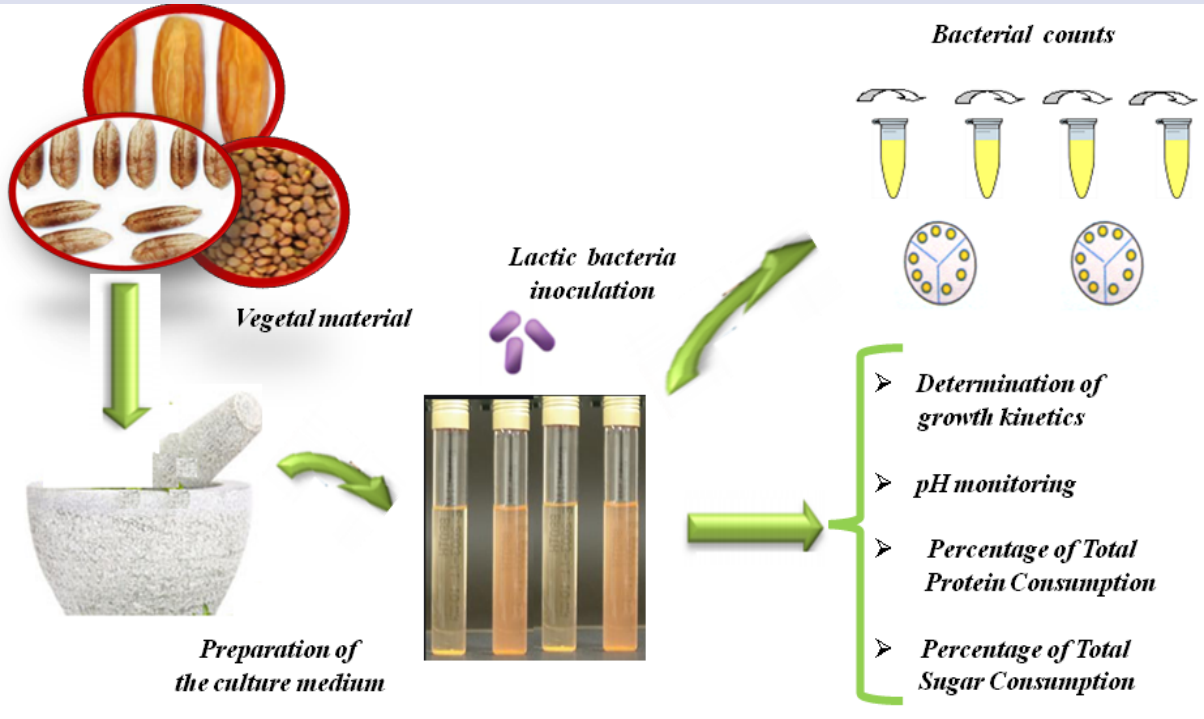
GRAPHICAL ABSTRACT



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