RESEARCH ARTICLE

Probiotication of *Bifidobacterium Spp.* in dairy and non-dairy mango juice medium – A Green approach

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Abstract: Mango (*Mangifera indica L.*) is a climacteric, tropical drupe of the *Anacardiaceae* family. This study aims at determining the ideal synbiotic combination using mango as a natural prebiotic source in two different mediums, dairy, and non-dairy, for two different probiotics *Bifidobacterium infantis* and *Bifidobacterium lactis*. Skimmed milk and sugarcane were observed on analysis to promote the growth of these probiotics and were the chosen dairy and non-dairy substrates used in combination with mango. The ideal synbiotic combination of Mango with dairy /non-dairy medium was optimized on the basis of sensory evaluation supported by total viable counts. Fermentation for 4 hours of the ideal synbiotic combinations with 0.1% inoculum of probiotic cultures was done. The 03:07 ratio of mango: skimmed milk and mango: sugarcane with 4 hours of incubation gave desirable results with increasing microbial counts, drop in pH and nominal increase in acidity as fermentation time progressed. This ideal combination of Probiotics with Mango in both dairy and non-dairy medium could be a promising blend in the food and beverage industry for formulating naturally functional products.

Keywords: Beverage, Green approach, Mango, Prebiotics, Probiotics, Synbiotics, Sugarcane juice, Skim milk

Introduction

Consumers show interest in ingesting diets to maintain the health and prevent the degenerative or chronic diseases such as diabetes, cancer, hypertension (Granato et al. 2010). The growing awareness of the relationship between diet and health has led to an increasing demand for food products that support health above and beyond providing basic nutrition i.e., functional foods. Functional foods comprise of conventional foods containing naturally occurring bioactive substances, foods enriched with bioactive substances and synthesized food ingredients introduced to traditional foods (Grajek et al. 2005). Prebiotics and probiotics are becoming leaders in the functional foods category as it is considered to exert positive effects on our body (Prado et al. 2008).

Probiotics have been a functional food of interest since the earliest of times. Probiotics are nonpathogenic microorganism which improves the intestinal microbial balance, provides health benefits and prevents some diseases (FAO, 2002). Prebiotics are non-digestible dietary components that pass through the digestive tract to the colon and are a potential substrate for fermentation by the microbiota. High potential is attributed to the simultaneous use of probiotics and prebiotics. The term “synbiotic” was first used in 1995, to describe a combination of synergistically acting probiotics and prebiotics (Bengmark, S., 2005).

Fruits & vegetables such as banana, asparagus, beans, cereals, onion, garlic, Jerusalem artichoke, chicory, are natural prebiotic sources. Apart from being a prebiotic source, fruits and vegetables also have other functional properties which is an added benefit. Moreover, the fruit juices also have a good refreshing taste and are a consumption choice for people of all age groups since they are perceived as healthy foods. (Jankovic, Sybesma et al., 2010).

With the idea of utilizing locally available indigenous fruit as a natural prebiotic source, this study explores the use of mango in

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dairy and non-dairy medium as a substrate for growth of *Bifidobacterium* species. Mango could be a better, greener and safer alternative as a prebiotic for use in the ideal synbiotic combination.

This study aims at determining the ideal synbiotic combination using mango as a natural prebiotic source in two different mediums, dairy, and non-dairy, for the probiotics *Bifidobacterium infantis* and *Bifidobacterium lactis*

Materials and Methods

Materials

Fresh mangoes were procured from the local market (Banganapalli variant). Fresh sugarcane juice was purchased from the local vendor and stored under refrigeration condition prior to use. Branded skim milk in tetra pack was procured from the supermarket and used for the study.

Probiotic strain

Proven probiotic strains of *Bifidobacterium infantis* (UBBI-01) & *Bifidobacterium lactis* (UBBLa-70) was obtained in the lyophilized forms from Unique biotech, Hyderabad for the study.

Probiotic Culture

The cultures were prepared by incubating the probiotic strains in *Bifidobacterium* broth (Himedia No. M1395) overnight at 37°C under anaerobic conditions to obtain the working culture. This was maintained at 5°C and sub cultured in *Bifidobacterium* broth.

Total Plate Count-TPC

Dilutions were placed on selective *Bifidobacterium* agar (Himedia No. M1396) and grown under anaerobic conditions in Anaerobic jars (GasPak System) for 48 hours at 37°C (Vinderola & Reinheimer, 1999).

Turbidimetric method

Turbidimetric method is an indirect, rapid method used for following growth of bacterial cultures. A spectrophotometer can be used for measurement of cell mass and in this indirect determination method the absorbances were estimated before

and after incubation (Pelczar M. J. et al., 2003). The optical density of the samples was checked at the wavelength of 600nm

Gram staining

Representative colonies were taken and subjected to Grams staining techniques (Rajan S. & Christy R., 2010) and observations were recorded.

Assessing the chosen dairy and non-dairy substrates for promoting Probiotic growth

Skimmed milk and sugarcane juice were checked for their suitability as a potential dairy and non-dairy substrate for *B. lactis* and *B. infantis*, by inoculating the probiotic cultures (100 µl in 10 ml of substrate), incubating for 24 hours and determining the bacterial viable counts and change in absorbance.

Assessing prebiotic efficacy of mango

The prebiotic potential of mango was observed by inoculating probiotic cultures (100 µl in 10 ml of substrate), incubating for 24 hours and determining the bacterial viable count and change in absorbance.

Sample Preparation

The selected mango fruits (Banganapalli variant) were washed and cleaned with potable water. The inedible parts like seed, rind and peel were removed. The fruit pieces were ground in a blender and the pulp of the fruits were used in the study. The pulp was stored frozen for further use. Mango pulp, skimmed milk, sugarcane juice were individually heated at 80 °C for 20 minutes and were used to prepare different combination.

Optimization of the ideal blend ratio

The dairy blend was prepared with skimmed milk and mango pulp, while the non-dairy blend was prepared with sugarcane juice and mango pulp in different ratios as given in Table 1

Sensory evaluation

The different ratios of fruit and the dairy medium/fruit and non-dairy medium were subjected to sensory evaluation using a five points hedonic scale. The sensory attributes were evaluated by

Table 1: Blend ratios

Blend ratio	Blend ratio of mango based dairy medium			
	D1	D2	D3	D4
Mango: Skimmed milk	01:09	02:08	03:07	04:06
Blend ratio	Blend ratio of mango based non-dairy medium			
	ND1	ND2	ND3	ND4
Mango: Sugarcane juice	01:09	02:08	03:07	04:06

30 panelists. The best combination acceptable to the consumers was determined and the ideal ratio of mango: dairy, mango: non-dairy was chosen for further study.

Assessing the chosen substrates for promoting probiotic growth

The viable count was determined by plate count technique. 100 µl of the *B. infantis* was inoculated into all the combinations. The blends were enumerated on selective Bifidobacterium agar & grown under anaerobic conditions in for 48 hours at 37°C (Vinderola & Reinheimer, 1999). Turbidimetric technique was also used as the indirect method of measuring the growth (Pelczar et al. 2003). The viable count of *B. lactis* was also similarly determined by total plate count and turbidimetric technique.

Analysis of the ideal synbiotic combination

Assessing the Growth pattern

The ideal combination was freshly prepared and 0.1 % of each probiotic culture was inoculated for the study. Viable counts and turbidity were determined at 0 hours and 4 hours.

Measurement of specific chemical changes produced

pH and acidity were determined by pH meter, titration (AOAC, 2016) respectively. Both pH and TA values of the formulated beverages was measured at 0, 2 and 4 hours during the incubation period.

PCR

PCR is used a novel approach to confirm the probiotic growth in mango-based combinations.

Genomic DNA from the culture & probiotic product was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA prepared was used as a template for PCR amplification. The amplification was done as mentioned by Yeon S, 2007 & the amplified products were analyzed by electrophoresis on a 2% agarose gel containing 1 µl of ethidium bromide, and the DNA bands were visualized under UV illumination and photographed.

Results and Discussion

Turbidimetric method

The optical density of *B. infantis* and *B. lactis* before incubation was found to be 0.377 ± 0.039 , 0.53 ± 0.022 and after incubation was found to be 0.721 ± 0.108 , 0.928 ± 0.017 respectively (Figures 1 & 2).

TPC

Using the plate count method, *B. infantis* and *B. lactis* was found to have 9.9242 ± 0.029 , 9.0492 ± 0.062 log cfu/ml in the working culture used in the study.

Gram staining

Representative colonies were taken and subjected to Grams staining techniques and it was observed that both *B. infantis* and *B. lactis* were stained purple, indicating that both are gram positive and rod shaped.

Assessing the chosen substrates for promoting probiotic growth

The ability of the selected dairy and non-dairy substrates (milk and sugarcane juice) to promote the growth of probiotics *B. infantis* and *B. lactis* individually was evaluated. It was observed that the viable count of *B. infantis* and *B. lactis* in the substrate milk, after 24 hours, was found to have increased from initial values of 9.9242 ± 0.029 , 9.0492 ± 0.062 log cfu/ml to 11.1289 ± 0.019 and 10.3388 ± 0.019 log cfu/ml respectively. This was also supported by the increase in OD values determined by turbidimetric determination. The optical density of *B. infantis* and *B. lactis* in the substrate milk, before incubation was found to be 0.514 ± 0.016 , 0.512 ± 0.04 and after incubation was 1.212 ± 0.012 , 1.123 ± 0.01 respectively. Milk, the dairy medium used in Mango-dairy combination is a proven substrate which promotes probiotic growth. Dairy-based fermented products and yogurts have been utilized as potential probiotic products since the ancient times (Bansal S et al. 2016).

The viable count of *B. infantis* and *B. lactis* in the substrate sugarcane was found to increase to 11.5021 ± 0.080 log cfu/ml and 10.7695 ± 0.008 log cfu/ml respectively. Similarly there was also an optical density of *B. infantis* and *B. lactis* in the substrate sugarcane, from 0.521 ± 0.005 to 1.618 ± 0.006 and 0.502 ± 0.008 to 1.311 ± 0.005 respectively.

An increase in the viable count of Bifidobacterium strains in native soymilk medium was observed by Havas et al. (2015). The fermentation processes cultured with initial cell concentrations in 10^5 - 10^7 cfu/ml resulted in 10^8 cfu/ml after 8–12 h of incubation in soymilk, and were kept viable up to the end of fermentation (48 h). Volumetric productivities of *B. bifidum* (B-3.2), *B. bifidum* (B-7.1) & *B. breve* (B-9.14) were 1.6×10^{10} cfu/L.h, 4.5×10^{10} cfu/L.h and 7.6×10^9 cfu/L.h, respectively. The values of *B. lactis* (Bb-12) & *B. longum* (Bb-46) probiotic strains were 2.7×10^9 cfu/L.h and 1.0×10^{10} cfu/L.h.

Assessing prebiotic efficacy of mango

The exact component responsible for mango's prebiotic capacity is unknown. Its prebiotic potential could due to the presence gallic acid, dietary fiber, resistant starch & also pectin content (Yadav et al. 2010; Ramulu et al. 2003; Chatterjee et al. 2016; Holscher, 2017; Slavin, 2013). The Prebiotic potential of mango

Fig. 1 Sensory evaluation of Dairy Blend

was observed by bacterial viable count and turbidimetric method. The viable count of *B. infantis* and *B. lactis* was found have increased to 11.2508 ± 0.024 and 10.3660 ± 0.012 log cfu/ml on incubation. This was also supported by the increase in OD values which was found to increase from 0.934 ± 0.002 to 1.458 ± 0.006 for *B. infantis* and from 1.222 ± 0.01 to 1.423 ± 0.01 for *B. lactis*.

Optimization of the ideal blend ratio used in the study

Sensory evaluation

Fruit juices have been suggested as an ideal medium for the functional health ingredients because they inherently contain beneficial nutrients, they have taste profiles that are pleasing to all the age groups (Vasudha and Mishra, 2013). It could be clearly observed that the panelists showed more preference as the proportion of mango juice increased (figure 1).

Statistical analysis of the results revealed that significant difference existed between the overall acceptability score ($p < 0.05$) of samples between the different blend ratios in both dairy and non-dairy mediums.

Assessing the ability of the probiotic culture to utilize the blends as a prebiotic substrate

Turbidimetric method

The change in optical density before and after incubation for 24 hours, in the different mango-skim milk and mango-sugarcane juice blends inoculated with *B. infantis* was observed to increase and the highest of variations was found in D3I, ND3I.

Similarly the change in optical density in different mango-skim milk and mango-sugarcane juice blends inoculated with *B. lactis*

was observed and the highest variations was seen in ND3I, ND3L (Table 2).

TPC method

After 24 hours increase in total viable count of *B. infantis* inoculated in the different mango-skim milk and mango-sugarcane juice blends was observed. It can be observed that D3I and ND3I show higher log cfu values (11.9173 ± 0.003), (12.0816 ± 0.004). Statistical analysis showed that the viable count of dairy and non-dairy combination inoculated with *B. infantis* was found to have no significant difference, hence comparable.

Similarly, after 24 hours an increase in total viable count of *B. lactis* inoculated in the different mango-skim milk and mango sugarcane juice blends was observed. D3L and ND3L showed higher log cfu values (10.6117 ± 0.003), (11.0792 ± 0.045) when compared to other samples (see Table 2). Statistical analysis showed that the viable count of dairy and non-dairy combination inoculated with *B. lactis* is found to have no significant difference, hence comparable.

The results of this study were similar with the results of study by Bujna et al. (2018) which showed that all investigated *Bifidobacterium* and *Lactobacillus* strains were able to grow well in the apricot juice without supplement of any nutrients meaning this matrix in itself was suitable medium for propagation of probiotic bacteria. In all cases the cell numbers at 24 h of fermentation were higher than 10^8 cfu/mL, and cell yields varied from 1.15×10^{10} cfu/L h to 1.78×10^{10} cfu/L h.

Analysis of the ideal synbiotic combination

The ideal synbiotic combination of 3:7 mango with dairy /non-dairy medium was optimized on the basis of the sensory score, supported by highest total viable counts in this ratio.

Fig. 2 pH & TA of D3I, ND3I, D3L and ND3L

D3I- 03:07 mango skim milk blend inoculated with *B.infantis*; ND3I-03:07 mango sugarcane blend inoculated with *B.infantis*; D3L- 03:07 mango skim milk blend inoculated with *B.lactis*; ND3L-03:07 mango sugarcane blend inoculated with *B.lactis*

Table 2 Total plate count and variation in OD values of the dairy and non-dairy blends

	TPC	OD
Dairy and non-dairy mango blends inoculated with <i>B. infantis</i>		
D1I	10.6018±0.072	0.167±0.002
D2I	10.6086±0.030	0.234±0.003
D3I	11.9173±0.003	0.808±0.007
D4I	11.3848±0.018	0.661±0.004
ND1I	10.5433±0.032	0.287±0.002
ND2I	10.5289±0.057	0.577±0.001
ND3I	12.0816±0.004	0.978±0.005
ND4I	10.5595±0.035	0.554±0.021
Dairy and non-dairy mango blends inoculated with <i>B. lactis</i>		
D1L	9.2709±0.014	0.316±0.013
D2L	9.3603±0.031	0.451±0.006
D3L	10.6117±0.003	1.136±0.004
D4L	10.4493±0.033	0.516±0.02
ND1L	9.4771±0.002	0.42±0.011
ND2L	9.4722±0.008	0.21±0.003
ND3L	11.0792±0.0457	0.489±0.005
ND4L	9.4771±0.001	0.374±0.007

The values are the means of triplicate determination. ± indicates the standard deviation of the mean

Table 3 OD values at 0th hour and after 4 hours

	0 Hour	4 Hour
D3I	1.486±0.002	1.726±0.004
ND3I	1.444±0.003	1.716±0.002
D3L	1.536±0.002	1.8775±0.001
ND3L	1.417±0.001	1.785±0.005

The values are the means of triplicate determination. ± indicates the standard deviation of the mean

Assessing the growth pattern

The growth pattern of the culture in the combinations during 4 hours of incubation was assessed by determining colony forming unit and optical density at various time intervals. The colony count of D3I increased from 10.1785±0.024 to 10.4413±0.010 & for ND3I the colony count increased from 10.150±0.007 to

11.4771±0.001. Similarly, the colony count of D3L increased from 9.7898±0.017 to 10.2936±0.013 & for ND3L the colony count increased from 9.7505±0.019 to 10.3658±0.020. The change in optical density of D3I, ND3I, D3L, ND3L from 0 hour to after 4 hours is shown in table 3.

In the study by Nguyen BT et al. (2019) pineapple juices were inoculated with different probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Bifidobacterium lactis*) at an initial cell density of about 10⁶-10⁷ cfu/ml. All investigated *Lactobacillus* and *Bifidobacterium* strains were able to grow well in the pineapple juice. In the case of *bifidobacterium* it reached a level of 10⁹ cfu/ml after 24 of fermentation.

Physiochemical analysis: pH & titrable acidity

Fig. 3 PCR

Both pH and titrable acidity values of the formulated combinations were measured at 0, 2 and 4 hours during the incubation period. The pH of all the four combinations was found to decrease gradually over the hours (shown in figure 2). The percentage TA of mango in dairy and non-dairy medium (*B. infantis*) observed at 0, 2, 4 hours recorded an increase and for mango in dairy and non-dairy medium (*B. lactis*) the percentage TA was found to increase as incubation time increases (figure 2). Andrade et al (2020) has also reported a similar decrease in pH from the initial 5.28 & 6.47 of jerivá and macaúba palm fruit pulps to 4.76 and 5.20 respectively during the fermentation with *B. lactis*

The results obtained of increased log cfu/ml and acidity combined with drop in pH are indicative that 3:7 ratio of mango:skimmed milk and mango: sugarcane were utilized as a prebiotic source for growth by *B. lactis* and *B. infantis*.

PCR

The presence of probiotic culture *B. lactis* was confirmed using specific primers, by conventional PCR. The sequence of the *B. lactis* primer used is forward primer: CCCTTTCCACGGGTC-CC and reverse primer: AAGGGAAA-CCGTGTCTCCAC The PCR product was represented by a single band in the corresponding region of the DNA marker ladder (figure 3).

Freeze dried powder of the ideal symbiotic combinations

Freeze-drying is a technique adopted for successful long-term preservation of material. The 3:7 blend of the ideal synbiotic combinations were freeze dried and preserved for further use.

Conclusion

Mango as a prebiotic source in dairy and nondairy medium showed promising and comparable results for both the bifidobacterium species. The blend ratio of 3:7 for the both the mediums was observed to be the ideal synbiotic combination which was optimized on the basis of the sensory score, supported by highest total viable counts in this ratio. It was observed that the ideal synbiotic combination of mango: dairy and mango: non-dairy medium containing 0.1% of *B. infantis* or *B. lactis* with a fermentation time of 4 hours provided desirable results for viable growth of probiotic culture and activity in terms of pH and acidity and hence can be used in probiotic product formulations using a green approach.

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