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Original Research

Efficacy and Stability studies of microbial folate fortified fruit juices prepared using probiotic microorganism

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Abstract: Folate, natural form of water soluble vitamin folic acid, is significant for humans as involved in most important metabolic reactions i.e. nucleotide synthesis and amino acid inter conversions. Thus its deficiency causes neural tube defects in newborns and cardiovascular diseases, and cancers. Humans cannot synthesize folate *de novo* so consumption through diet is essential. Natural food sources, supplements and fortified food products are the choices available to complete the Daily recommended intake. However microbial fortification using probiotics recently gained wide attention due to dual advantage of natural food matrix with enhanced folate content along with the probiotics benefits. Current study was focused on the microbial fortification of fruit juices and their efficacy and stability studies. Freshly filtered orange and tomato juice was prepared and inoculated with *Streptococcus thermophilus* NCIM 2904. Incubation was done at 40°C and samples were collected at different time interval. Folate extraction was done using human plasma and content was measured by microbiological assay using *Lactobacillus casei* NCIM No. 2364. Efficacy and stability studies were carried out to ensure the quality of juices to be consumed in terms of folate content, viable cell count and pH after 4 weeks of storage at low temperature. Positive results were observed as folate content was quite stable whereas viable cell count was also found to be significant till some time without adding any preservatives. The results indicated that fortified fruit juices could be used as probiotic beverages with enhanced folate content.

Key words: Folate; Microbial fortification; Probiotics; Viable cell count; Efficacy.

Introduction

Folate is an essential B vitamin, also known as B9, pteroyl-L-glutamic acid, folacid, which has significant role in the growth, metabolism and reproduction of human beings. Recent findings on the preventive role of folate in the reduction of neural tube defects in newborns (2, 24), cardiovascular diseases (4, 25) and certain forms of cancer such as breast cancer, cervical cancer (6, 11, 14, 18) have grab the attention of researchers now a days. Thus renewal of research on folate supplementation by any means to combat its deficiency came into the existence. Folate has various significant roles in our body such as methyl group biogenesis, nucleotide and amino acids synthesis, and proliferation of rapidly dividing cells like leukocytes, erythrocytes and enterocytes (16). Daily recommended intake of dietary folate varies from 200-400 µg for an adult to 400-600 µg for pregnant women (1). Humans are dependent on exogenous supply of folate from supplements or food sources as they are auxotrophic for folate synthesis. Major dietary sources of folate are leafy greens mainly spinach, legumes like beans and nuts, some fruits juices, milk and fermented dairy products (10). Folate is synthesized by various bacteria and yeast. Mainly the bacteria used in the fermentation of food items generally possess the biosynthetic ability to produce folate because folate is an essential cofactor in bacterial metabolism and growth (15, 23).

Important methods to increase the folate level in the human diet are to enhance the folate level of food either through fortification of food products and fermentative fortification besides the folate supplementation. Natural capacity of microorganism for vitamin production in fermented foods needs to be exploited for the fermentative fortification (5). The strain used in the fermentative fortification should exhibit the probiotic properties for the safe and direct consumption of the fermented products. Probiotics, mainly the strain of genera *Lactobacilli* and *Bifidobacterium*, are the beneficial microorganism which exerts beneficial effects on human health. Already much work has been done for the folate production or fermentative fortification in the milk or other dairy products (12, 19, 21). Fermentative fortification was also performed in the reconstituted skim milk medium by the *S. thermophilus* NCIM 2904 and *L. helveticus* NCIM 2733 in our previous study (7). Both of these strain produced the folate at significant level of folate which was further increased to the maximum level after optimization of cultural conditions. Now the recent need is to try some other food products for the fermentative fortification.

This paper deals with the introduction of microbial or fermentative fortification in fruit juices like orange and tomato juice by using the *S. thermophilus* NCIM 2904. This strain was chosen for the study due to the probiotic efficiency of the strain which was reported earlier in our study (8).

Materials and Methods

Microorganisms, chemicals and culture conditions

Yoghurt starter cultures *Streptococcus thermophilus* NCIM 2904 was procured from National culture Laboratory, Pune for the fortification based on the ability to produce folate in the fermented milk. Microbiological assay for the quantitative estimation was performed using *Lactobacillus casei* as an assay organism. *Lactobacillus casei* NCIM 2364 was also obtained from NCL, Pune. All strains were grown and subcultured in MRS media (Himedia, Mumbai). Composition of MRS media is as follows: Peptone- 10.0 g/L, Yeast Extract- 5.0 g/L, Beef Extract- 10.0 g/L, Dextrose- 20.0 g/L, Ammonium Citrate- 2.0 g/L, Sodium Acetate- 5.0 g/L, Magnesium Sulphate- 0.1 g/L, Manganese Sulphate- 0.05 g/L, Dipotassium Phosphate- 2.0 g/L. For the Extraction of folate from the sample, Human Plasma was purchased from Sigma.

All the procured strains were initially maintained in the semi stab prepared with MRS media at 37°C for 24 h and stored at 4°C±1°C and these stock cultures were continuously transferred in fresh MRS semi stab in every 3-4 weeks. Subculturing of all the strains was done serially at least three times prior to use for production to minimize the lag phase of the culture.

Fermentative or microbial fortification of orange and tomato juice

Orange and tomato were procured from the local market. The juice of orange and tomato was prepared using a kitchen blender and filtered through 0.45 µm filter (Himedia, Bangalore). *S. thermophilus* was allowed to grow in folic acid assay medium and incubated for 8 h at 37°C at static condition. Fermented broth was centrifuged at 10,000 rpm for 20 min to obtain the cell pellet and cell pellet was washed twice with 0.85% saline and re-suspended in it. This cell suspension is used as inoculum for the fermentative fortification of orange and tomato juice. 5% of inoculum was added in both the juice and incubated at 40°C for 24 h at static condition. Fermentative fortification was also carried out with the addition of PABA and glutamate (50 µM) in orange juice and tomato juice. Samples were taken at different time intervals and processed for analysis of folate by microbiological assay after extraction.

Extraction of folate from fruit juices

Modified extraction procedure was followed for the extraction of folate from the fruit juices for the total folate (extracellular and intracellular) estimation (19). For this first of all human plasma solution was prepared according to the method described earlier (3). For the extraction procedure, 6 mL of the fermented juice was taken and 10 mL of extraction buffer (0.1 M phosphate buffer containing 0.5% sodium ascorbate as reducing agent) was added. The mixture was kept in a boiling water bath for 15 min and then centrifuged at 4000 rpm for 10 min. The mixture was allowed to cool down and then 0.4 mL of human plasma solution was added into it. Polyglutamyl deconjugase enzyme present in the Human plasma converts the polyglutamyl forms of folates to monoglutamyl forms. The mixture was then incubated at 37°C for 1 h under continuous rotation. Finally the reaction was stopped by placing the samples in boiling water for 5 min. Then, the extract was centrifuged at 10000 rpm for 20 min. Supernatant was then filtered through a 0.45µm filter and used directly for further quantification by microbial assay.

Microbial assay for folate Analysis

MRS broth was prepared and 10 ml of MRS broth was inoculated with the *Lactobacillus casei* from an agar stab culture. Culture was incubated at 37^0C for 24 h. After incubation, cultured broth of *L. casei* was taken and centrifuged to obtain cell pellet. Washing of cell pellet was done twice with sterile saline solution (0.85% w/v NaCl solution). Finally cell pellet was re-suspended in sterile saline solution and it was used as an inoculum for microbial assay of folic acid. 9.4 gm of folic acid *casei* media and 50 mg of ascorbic acid was added in 100 ml of distilled water. It is dissolved by heating. Final pH was maintained in the range of 6.2 -6.7. Then 5 ml of media was added in each test tube. Standard folic acid solution (0.2ng/ml) and unknown samples were added into the media. Distilled water was added to test tube to make up total volume 10 ml. Media was sterilized by autoclaving at 15 lbs (121°C) for 5 min. Media was cooled immediately. Now one drop of prepared inoculum was used to inoculate the folic acid *casei* media. This media was incubated at 37°C for 18-24 h. After incubation, absorbance was taken at 600 nm for each sample. Concentration of folate was calculated from the standard curve plotted between the concentration of folate and absorbance at 600 nm.

Efficacy and stability studies of folate fortified fruit juices

All the microbial folate fortified food products were checked for the stability on long term storage and viable cell count for the probiotic efficiency. Probiotics inoculated fruit juices were incubated at 40°C for 6 h. After that samples were stored in cold room at low temperature at 4°C for 28 days. Subsequently after each one week stability and efficacy of orange and tomato juices were checked in terms of folate content, viable cell count and pH to determine the shelf-life of the product for the intension of human consumption. Folate content was measured by performing microbiological assay with *L. casei* and pH was determined by pH meter. Viable cell count (log cfu/ml) was determined by plating the serially diluted sample on MRS agar plate at 37°C for 48 h.

Results and discussion

Fermentative fortification of orange juice

Folate is already present in the orange juice in small quantity that is not sufficient enough to meet the daily recommended intake in small serving. So in this study microbial bio-fortification was tried in the orange juice. It has been observed that folate content was found to be maximum at 6 h i.e. 45.3 µg/100mL (Figure 1). Thus folate concentration was enhanced up to 2.35 fold of the initial value of folate in orange juice (19.23 µg/100mL) in 6 h. Afterwards a decline was observed in the folate content may be due to the behavior of yogurt starter culture as the folate producers and also the folate consumers for their own growth and metabolic activi-

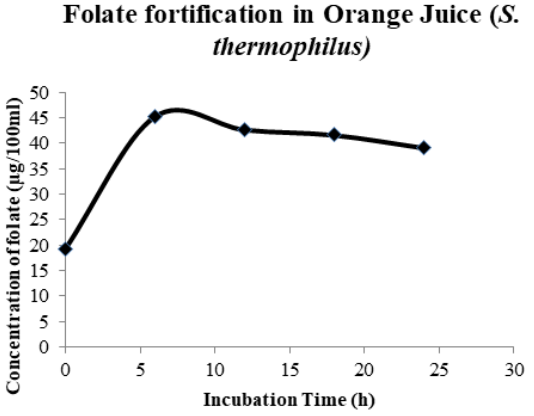

Figure 1. Folate Fortification profile in Orange juice by *S. thermophilus*.

ties (22). Folate concentration decreased by 13.48 % (39.13 µg/100mL) till 24 h. The results are in agreement with the study reported earlier with *L. lactis ssp. cremoris* which is used for the fermentative fortification of cucumber and melon juice. In cucumber juice folate content was increased to 60±1.9 ng/mL from the initial level of 10±0.2 ng/mL whereas folate content enhanced to 26±1.6 ng/mL than initial level of 18±0.9 ng/mL in melon juice (13).

PABA and glutamate (50µM) were also added in orange juice to study its effects on the folate fortification. Folate concentration was increased to 53.5 µg/100mL (2.78 fold) on PABA addition and57.67 µg/100mL (2.99 fold) on glutamate addition (Figure 2). Combination of PABA and Glutamate addition increased the folate concentration to the maximum level of 64.8 µg/100mL (3.36 fold) than the initial folate level of orange juice (19.23 µg/100mL). It has also been noticed that reduction is folate concentration till 24 h is reduced to a lesser extent after the addition of PABA and glutamate. Only 3.53% reduction in folate concentration till 24 h was noticed on both PABA and glutamate addition. The result was somehow supported by the earlier study (13). In this study, PABA and glutamate (25 µmol/L) was added in cucumber and melon juice which is inoculated by *L. lactis subsp. cremoris*. Folate level of cucumber juice did not show significant results on PABA and glutamate addition whereas folate content was enhanced to 36±2.3 ng/mL from the initial level of 10±0.2 ng/mL water melon juice. PABA and glutamate may exert the

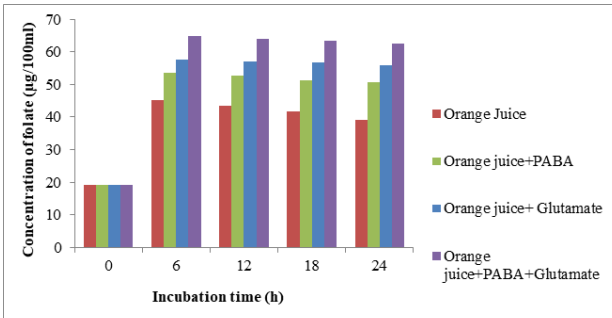

Figure 2. Comparative profiles of folate fortification in orange juice by *S. thermophilus* without any additive, with PABA and Glutamate addition.

increased production due to the role of these as precursor for folate synthesis (23)

Microbial folate fortification in tomato juice

It has been noticed that folate content was enhanced to 33.56 µg/100mL than the initial folate level of 19.83 µg/100mL in 6 h (Figure 3). Thus folate concentration was increased by the 1.69 fold to the initial value. After that a decline of 24.55% (25.32 µg/100mL) was observed in folate concentration at 24 h.

PABA and glutamate (50µM) were also added in the tomato juice to evaluate its effect on folate production. It has been observed that folate concentration was increased to 42.39 µg/100mL (2.13 fold) and 42.86 µg/100mL (2.16 fold)respectively on PABA and glutamate addition in 6 h (Figure 4). Mixture of PABA and glutamate resulted in the increase of folate concentration to the maximum level of 43.7 µg/100mL (2.20 fold) than the initial folate level of tomato juice 19.83 µg/100mL at 6 h. Decline in folate concentration till 24 h is reduced on addition of PABA and glutamate. However, it has been noticed that glutamate addition did not affect the folate production in tomato juice at 6 h. The main reason behind it is unclear. Although it is not necessary that precursors exert induced response in every condition. It may also be said that higher or lower concentration than the 50 µM might exert better effects on fermentative folate fortification in tomato juice.

Efficacy and Stability studies of fortified food products

During the whole study folate production or micro-

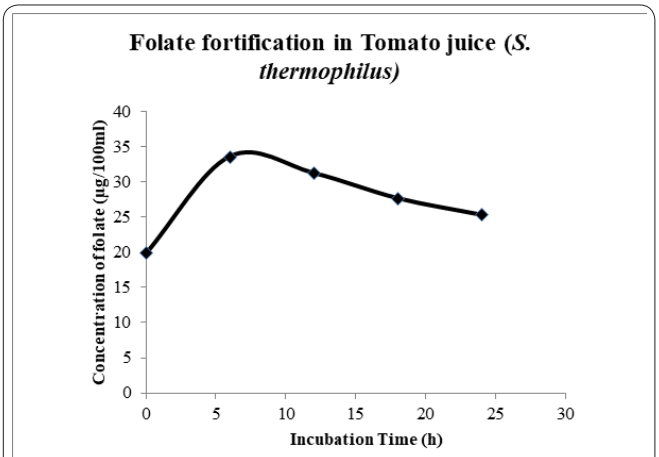

Figure 3. Folate fortification profile in tomato juice by *S. thermophilus*.

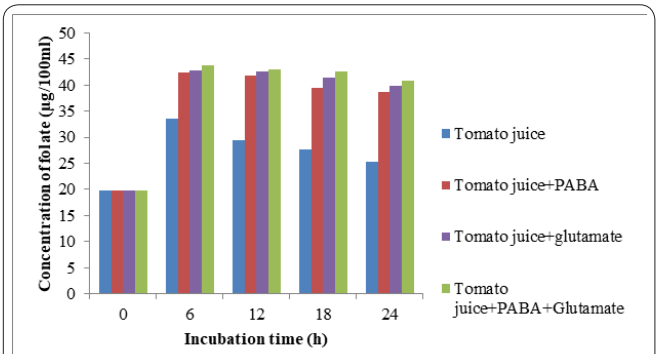

Figure 4. Comparative folate fortification profiles in tomato juice by *S. thermophilus* without any additive, with PABA and glutamate addition.

Table 1. Efficacy and stability studies of folate fortified orange juice with added PABA and glutamate by *S. thermophilus*.

Days	0	7	14	21	28
Folate content (µg/100mL)	64.32	62.34	59.46	57.34	53.48
Viable cell count (Log cfu/mL)	9.35	8.92	7.29	6.89	4.67
pH	3.7	3.4	3.3	3.1	3.1

Table 2. Efficacy and stability studies of folate fortified tomato juice with added PABA and glutamate by *S. thermophilus*.

Days	0	7	14	21	28
Folate content (µg/100mL)	43.28	42.9	40.67	37.45	36.6
Viable cell count (Log cfu/mL)	8.67	8.23	7.22	6.56	3.12
pH	4.6	4.58	4.2	4.1	3.95

bial folate fortification was studied in the orange juice and tomato juice. All these folate rich probiotic fermented food stuffs can be consumed if prepared using all the safety criteria at all the level as such without any purification needed. For the market purpose, long term storage of these products is also an important issue to be discussed. So in this phase of study, efficacy and stability studies of these folate rich probiotic food stuffs has been carried out.

Folate fortified orange juice and tomato juice supplemented with PABA and glutamate has been evaluated for the shelf life in terms of folate content, viable cell count and pH of the juice after some time of storage. From the table 1, it has been detected that folate content of supplemented orange juice was almost stable till 28 days of storage. Initially the folate content was 64.32 µg/100mL which was reduced to 53.48 µg/100mL thus there is not a major degradation in folate content. Only the viable cell count decreased continuously and remains low 6.89 log cfu/mL at 21 days and became 4.67 log cfu/mL at 28 days. For the probiotic food viable cell count must be the 7 log cfu/mL (20). pH of the juice is already low as juices are acidic in nature. However, it tends to almost stable and varies in the range of 3.1- 3.7. After the analysis of all the required studies, it may be concluded that orange juice with PABA and glutamate additives can be stored for maximum 21 days. The study was evidenced by a study in which changes in microbial population, pH and some components were observed in carrot juice after fermentation using some *Bifidobacterium* strains (17).

Shelf life of folate fortified tomato juice with added PABA and glutamate has also been evaluated in the similar manner. Folate content was found to be stable somehow till the four week of storage. Folate content was 43.28 µg/100mL just after the completion of incubation time which was reduced to 36.6 µg/100mL (Table 2). pH of the folate rich tomato juice was also quite stable during the storage and varies between 3.95-4.6 however the decline in viable cell count was observed till the 28 days and became very low as 3.12 log cfu/mL. Although decline till 28 days was prominent but it was somehow alright till the 21 days of storage i.e. 6.56 log cfu/mL. Thus, it may be concluded that tomato juice supplemented with PABA and glutamate had the shelf life of maximum 21 days.

Contribution of the folate fortified food products in the % daily value

Percent Daily value is the percentage of any nutrient provided in the one serving of food with respect to the daily need of that nutrient. This is generally mentioned on the nutrition fact label of the food. If it refers as 25 percent for folate, it means that one serving will provide the 25 percent of the folate you need every day. In this section % DV of all the folate fortified products of this work was calculated by considering the daily recommended intake of folate for an adult i.e. 400 µg/day. By calculation of % DV, we can have the approximate idea of how much folate we will consume each day in one serving of these folate fortified fruit juices. Initially orange juice provided the 46.91 µg/cup folate equivalent to 11.72% DV of folate which increased to 153.30 µg/cup corresponding to 38.32% DV of folate after fermentative fortification. Similarly microbial folate fortification of tomato juicewith added PABA and glutamate contributed to 25.84% DV (103.38 µg/cup) which was initially 11.37% DV (45.49 µg/cup).

Conclusion

Fermented dairy products with enhanced nutritional value have been consumed since centuries due to health inducing effects. However fermentative or microbial fortification to produce functional foods with enhanced nutritive value except the dairy products is the current demand in food industry mainly for the lactose intolerant persons. The elevated levels of folate in the orange and tomato juice with added PABA and glutamate opens the door for the possible fermentative fortification of other fruit and vegetable juices also by acquiring proper hygiene and technology. Positive results of stability and efficacy studies also indicated the hope in this direction. Based on the present result, nondairy probiotic food or beverages can be developed for the lactose intolerant persons by using either *S. thermophilus* or other probiotics or mixed population of probiotics.

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