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Production of Lactic Acid by lactobacilli using Legume Flours

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Abstract

Due to their high nutritional value, richness in carbohydrates and good source of protein, legumes are considered essential for human diet. Legumes flours were used as fermentable material for lactic acid production. Hence, previously isolated lactobacilli strains were used in this study. The strains showed strong microbial growth and their survival in glucose-containing MRS medium and were described using a modified Gompertz equation. Lactobacilli exhibited the shortest latency phase in MRS-glucose medium. While the highest lactic acid produced was 15.40g/l by *Lpb. pentosus* U1 isolate after 48hours. Furthermore, these strains were evaluated in flours samples for acidification capacity. The results showed a decrease in pH values after 12hours of fermentation, indicating the fastest acidification with maximum lactic acid generation of 31.66, 25.33, 31.00 and 31.66g/l for black gram flour by *Lpb. plantarum* M1, *Lpb. pentosus* U1, *Lpb. plantarum* VIP1 and *Lev. brevis* TIP1 isolates. Black gram flour was considered the best among the used substrates for enhancing lactic acid production. Overall, this study can be termed as a preliminary step for fermentation using the desired organism in preparing legume-based food at the industrial level and benefiting consumers.

Keywords: Lactobacilli; Legumes; Fermentation; Lactic acid; Growth curve; Fermented food

1. Introduction

Lactic acid (LA) is a principal organic acid and is included in the Generally Recognized as Safe (GRAS) category, produced by lactic acid bacteria (LAB). It is an important chemical with many potential applications in pharmaceuticals, textiles, chemical industries, cosmetic industries and recently in producing biodegradable plastics such as poly-lactic acid (PLA) [1]. LA can be synthesized artificially or by fermentation [2]. However, production of LA using fermentation pathway has become more successful. About 95%, generate optically pure lactate and its process is eco-friendly. The major factor during lactic fermentation is the decrease in pH due to the conversion of carbon substrate [3]. That is why it becomes necessary to determine the acidity level and, cell population for nutritional reason that imparts sensory attributes in food products [11].

Legumes are an important food source produced worldwide, supplying considerable portion of nutrition required for growth and maintenance of body. Legume belongs to *Leguminosae* family, serving major amount of nutrition in the form of proteins and carbohydrates in diet. Nowadays, nutrition is considered as a tool in preventing diseases [4]. As result of this, attention has been made on food and feed ingredients, and in developing so-called functional foods. Functional foods can be classified as naturally fortified food or food with additional nutritive

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components, that are linked to diseases prevention [5]. Due to their huge resources and easy storage, legume-based foods are becoming new frontier. Previous reports suggest good growth of lactobacilli in legume matrices, indicating incorporation of strains into legume substrate resulting in production of fermented food that attributes in development and well-being of consumers [6]. Hence, legume flours are the best example of fermentable substrate with vast composition. Indeed, legumes flour is a rich source of nutrients like protein, carbohydrates such as resistance starch, sucrose, and non-digestible oligosaccharides, fibers, fats and mineral [4]. Fermented legume flours can be taken as synbiotic, where in, both probiotic and prebiotic activities are present [7]. Therefore, a lab-scale experiment was set-up for legume flour fermentation using lactobacilli. The aim of this study was to evaluate the maximum production of lactic acid during fermentation process of this functional food using legume flours. However, reduction in anti-nutritional factors and sensory attributes are yet to be investigated during fermentation.

2. Materials and Methods

2.1 Microorganisms

Lactiplantibacillus (Lpb.) plantarum M1, *Lactiplantibacillus (Lpb.) pentosus* U1, *Lactiplantibacillus (Lpb.)* VIP1, and *Levilactobacillus (Lev.) brevis* TIP1, microaerophilic organisms, isolated from legume based flour, identified and maintained in our laboratory, showed excellent capacity by producing relevant amount of lactic acid in MRS medium characterized by utilizing non-oligosaccharides via fermentation from legumes [8].

2.2 Materials

Dried split beans of red lentil (RL) (*Lens culinaris*), black gram (BG) (*Vigna mungo*), pigeon pea (PP) (*Cajanus cajan*), and lima bean (LB) (*Phaseolus lunatus*) were purchased from grocery markets, in Vadodara, Gujarat.

2.3 Preparation of Lactobacilli Starters

Lactobacilli were grown in a selective MRS broth (HiMedia, Mumbai, India) and incubated at 37˚C for 24hours. Lactobacilli starter with the final concentration of 10^7 CFU/ml in MRS broth was centrifuged at 10,000 rpm for 10 mins before removing the supernatant.

2.4 Utilization of Glucose for Lactic Acid Production and Kinetic Parameters of Bacterial Growth and Acidification

The experiment was conducted in 250 ml flask with 35 ml of production medium containing 20g/l glucose with other ingredients of MRS medium. Cultures were enriched in the glucose containing medium with an addition of 1% inoculum. The supernatant was further analyzed for bacterial growth, pH, titratable acidity and lactic acid in the fermented medium. Total titratable acidity (TTA) was determined on the amount of 0.1N NaOH required to neutralize 10g of the fermented sample [9]. The growth of bacteria was expressed with modified Gompertz equation [10, 11]. Similarly, kinetics of acidification were determined by a pH-meter.

2.5 Preparation and Inoculation of Flour

Legume beans were cleaned and milled using mortar and pestle to obtain fine fraction of flours. The flours were sieved through 200µm sieve and stored in air tight plastic containers. Flours prepared were coded as followings: RL, BG, PP and BB.

Flours were separately mixed with sterile distilled water (1:2w/v), in sterile containers. Each one of the coded flour (RL, BG, PP and BB) was inoculated with the four lactobacilli

isolates (M1, U1, VIP1 and TIP1) separately and incubated at 37˚C for 12hours. Controls were RL, BG, PP and BB flours without inoculum.

2.6 Determination of pH and Lactic Acid

The pH value of MRS-glucose medium and fermented batter was measured using pH meter. The spectrophotometric method for lactic acid quantification was followed as described by Borshchevskaya *et al.* [12]. The amount of lactic acid in the samples was calculated based on the standard calibration curve. The obtained data was divided into the sets of experiments done in triplicates.

2.7 Statistical Analysis

All statistical analysis was subjected to analysis of variance (ANOVA) using a software Graph pad 8.0. Experiments were conducted in triplicates and values were calculated as the mean ± standard error.

3. Results and Discussion

Legumes are an excellent source of carbohydrates consisting of 25-65% of the component [13]. As previously reported, lactobacilli isolated from legume based fermented batters was used [8]. The evaluation of metabolites like LA acts as a conservative that can be useful for legume-based bioprocessing [14]. Preliminary, these trials were made to select the best candidate to be used as a starter for legume batter fermentation.

According to the literature, lactobacilli are known to produce lactic acid from various carbohydrate sources [15]. In particular, the isolates were tested for their ability to generate maximum lactic acid using MRS medium containing glucose. Using glucose as a sole carbon source, the maximum titer reached 15.40g/l and minimum 14.80g/l, was recorded for *Lpb. pentosus* U1 and *Lpb. plantarum* M1 isolates, respectively. Whereas, *Lpb. plantarum* VIP1 and *Lev. brevis* TIP1 isolates managed to produce 13.91 and 11.70g/l of LA after 48hours. Fermentation with glucose led to a reduction in pH from 6.56 to 4.50 after 48hours of fermentation at 37˚C for all isolates (Figure 1). Furthermore, cell densities of 9.91, 9.99, 8.92 and 9.81 log CFU/ml were recorded for *Lpb. plantarum* M1, *Lpb. pentosus* U1, *Lpb. plantarum* VIP1 and *Lev. brevis* TIP1 isolates. Previous reports stated that the concentration of LA increased once the growth ceased. This can be due to the limitation of nutrients other than carbohydrate, which results in energy uncoupling between bacterial growth and lactic acid [16]. The acidity of medium increased during fermentation reaching values in the range of 12.61 to 16.01g/l after 48hours. The rapid incline in acidity observed during the fermentation minimizes dominance of spoilage bacteria [3].

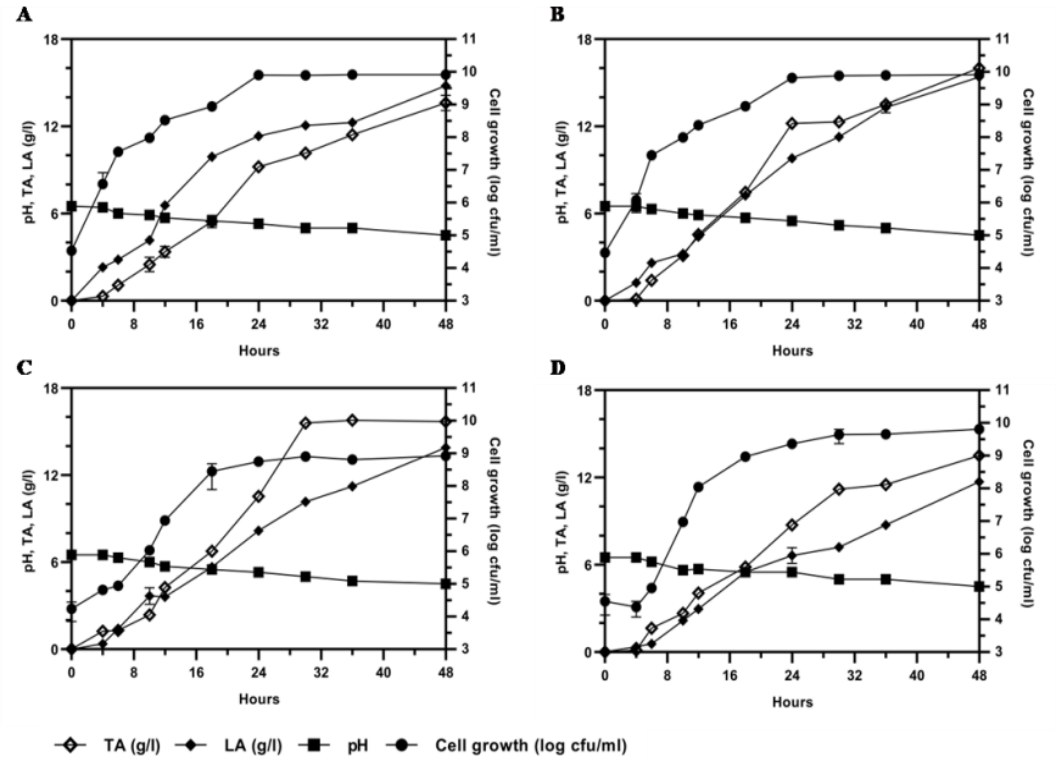

Figure 1: Profile of microbial growth, lactic acid, total titratable acidity and pH during fermentation with (A) *Lpb. plantarum* M1 (B) *Lpb. pentosus* U1 (C) *Lpb. plantarum* VIP1 (D) *Lev. brevis* TIP1 using glucose (20 g/l) in MRS medium at 37 ˚C.

Further, investigation was conducted on the maximum production and productivity of LA from MRS-glucose medium with an initial concentration of 20g/l at pH 6.5. Lactobacilli spp. exhibited 4 hours of lag phase with highest increment in biomass of 1.50 at OD_{600} nm by *Lpb. pentosus* U1 isolate. A longest stationary phase was observed from 24 to 40hours with maximum LA production in the obtained lactobacilli. LA productivities using 20g/l of glucose ranged from 0.24 to 0.32g/l/h, while the maximum LA productivities obtained were between 1.80 to 3.70g/l/h. *Lpb. pentosus* U1 isolate indicated highest for maximal LA productivities among all isolates (Table 1).

Table 1: Kinetic parameters of lactic acid in MRS-glucose medium by lactobacilli

Isolates	Conc. of glucose (g/l)	OD 600 nm [a]	LA conc. (g/l)[b]	P_{LA} (g/l/h)[c]	Max P_{LA} (g/l/h)[d]
Lpb. plantarum M1,	20	1.49 ± 0.01	14.8 ± 2.17	0.30	3.73 (6h)
Lpb. pentosus U1	20	1.50 ± 0.01	15.40 ± 0.0	0.32	1.8 (6h)
Lpb. plantarum VIP1	20	1.29 ± 0.09	13.91 ± 0.0	0.28	2.2 (6h)
Lev. brevis TIP1	20	1.48 ± 0.20	11.70 ± 0.26	0.24	2.4 (6h)

[a]OD, highest optical density; [b]Maximum lactic acid production after 48 h; [c]Maximum productivity of lactic acid at the end of 48 h using initial glucose concentration of 20 g/l; [d]Highest lactic acid productivity at desired time.

LA and its derivatives are important organic acids with widespread applications in industries [15] that are produced through fermentation by many known microorganisms using

a variety of saccharides owing to their rapid proliferation and high productivity [17]. Since lactobacilli are highly capable of converting carbohydrates to LA [18], and are also known heterofermenters requiring complex nutrients, it is imperative to use the stain selected to determine the medium composition [19]. However, in this study the process of decoupling was observed where the energy obtained in generating LA is not utilized for bacterial growth, instead is wholly utilized in maintaining pH [20]. On the other hand, reduction in cell growth was found in some strains at 48hours which was probably due to the accumulation of high amount of lactate that resulted in growth inhibition [17]. Although, this inhibition was noted because of the disruption in cell membrane of organism, the deposition of anion due to acidification in cytosol [21]**.**

Moreover, this study examined the growth kinetics of lactobacilli isolates in a MRS-glucose medium and fitted the Gompertz equation derived by Zwietering *et al.* [10] to describe the growth pattern of an organism. Since microorganisms during log phase grow faster, thus plotted as $y = \ln(N/N_0)$ which is the relative population size against time (Figure 2). The bacterial growth parameters were determined using the equation λ, μ_{max} and A [$A = \ln(N/N_0)$]. Table 2, shows highest growth rate of *Lev. brevis* TIP1 isolate (1.18 per hour), where organism consumes carbohydrates rapidly to achieve maximum growth. Whereas, the shortest doubling time observed was 3.12hours. A clear 3hours of lag phase was observed after which the organism started to proliferate speedily, probably due to the less starting inoculum that caused increase in the duration of lag phase. Change in the physiological environment in the growth medium could be another influencing factor [22]. Variation in cell growth and experimental data, when compared with modified equation displayed suitability of model in relating the behavior of growth in MRS-glucose medium following the exponential law [11]. However, the modified equation says that microbial population is directly proportional to the growth rate which decays significantly with time owing to death in bacteria [11]. Therefore, this model is most frequently used for microbial growth and is presently the most common among several organisms.

The kinetics of acidification were calculated by Gompertz equation. A similar outcome behavior was observed in glucose medium for lactobacilli. With the change in pH several factors including bacterial growth rate, utilization of substrate, acidity and fermentation pattern were significantly influenced during the process [23]. The kinetic parameters, ΔpH and V_{max} depend on the bacterial concentration of log phase, where lag phase lasted for 6hours (λ), and V_{max} measured was 0.1dpH/h, highest ΔpH obtained was 0.6. While a similar variation in pH was obtained among lactobacilli.

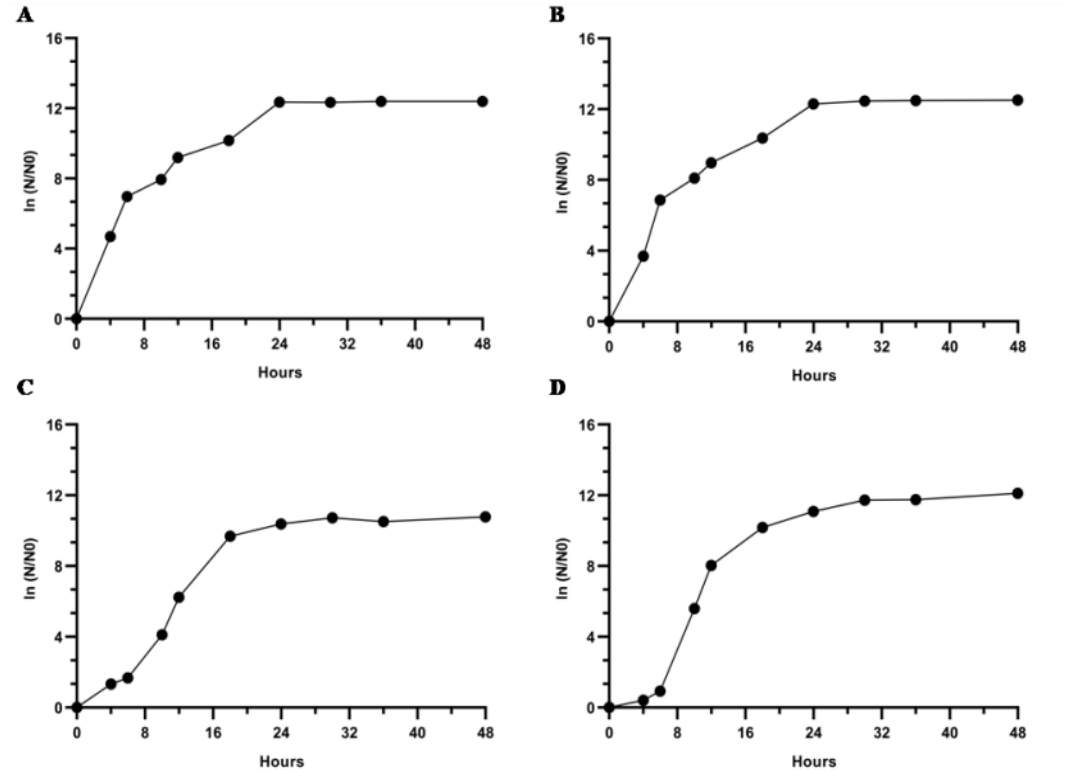

Figure 2: Growth kinetics fitted with modified Gompertz equation, during fermentation with (A) *Lpb. plantarum* M1 (B) *Lpb. pentosus* U1 (C) *Lpb. plantarum* VIP1 (D) *Lev. brevis* TIP1 in MRS-glucose medium.

Table 2: Kinetics parameter of bacterial growth curve in MRS-glucose medium using lactobacilli

Parameters	*Lpb. plantarum* M1	*Lpb. pentosus* U1	*Lpb. plantarum* VIP1	*Lev. brevis* TIP1
μ_{max} (h^{-1})	0.37	0.28	0.76	1.18
λ (h)	12	18	12	12
ε (h)	24.83	37.03	8.19	6.81
A	9.19	10.37	6.23	8.04
Doubling time (h)	7.45	7.14	7.96	3.12

* Values were obtained using modified Gompertz model for experimental data done in triplicates.
D_0, density at $t = 0$; D_t, optical density (OD_{600nm}) at time t; t, growth at particular time (h); A, the asymptotic value (D_0 and OD_{600nm}); μ_{max}, maximum growth rate per hour; λ, duration of lag phase (h); ε, exponential growth time, calculated as $\varepsilon = A/\mu_{max}$.

In order to pick lactobacilli with best technological properties relevant for legume bioprocessing, four selected representatives lactobacilli isolated from fermented batter were evaluated for the acidification capacity to check decline in the pH of different legume flour samples. The results of this acidification activity are shown in Figure 3. It was observed that all tested lactobacilli strains confer excellent acidifying abilities. Isolates effectively decreased pH significantly ($p < 0.05$) to 4.5 after 12hours of fermentation. The initial pH value (t0) was close to 6.3 in four isolates for all the flours, and nearly 4hours of lag time was observed for all isolates. The value of pH remained almost constant. However, it pH gradually decreased during

fermentation. After 12hours the lowest values recorded were 4.76, 5.0, 4.73 and 4.50 for *Lpb. plantarum* M1, *Lpb. pentosus* U1, *Lpb. plantarum* VIP1 and *Lev. brevis* TIP1 isolates using BG flour (Figure 3). Except *Lpb. pentosus U1* isolate, the remaining strains acidified faster revealing ΔpH, i.e., difference in pH before and after inoculation of the strains. Although, initial pH was recorded before incubation, where *Lev. brevis* TIP1 showed fastest acidification ability to decrease pH of BG flour, rest of flours pH value were approximately 5.5. However, *Lpb. plantarum* M1, and *Lpb. plantarum* VIP1 isolate significantly increased acidity. The lowest acidity was produced in BG flour, which was probably because black gram is the most favorable flour for enrichment of lactobacilli. These findings are also in a line with Ventimiglia *et al.* [24] who reported that *Lpb. plantarum* was the highest acid producer by converting raw materials into organic acids, mainly lactic acid. Indeed, faster lowering of pH by organism improves safety and high organoleptic properties of the fermented products [25]. The quantity of lactic acid recorded is shown in Figure 4, where no LA was produced in the initial hours of fermentation. An almost linear analogue of LA production was obtained after lag phase (~7h) until the end of fermentation. Maximum values of 31.66, 25.33, 31.00 and 31.66g/l were measured for BG flour by *Lpb. plantarum* M1, *Lpb. pentosus* U1, *Lpb. plantarum* VIP1 and *Lev. brevis* TIP1 isolates. Similarly, lower levels of LA were also reported for LB (14.00g/l) by *Lev. brevis* TIP1 isolate, PP (19.70, 20.33g/l) by *Lpb. plantarum* M1, and *Lpb. pentosus* U1, isolate. Generation of LA could be directly linked to the lactobacilli growth. Several studies related to cereal fermentation noted an increase in lag time phase, due to addition of water in cereal suspension such as in wheat flour. While low water level during the start of fermentation can prevent the growth of bacteria [26]. However, the maximum LA measured was 3.1 g/l from rice flour. Therefore, the future perspective was to test different concentrations of legume flours singly or in mixed combinations using same isolates to check, whether they can give same results in terms of cell growth, pH and lactic acid production.

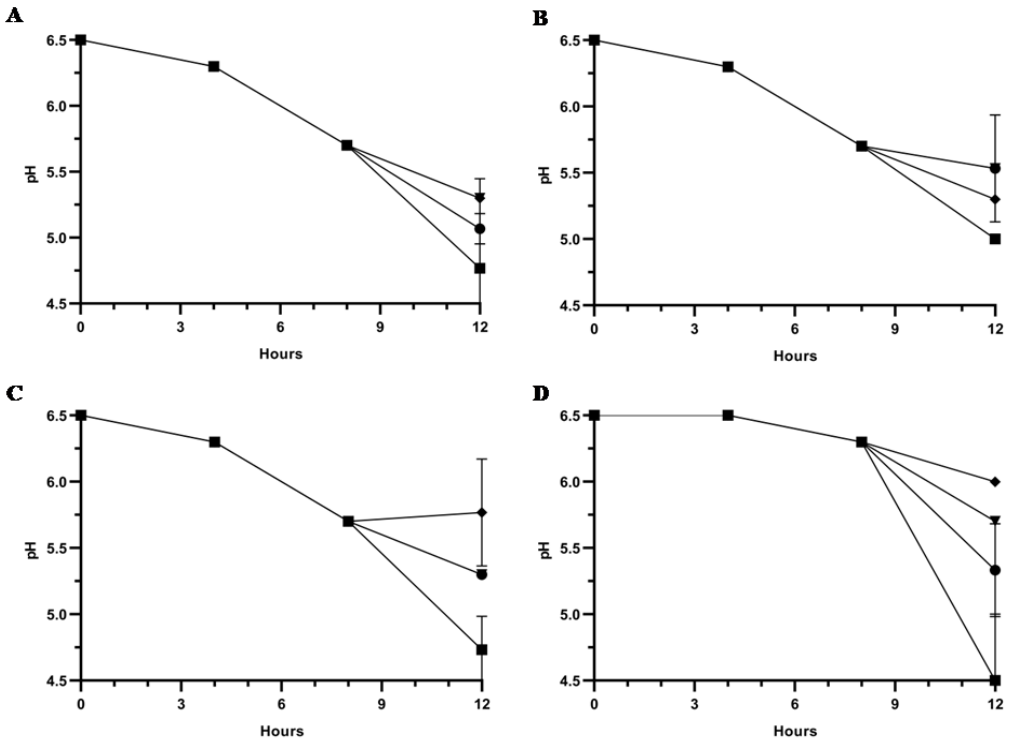

Figure 3: pH of different legume flours inoculated with respective (A) *Lpb. plantarum* M1 (B) *Lpb. pentosus* U1 (C) *Lev. brevis* TIP1 (D) *Lpb. plantarum* VIP1 isolates.

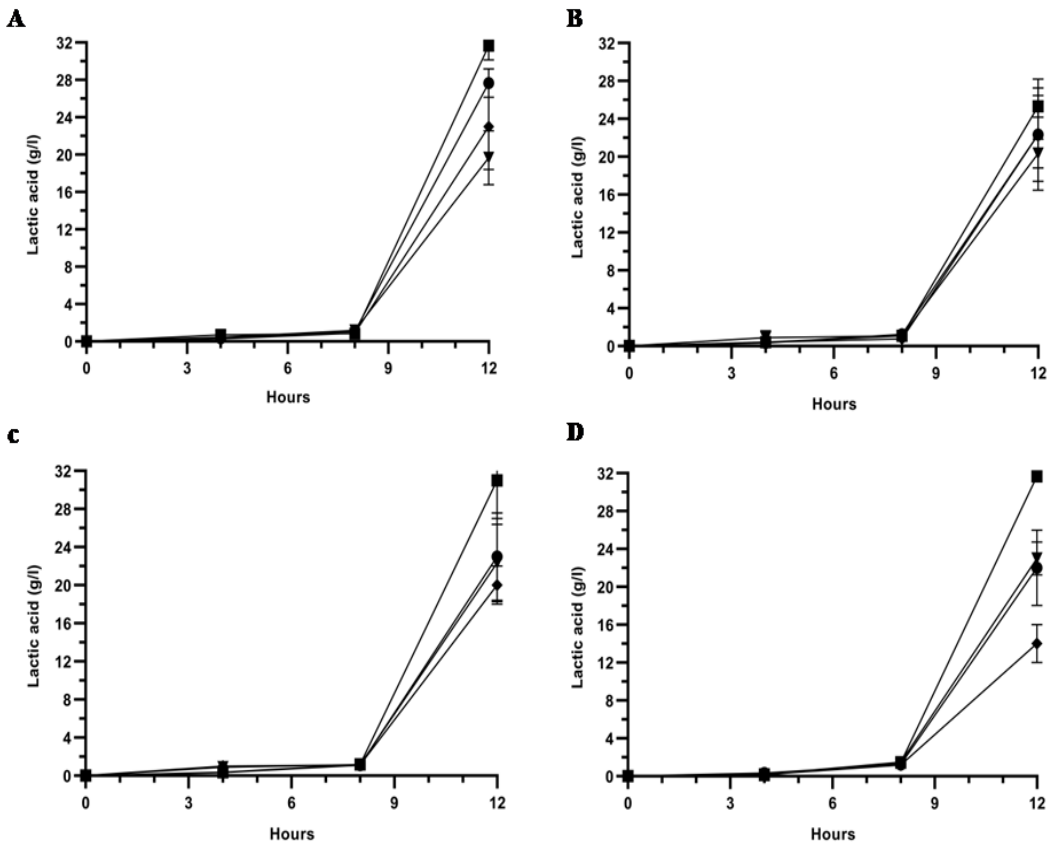

Figure 4: Lactic acid production in different legume flours inoculated with respective (A) *Lpb. plantarum* M1 (B) *Lpb. pentosus* U1 (C) *Lev. brevis* TIP1 (D) *Lpb. plantarum* VIP1 isolates.

1. Conclusion

Lactobacilli is one of the most illustrative and popular GRAS organism observed in the fermented food and thus it becomes necessary to address its effects in producing LA which is considered to play an important role in reducing the risk of contamination during fermentation with other microbes. This study was an initial step to focus on fermentation of legume flours using lactobacilli as starters during function foods production. The results showed strong decrease in pH, a raise in cell count and at the same time increased acidification as a consequence of production of LA occurs. Based on results, black gram flour seemed to be the best legume flour for fermentation. Although, red lentil, pigeon pea and lima beans seemed to be good substrates, but their fermentation process needs to be optimized. Sufficient microbial density was achieved in all the fermented samples which was proved by *in vitro* experiments done to demonstrate its functionality. The results indicated possibilities of bacteria to adapt easily in different environments and being successfully capable in reducing sugars into palatable form. Fermented flours could be employed as finished and semi-finished products as *idli* batter and baby foods to further develop properties like immunomodulatory activity and proper digestibility after consumption. Furthermore, the mixtures of flours could be used to verify the effects of other flours on microbial growth, pH and lactic acid production. However, the process can be improvised by controlling pH during fermentation or by suppressing the concentration of oxygen. Hence, this provides an excellent opportunity for food related industries to develop low-cost foods filled with nutrition that can be directly consumed.

2. Conflict of Interest

The author declares no conflict of interest.

3. Acknowledgement

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