PREPARATION OF ALMOND-BASED VEGAN CHEESE USING *LACTOBACILLUS CASEI* ISOLATED FROM THE PROBIOTIC DRINK - YAKULT, AND CHARACTERIZATION OF ITS NUTRITIONAL VALUES.

Glory Angel S[1] and Gomathi N[2]*

1- PG Student, Department of Microbiology, Valliammal College for Women
2 - Corresponding Author, Assistant Professor, Department of Microbiology, Valliammal College for Women, E – 9, Anna Nagar East, Chennai – 102, Tamil Nadu, India*

ABSTRACT

Vegan cheese is a category of non-dairy cheese that does not use any animal products. Vegan cheese can be made with components derived from vegetables, such as proteins, fats, and milk. It also can be made from seeds, such as sesame, sunflower, nuts (cashew, pine nut, peanuts, almond), and soybeans. Vegan cheese is a safe and healthy alternative for those with allergies or lactose intolerance. In the present study, vegan cheese was prepared by fermenting the coagulated product of almond milk using the bacterium isolated from the probiotic drink, Yakult. 5 Lactobacillus isolates were obtained from different dairy products like Yakult, Hatsun curd, Heritage curd and Yoghurt. The isolates were tested for antioxidant activity by DPPH assay. The isolate which had shown good antioxidant activity was selected for the preparation of vegan cheese. The bacterial isolate, Lactobacillus casei obtained from the probiotic drink, Yakult had shown good antioxidant activity. To optimize the almond and water ratio for milk extraction, different almond: water ratios were tested (1:3, 1:5, 1:7, and 1:9). The solid content of milk was observed to decrease significantly with an increase in the water content of milk. The almond milk was coagulated using 0.5% Magnesium chloride. The coagulated product of almond milk was fermented using Lactobacillus casei for 24 hours at 37°c. The concentration of protein, lipid, and carbohydrate was estimated in the unfermented coagulated product, fermented almond-based cheese and cheese mixed with beetroot extract. There was a significant decrease in the concentration of protein and carbohydrate in the fermented almond cheese due to the proteolytic activity and fermentation of sugars by Lactobacillus casei but there was no significant change in the concentration of lipid. The carbohydrate concentration was slightly increased after mixing the fermented cheese with beetroot extract and this could be due to the sugar content present in the beetroot extract. When the beetroot extract is mixed with fermented almond cheese, it increases the nutritional value of the cheese product as it contains carbohydrates, protein, iron, vitamin C, vitamin A, magnesium, potassium, and folate. Consequently, further studies are required from many perspectives to widen the range of nutritious end products.

Keywords: *Lactobacillus casei, Vegan cheese, Yakult, Almond, Probiotic drink*

1. INTRODUCTION:

Vegan cheese is a safe and healthy alternative and is also a good alternative for those with allergies or lactose intolerance. It also can be made from seeds, such as sesame, sunflower, nuts (cashew, pine nut, peanuts, almond), and soybeans. Vegan cheeses range from soft fresh cheeses to aged and cultured hard cheeses like plant-based Parmesan. The nutritional value of vegan cheese varies. Most vegan cheese contains no cholesterol and less saturated fat than dairy cheese. The vegan cheese had higher riboflavin and vitamin B12, making it an acceptable replacement for cheddar cheese in terms of those nutrients.

Plant-based cheese alternative (PBCA) is one of the many new emerging dairy-free products responding to the requirements of people who choose to predominantly eat plant-based (PB) food. In 2016, the global market value of vegan cheese amounted to approximately 2.06 billion US dollars and this is predicted to increase to 3.90 billion dollars by 2024 while sales of vegan cheese in the USA increased by 43% from 2009 to 2018. (Munekata *et al.*,2020). Certain developing countries such as India are in urgent need of alternate sources of proteins because animal proteins are becoming more expensive and getting beyond the reach of middle-class people.

Food industries are fortifying food products with probiotic strains as they improve the food's digestibility, texture, or shelf life. They also contribute to the sensory profile of final products by improving flavor and texture. Regular consumption of probiotic microorganisms improves lactose digestion, regulates bowel function, stimulates the immune system, and inhibits the growth of pathogens (Ouwehand *et al*., 1998)

The almond is a nutritionally dense food, providing a rich source of B vitamins - riboflavin and niacin, vitamin E, and essential minerals like calcium, copper, iron, magnesium, manganese, phosphorus, and zinc. Almonds can be processed into a milk substitute called almond milk; the nut's soft texture, mild flavor, and light coloring (when skinned) make for an efficient analog to dairy and a soy-free choice for lactose-intolerant people and Vegans. Almond cheese contains lots of healthy fats, fiber, protein, magnesium, and vitamin E. The health benefits of almonds include lower blood sugar levels, reduced blood pressure, and lower cholesterol levels. They can also reduce hunger and promote weight loss.

Almonds have high anti-oxidant activity owing to the α-tocopherol and polyphenolic constituents which might improve the bioavailability of dietary iron. As it has been reviewed, although non-heme iron is easily oxidized (not bioavailable) due to the rise of pH in the lumen, the presence of reductants from food can maintain this micronutrient in its reduced form and, therefore, positively improve its absorption. Moreover, almond milk is considered an appropriate alternative to cow milk, since, besides the healthy lipid profile, it has a low ratio of Na/K and a balanced ratio of Ca/P. (Chen *et al.,* 2006)

Almond nuts are rich in mono- and polyunsaturated fatty acids (mainly oleic and linoleic acids), vegetable proteins, dietary fibre, phytosterols, polyphenols, vitamins, and minerals, most of which compounds have antioxidant properties and a proven beneficial effect on the plasma lipid profile, low-density lipoprotein oxidation and inflammatory processes, among other things (Jones *et al.,*2011). Although around 50% of almond composition is fat, intakes of 7g per day of this nut have been shown to reduce low-density lipoprotein cholesterol concentration by 1%, and up to 84 g per day can be consumed without weight gain. These nuts have a low glycemic index (they do not adversely impact insulin sensitivity) and have been found to possess prebiotic effects since they stimulated the growth of gut *Bifidobacteria* and *Eubacterium rectale*. Hence, considering the health benefits of almond intake, almond milk might be considered a good food matrix with which to obtain healthy fermented products. Moreover, if probiotic bacteria carry out the fermentation process, the developed fermented product could be useful in preventing some immunomodulatory diseases, such as allergies. (Sabate *et al* 2003)

Probiotics are live microorganisms that, when administered in adequate amounts in edible matrices (i.e., food products), confer health benefits on the host (FAO/WHO, 2001), such as the reduction of hypercholesterolemia, the host immune modulation, the alleviation of constipation, the protection against traveller's diarrhoea, the protection against colon and bladder cancer, the prevention of osteoporosis or the modulation of food allergies (Saad *et al.,*2013).

Lactobacillus casei is a homofermentative microorganism. It is acid-tolerant and could thus survive during cheese ripening ((Kourkoutas *et al.,* 2005). This bacteria has been identified as facultatively anaerobic or microaerophilic, acid-tolerant, non-spore-forming bacteria. *Lactobacillus casei* is an organism that belongs to the largest genus in the family *Lactobacillaceae*, a lactic acid bacteria (LAB). Lactic acid bacteria (LAB) are widely exploited for its probiotic and fermenting properties. This species is a non-sporing, rod-shaped, gram-positive microorganism found within the human body's reproductive and digestive tract. In mouse experiments as well as in human studies, the oral intake of Lactobacilli resulted in stimulation of macrophages, lymphocytes, and natural killer (NK) cells, higher production of g-interferon, and significantly higher secretory IgA responses against pathogenic agents *(Salmonella, Rotavirus*). (Kaila *et al.,* 1992).

The study aimed to prepare vegan cheese (non-dairy) from almond milk using a Probiotic strain with best Antioxidant activity screened from dairy products and to characterize its nutritional values.

2. MATERIALS AND METHODOLOGY:

2.1 Collection of samples:

Probiotic drink – Yakult and Dairy products like Curd and Yoghurt samples were purchased from the Supermarket.

2.2. Isolation of Lactobacillus from Probiotic Drink and Dairy Products:

The bacteria *Lactobacillus spp*. was isolated using MRS broth and MRS agar media. The Probiotic drink, curd, and yogurt samples were dissolved in sterile distilled water and serially diluted and were plated into selective medium *Lactobacilli* MRS agar by Spread Plate Technique and incubated for 24 hours at 37°C.

2.3 Preparation of Lactobacilli strains for DPPH Assay:

Preparation of samples was performed according to Chen *et al* (2014). The Lactobacilli strains were cultured in MRS Broth at 37°C for 18 h. The cells were harvested by centrifugation at 2010g for 10 minutes at 4°C. Cell pellets were washed twice with PBS. And suspended in PBS.

2.4 Screening Lactobacilli strains for Antioxidant Activity by DPPH Assay:

Free radical scavenging ability for Lactobacilli isolated from dairy products was done using a stable DPPH radical (1,1-diphenyl-2-picrilhydrazyl). The effect of given samples on DPPH radical was estimated according to the procedure described by Von Gadow *et al.* (1997). Two ml of 6×10^{-5} M methanolic solution of DPPH was added to 50 µl of a sample. The absorbance was read at 515nm. The decrease of absorbance at 515 nm was continuously recorded in a spectrophotometer for 16 min at room temperature. The samples were tested at 1 mg/ml concentration. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time and the percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 min duration as follows:

All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula of Yen and Duh (1994).

$IP = [(A_{C\,(0)} - A_{A(t)} / A_{C\,(0)})] \times 100$

Where $A_{C\,(0)}$ is the absorbance of the control at $t = 0$ min; and $A_{A(t)}$ is the absorbance of the antioxidants at $t = 16$ min.

2.5 Characterization of Lactobacillus species:

Based on the Antioxidant activity of Lactobacilli isolates, the best isolate was chosen for the study. The Morphological and physiological characterization were done for the selected *Lactobacillus* sp. The biochemical tests were performed as suggested by Garrity *et al.,* 2003 which include the following tests like Citrate utilization test, Methyl red test, Oxidase test, Voges Proskauer (VP) test, Urease test, Indole production test, Catalase test, Starch hydrolysis test, Nitrate reduction test, Gram's staining, and Triple sugar iron agar (TSI) test.

2.6 Optimization of the amount of water used for extraction of Almond Milk:

Almonds (80 g) were soaked in water (1 liter) at room temperature for overnight. The soaked nuts were drained, and the outer layer was removed and rinsed with running tap water. Almonds were ground with water in a high-speed blender in different ratios (1: 3, 1: 5, 1: 7, and 1: 9). The Almond slurry was indirectly heated in a water bath at 85°C for 45 min (Rekha *et al*., 2013) and then filtered through a double layer of cheese muslin cloth to separate Almond milk from residues.

2.7 Coagulation of Almond protein:

Almond milk was heated at 95°C for 5 min and then cooled at room temperature to 80°C with a constant stirring. Magnesium chloride (MgCl2) solutions (0.5%) were added to the Almond milk and stirred for 10 min. Milk was allowed to coagulate for 15 min. The coagulated milk was filtered through a cheese cloth, and the curd was pressed with 500g weight for 15 min. After pressing, the cloth was removed, and the pressed curd was stored at 4°C.

2.8 Estimation of protein using Bradford method:

The protein content in the coagulated product of almond milk was estimated according to the dye binding method of Bradford (1976). 100 mg of the dye CBB G- 250 was dissolved in 50ml of ethanol and 100 ml of orthophosphoric acid. The mixture was diluted to 1000 ml with distilled water. The dye was checked for absorbance at 595 nm in a spectrophotometer and adjusted until to get the optical density (O.D) value 1.18 with dye or distilled water. The dye was filtered through WhatmanNo.1 filter paper and stored in an amber colored bottle at 4ºC. 0.1 g of coagulated product was mixed in 1ml of distilled water. 100µl of the suspension was added with 1 ml of the Bradford's reagent (CBB) and incubated for 20 minutes at room temperature for the development of blue color developed was read at 595 nm in a spectrophotometer. The amount of protein was determined using bovine serum albumin as the standard.

2.9 Estimation of Total Carbohydrate (Dubois, 1956):

About 0.1g of extract was dissolved in 1ml of distilled water. To estimate the carbohydrate content in given extract, 0.5ml of 5% phenol added to the 100µl of extract solution, followed by 2.5ml of concentrated H_2SO_4. The absorbance was measured after 10 minutes at 500nm against blank using Spectrophotometer. Glucose was used as a standard.

2.10 Estimation of Total Lipid:

About 1g of coagulated product was weighed and dissolved in 20ml of Folch's reagent(40ml of chloroform and 20ml of methanol). The mixture was filtered and the filtered solution was added to the preweighed petriplate. The petriplate was incubated until the solvent completely evaporates and the petriplate was allowed to dry. After drying, the petriplate was reweighed. (Bligh and Dyer,1959).

2.11 Preparation of probiotic cheese spread:

Following the method described by Sharma and coworkers (2017), the growth profile of probiotic strains (*Lactobacillus casei*) in MRS medium was performed. Optical density was measured at 600nm. The whole cell culture fluid was centrifuged at 10000 rpm for 10 minutes at 4°C, washed twice with sterile saline solution (0.85% NaCl) and resuspended in saline water to its original volume and added to a pressed curd. Fermentation was done at 37°C for 24 hours.

2.12 Preparation of Beetroot extract:

Beetroot extract was prepared and 0.5ml of extract was added to fermented vegan cheese under aseptic conditions as flavouring agents and stored at 4ºC in airtight bottles for further analysis.

2.13 Physico-chemical analysis of fermented Almond cheese analysis:

After 24 hours, protein, lipid and carbohydrate content were again tested for the fermented vegan cheese prepared from Almond milk and beetroot extract using *Lactobacillus* species.

3. RESULT:

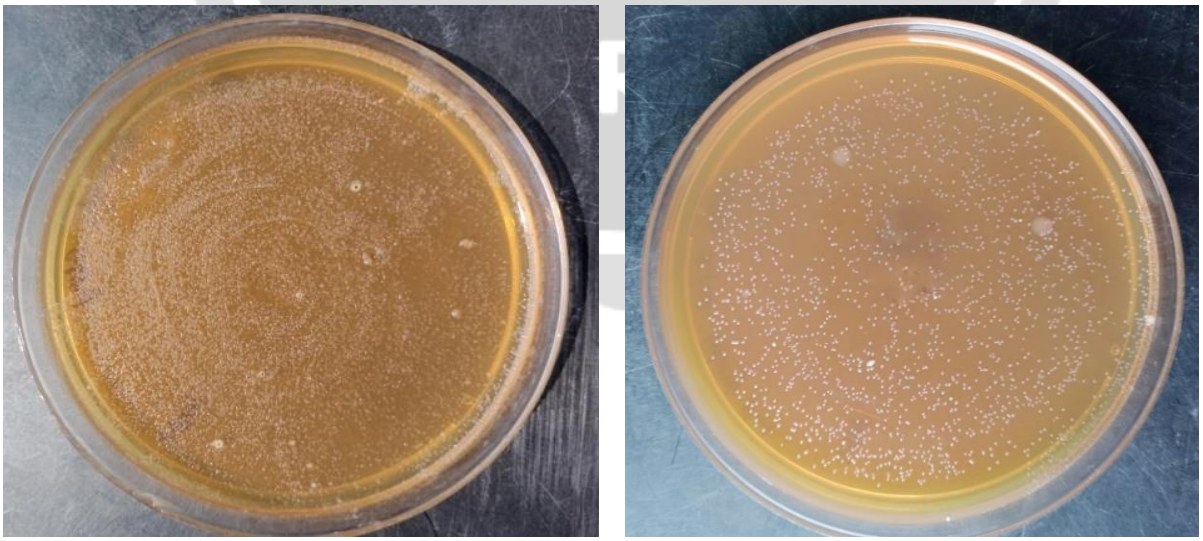

Fig1: Lactobacillus colonies on MRS agar

Fig2: Pure culture of Lactobacillus on MRS agar

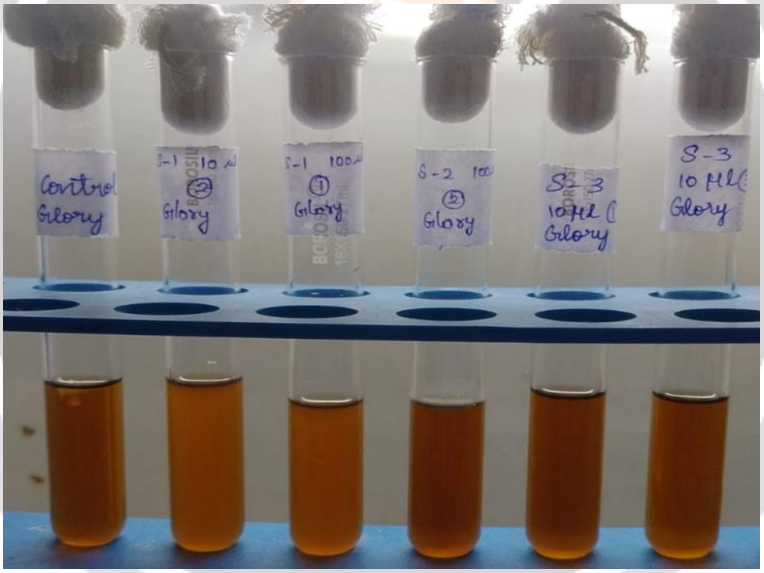

Fig 3: Cultivation of Lactobacilli isolates in MRS broth

Sample	Isolates	%Inhibition in different concentration
Yakult	L1	50.55%
Hatsun curd	L2	39.69%
Heritage curd	L3	38.39%
Heritage curd	L4	34.87%
Yoghurt	L5	27.63%

Table 1: The percentage inhibition of DPPH radical by the Lactobacilli isolates

S.NO	BIOCHEMICAL TEST	RESULT
1	Indole production test	Negative
2	Methyl Red	Negative
3	Voges proskauer	Negative

4	Nitrate reduction test	Positive
5	Citrate utilization test	Negative
6	Oxidase test	Negative
7	Urease test	Negative
8	Triple sugar iron test	Negative
9	Catalase test	Negative
10	Starch hydrolysis test	Positive
11	Gram's Staining	Gram positive Rod

Table 2: Biochemical characterization of *Lactobacillus casei* isolated from Yakult

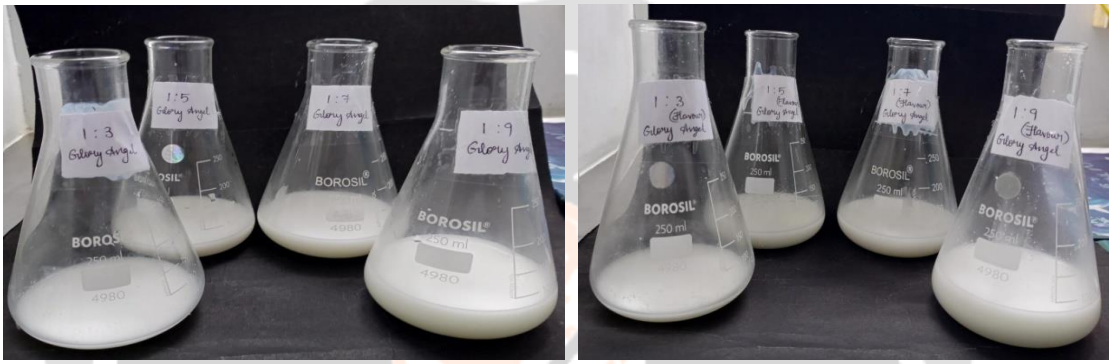

Fig4: Optimization of amount of water used for Almond milk extraction

Fig5: Coagulated product of Almond milk

Fig 6: Fermented Cheese

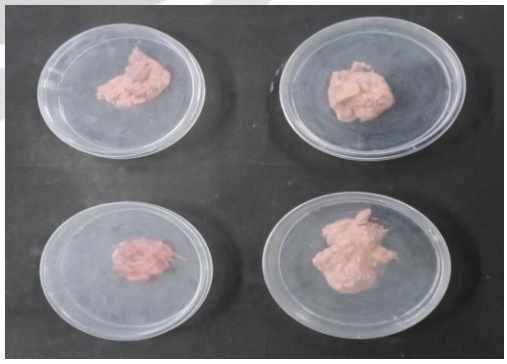

Fig 7: Fermented Cheese with Beetroot extract

Fig 8: Frozen cheese with beetroot extract flavour

Parameter	**Ratio of Almond and water**	**Concentration of protein in the unfermented coagulated product**	**Concentration of protein in the fermented cheese**	**Concentration of protein in the fermented cheese with Beetroot extract**
Protein	1:3	10.948mg/g	9.259mg/g	9.659mg/g
	1:5	10.586mg/g	9.365mg/g	9.865mg/g
	1:7	9.949mg/g	8.823mg/g	8.63mg/g
	1:9	9.099mg/g	8.495mg/g	8.995mg/g

Table 3: Estimation of Total Protein in Almond Cheese

Parameter	**Ratio of Almond and water**	**Concentration of lipid in the unfermented protein**	**Concentration of lipid in the fermented cheese**	**Concentration of lipid in the fermented cheese with Beetroot extract**
Lipid	1:3	20mg/g	19.8mg/g	20.2mg/g
	1:5	30mg/g	29.9mg/g	31mg/g
	1:7	50mg/g	49.8mg/g	50.5mg/g
	1:9	20mg/g	19.9mg/g	20.5mg/g

Table 4: Estimation of Lipid in Almond cheese

Parameter	Ratio of Almond and water	**Concentration of carbohydrate in the unfermented protein**	**Concentration of carbohydrate in the fermented cheese**	**Concentration of carbohydrate in the fermented cheese with Beetroot extract**
Carbohydrate	1:3	14.3mg/g	13.3mg/g	13.8mg/g
	1:5	13.2mg/g	12mg/g	12.6 mg/g
	1:7	11.8mg/g	10.1mg/g	10.7mg/g
	1:9	7.7mg/g	6.2 mg/g	6.7mg/g

Table 5: Estimation of Carbohydrate in Almond cheese

6. DISCUSSION:

Lactobacillus species were isolated on *Lactobacillus* MRS agar from different dairy products. The colonies were white creamy and translucent. A total of five *Lactobacilli* isolates were obtained. One bacterial isolate from Yakult (probiotic drink), 3 bacterial isolates were obtained from curd, and 1 bacterial isolate was obtained from Yoghurt.

A total of 5 bacterial isolates were subjected to DPPH assay for antioxidant activity. The antioxidant activity of lactic acid bacteria is associated with multiple health-protective effects. The percentage inhibition of the DPPH radical by the *Lactobacilli* isolates was calculated according to the formula of Yen and Duh (1994). All bacterial isolates exhibited Antioxidant activity and the *Lactobacillus* species (S1) isolated from Yakult exhibited the highest antioxidant activity when compared with other bacterial isolates (Table 1)

Some *Lactobacilli* strains are regarded as probiotic bacteria due to their ability to improve the health of hosts. Some *Lactobacilli* strains exerted antioxidant activity benefiting host health after they colonized and propagated in the human gastrointestinal tract.(Ren *et al* ., 2014). Mu *et al* reported that all 13 *Lactobacilli* strains tested showed antioxidant activity which correlates with my study.The Lactobacilli isolate L1 isolated from Yakult showed good antioxidant activity. Yakult is a fermented probiotic dairy drink prepared using *Lactobacillus casei. Lactobacillus casei* was selected for the preparation of Cheese analog. The vegan cheese was prepared from almond milk and beetroot extract was used as a flavouring agent.

The most important factor is the amount of water required to prepare milk as it affects the solid content of the milk, the quality, and the texture of coagulated proteins. The texture and moisture content of cheese are important factors as they affect the product acceptability (Rekha *et al.,* 2011). To optimize the almond and water ratio for milk extraction, different almond: water ratios were tested (1:3, 1:5, 1:7, and 1:9). The solid content of milk was observed to decrease significantly with an increase in the water content of milk which correlates with the study of Sharma *et al*. The optimum Magnesium chloride concentration for protein coagulation was 0.5%. (Sharma et al., 2018). Hence the same concentration of Magnesium chloride was added to the almond milk for coagulation.

The almond and water ratio of 1:3 was found to be optimum for the extraction of almond milk for cheese preparation. Due to the proteolytic activity of the probiotic strain, significant decrease in the protein content after fermentation(10.948 mg/g to 9.259 mg/g) was observed (Table 3). A slight increase in the protein content after mixing with the beetroot extract was observed. No significant change in the fat content was observed (Table 4) after fermentation which correlates with the study of Sharma *et al*.

The amount of carbohydraytes decreased significantly after fermentation from 14.3mg/g to 13.3mg/g (Table 5) after fermentation due to the utilization by microorganisms or production of organic acids by microorganisms. Kouroutas *et al*.(2006) observed similar results during the fermentation of cheese by *Lactobacillus casei*. The carbohydrate concentration was slightly increased after mixing the fermented cheese with beetroot extract and this could be due to the sugar content present in the beetroot extract. When the beetroot extract are mixed with fermented almond cheese, it would increase the nutritional value of the cheese product as it contains carbohydrates, protein, iron, vitamin C, vitamin A, magnesium, potassium and folate.

5. CONCLUSION:

Almond cheese prepared by fermenting the coagulated product of almond milk using *Lactobacillus casei* can be a good alternative to dairy products. The almond and water ratio of 1:3 was found optimum for the extraction of milk for cheese preparation from almonds. There is a significant decrease in the protein and carbohydrate content of the coagulated product of almond milk after fermentation. This is due to the proteolytic activity and fermentation of sugars by *Lactobacillus casei*. There is no significant change in the concentration of lipids in the almond-based vegan cheese. The beetroot extract added to the cheese will not only act as a flavoring agent but also enhance the nutritive value of the almond-based cheese as it is rich in iron, manganese, vitamin A, vitamin C, and potassium. Consequently, further studies are required from many perspectives to widen the range of nutritious end products.

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