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Development of a functional fermented peanut-based cheese analog using probiotic bacteria

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Abstract

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Cheese analogs are usually defined as products made using non-dairy proteins to produce a product similar to cheese. These products are increasingly popular due to their cost-effectiveness, health benefits, and simplicity of their manufacturing processes. Herein, attempts have been made to form a functional veg spread by using peanut and probiotic microorganism Lactobacillus rhamnosus NCDC18. The proportion of peanut seed and water for milk extraction was optimized based on the solid content of milk. Based on the water retention ability (WRA) and the solid and protein recovery of coagulated protein, the salt percentage for coagulation of peanut protein was optimized. Fermentation of coagulated protein by probiotic strain was done at 37°C for 24 h. A comparative analysis of physico-chemical properties such as moisture content, ash content, fat content, protein content, carbohydrate content, Vitamin C, antioxidant, titratable acidity, and pH was done before and after fermentation. For the extraction of milk with the desired amount of solid (5.5%), the optimum ratio of peanut and water was found to be 1:6. For the coagulation of peanut protein, the optimum coagulant (salt) was determined at 0.5%. The maximum solid recovery (51.7 ± 0.04) , protein recovery (69.21 ± 0.01) , and WRA (67.39 ± 0.03) were obtained with 0.5% magnesium chloride. No significant change was observed in the moisture, ash, fat, antioxidant, and vitamin C contents, while protein, carbohydrate, pH, and titratable acidity were found to change significantly before and after fermentation. Proteolysis of peanut protein by probiotic strain was found to be $61 \,\mu g/mg$. As the refrigerated storage period increased (0 to 15 days), a significant ($P \le 0.05$) decrease in cell viability and pH was observed. Key words: proteolysis, cell viability, peanut spread, probiotic, fermentation

Introduction

Cheese spread is a rich source of fats, which constitute around 40% of its dry mass, and, importantly, most of the fats in a cheese spread are saturated (Kong-Chan et al., 1991). These saturated fats in cheese and other dairy products have been associated with cardiovascular disease development, which is one of the leading causes of deaths globally (Bachmann, 2001). Due to its high nutritional value, functional properties, and affordable cost (Diarra et al., 2007), peanut (groundnut, *Arachis hypogaea*) is a rare crop with many potential uses. Biological effects of peanuts such as lowering blood cholesterol level, weight loss, anti-inflammatory, and anticancer have been recently studied and discussed (Rafiq et al., 2017). 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. Many thirdworld countries do not have an adequate supply of highquality proteins; therefore, the increasing demand for animal protein products, such as meat and milk, around the world has promoted the search of equivalent types of proteins (Diarra et al., 2007). In addition, the utilization of peanuts as a protein source offers the opportunity to alleviate protein deficiencies in vegan people. Peanuts can be consumed in raw, processed, and semiprocessed form. Peanut butter is an ideal substitute for milk butter having low calorie fat, high protein, and high

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fiber content. Peanut skins are a good source of phenolic compounds: they can be used to produce nutraceutical ingredients and antioxidants. Peanut proteins are equally nutritional as meat and egg proteins, but are affordable for most people (Zhao et al., 2012). Importantly, peanuts can also be a source of protein ingredients to serve in food fortification and food formulation (Wu et al., 2009).

Recently, probiotic-based foods are considered as the most up-surged area of research due to their health benefits (Sharma et al., 2017). Food industries are fortifying the food products with probiotic strains as they improve the food digestibility, texture, or shelf life (Wood 1997). They also contribute to the sensory profile of final products via improving flavor and texture (Pescuma et al., 2010). Regular consumption of probiotic microorganisms improves the lactose digestion, regulates the bowel function, stimulates the immune system, and inhibits the growth of pathogens (Ouwehand et al., 1998).

Peanut milk has already been used for the preparation of yogurt and cheese spread (fortified with animal milk) (Diarra et al., 2007). However, the preparation of functional cheese spread using 100% peanut milk and a probiotic microorganism has not been reported yet. Hence, the objective of this study was to prepare a cost-effective functional vegan cheese spread with a high biological potential in order to help overcome various problems such as lactose intolerance or protein deficiency diseases.

Materials and methods

Procurement of raw material, microbial culture, and growth medium

Peanut seeds used in the study were obtained from a local market of Jalandhar, Punjab, India, and stored in an airtight container at room temperature prior to processing. Lyophilized culture of probiotic strain (*Lactobacillus rhamnosus* NCDC18) was obtained from the National Dairy Research Institute, Karnal, Haryana, India. Lactobacillus MRS medium was procured from Himedia (India). For the revival of the lyophilized culture of probiotics, the medium was supplemented with 0.3 g L-cysteine/l (oxygen scavenging component to accelerate growth of probiotic strains) (Homayouni et al., 2008).

Optimization of the amount of water used for peanut milk extraction

Peanuts (200 g) were soaked in water (1 liter) at room temperature for 6 h. The soaked nuts were drained, and the outer layer was mechanically removed (dehulling) and rinsed with running tap water. Peanuts were ground with water in a high-speed blender in different ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, and 1:9). The peanut 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 peanut milk from residues.

Coagulation of peanut protein

Peanut 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 (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6%) were added to the peanut 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 500 g weight for 15 min. After pressing, the cloth was removed, and the pressed curd was stored at 4° C.

Determination of protein content, solid content, and WRA

Using the Kjeldahl method (Association of Official Analytical Chemists, AOAC 2010), protein content of the coagulated curd was determined. Based on the method described by Kao and coworkers (2003), solid content and WRA were determined.

Preparation of probiotic cheese spread

Following the method described by Sharma and coworkers (2017), the growth profile of probiotic strains (Lactobacillus rhamnosus NCDC18) in MRS-Cysteine medium was performed. The seed culture for inoculating peanut curd was prepared by inoculating lyophilized culture of Lactobacillus rhamnosus NCDC18 (1%) in MRS-Cysteine medium and then incubated statically at 37° C for 24 h. The primary seed culture (10% (v/v)) was then transferred into the secondary seed culture (MRS-Cysteine broth) and cultivated for 24 h at 37°C in static conditions. The secondary seed culture (10%, v/v) was again transferred to MRS-Cysteine broth and incubated at 37°C for 24 h and designated as a tertiary seed culture. After each 2 h interval, the whole cell culture fluid (1 ml) was withdrawn from the tertiary culture to monitor the growth profile of probiotic bacteria. Optical density was measured at 600 nm (OD of 0.6 corresponds to a ~9.00 log (10^9) CFU/ml according to Mc-Farland scale.

Fermentation of the tertiary seed culture was conducted for 12 h. After that, the whole cell culture fluid was centrifuged (10 000 rpm for 10 min 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 ressed curd. Fermentation was done at 37° C for 24 h. After 24 h, 1% salt and 1% black pepper solutions were added under aseptic conditions as flavoring agents and stored at 4°C in airtight bottles for further analysis.

Physico-chemical analysis of fermented peanut cheese analog

Fermented cheese analog was analyzed for various physico-chemical properties such as moisture content, ash content, total solids, pH, titratable acidity, fat, protein, and carbohydrates.

Using the methods given by AOAC (2010), moisture and ash contents were determined. The change in pH of the fermented cheese analog was determined using a digital pH meter (Thermo Scientific, Orion 2 Star pH Benchtop). Total solids were determined using a drying cheese sample (2 g) in an oven (100°C) for 16 h. Following the Soxhlet extraction method (AOAC 2010), fat content was determined. According to Kjeldahl method (AOAC 2010), protein content was determined.

The assessment of proteolytic activity of probiotic bacteria in the fermented peanut-based cheese analog

Using the *o*-phthaldialdehyde (OPA) test described by Pescuma and coworkers (2010), proteolytic activity of probiotic bacteria in the fermented sample was determined.

Storage study of fermented peanut cheese analog

After fermentation, the flavoring agents (black pepper and salt) were added to the fermented peanut-based cheese analog and packed in schott glass bottles. These samples were stored at refrigerated conditions (4°C) and assessed for microbial stability, pH, and titratable acidity during 15 days' storage.

Statistical analysis

Using a commercial statistical package, SPSS ver. 11.5 (SPSS Inc., Chicago, IL, USA), the analysis of a variance test was carried out. All results of the chemical analysis were recorded as a mean \pm SD of three replicates. Mean values were compared and significant differences were given using Duncan's LSD test ($P \le 0.05$).

Result and discussion

Optimization of water amount for peanut milk extraction

The most important factor is the amount of water required to prepare milk as it affects the solid content of milk, the quality, and the texture of coagulated proteins. Texture and moisture content of cheese are important factors as they affect the product acceptability (Rekha et al., 2011). To optimize the water and peanut ratio for milk extraction, different peanut:water ratios were tested (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, and 1:9). The solid content of milk was observed to decrease significantly with an increase in the water content of milk (Table 1). No significant change was observed in pH and titratable acidity of milk with an increased amount of water used. For the preparation of the cheese analog, a solid content (5.5%) of peanut milk prepared using 1:6peanut:water ratio was selected. Similar to our observations, a lower solid content led to a higher moisture content of coagulated proteins that resulted in a soft texture (Cai and Chang, 1997). Lim and coworkers (1990) reported that an increased solid content led to a decreased moisture content resulting in increased hardness of cheese.

Optimization of salt concentration (MgCl₂) for peanut protein coagulation

Based on several parameters such as the protein recovery rate, the solid recovery rate, and WRA, optimization of salt concentration (MgCl₂) for coagulation of peanut proteins was done. All tested parameters increased significantly as MgCl₂ concentration increased from 0.1 to 0.5%, but no significant change was observed as the concentration increased further to 0.6% (Table 2). The maximum protein recovery (69.21 \pm 0.01), solid recovery (51.7 \pm 0.04), and the highest WRA (67.39 \pm 0.03) were observed at 0.5% coagulant concentration. Therefore, 0.5% MgCl₂ was used for the coagulation of milk protein.

At lower $MgCl_2$ concentrations (0.1–0.4%), a lower protein and solid recovery can be related with the omission of non-coagulated proteins and other solids into the whey during the pressing step. Kao et al. (2003) observed similar results during tofu preparation for the coagulation of soy milk by calcium sulfate and reported that high concentrations of salt led to cross-linkage resulting in a too compact and porous structure of cheese

Peanut [gm]	Water [ml]	Milk [ml]	pН	TSS [°Bx]	Titrable acidity [%]
20	20	9 ± 0.7^{a}	$6.5\pm0.04^{\text{a}}$	$20\pm0.55^{\rm a}$	$0.61\pm0.04^{\text{a}}$
20	40	$25\pm1^{\rm b}$	$6.5\pm0.02^{\text{a}}$	$18\pm0.23^{\rm b}$	0.61 ± 0.06^{a}
20	60	$51 \pm 1.5^{\circ}$	$6.5\pm0.05^{\text{a}}$	$16\pm0.15^{\circ}$	$0.61\pm0.08^{\text{a}}$
20	80	$72\pm2^{\text{d}}$	$6.5\pm0.02^{\text{a}}$	$13\pm0.19^{\rm d}$	$0.61\pm0.02^{\rm a}$
20	100	94 ± 2.5^{e}	$6.5\pm0.06^{\text{a}}$	6 ± 0.45^{e}	$0.61\pm0.01^{\text{a}}$
20	120	$114\pm3^{\rm f}$	$6.5\pm0.02^{\text{a}}$	$5\pm0.27^{\mathrm{f}}$	$0.61\pm0.03^{\text{a}}$
20	140	$135\pm3.5^{\rm g}$	$6.5\pm0.03^{\text{a}}$	$4\pm0.21^{ m g}$	$0.61\pm0.03^{\text{a}}$
20	160	$157\pm3^{\rm h}$	$6.5\pm0.06^{\text{a}}$	$3\pm0.19^{\rm h}$	$0.61\pm0.02^{\text{a}}$
20	180	$178\pm2.5^{\rm i}$	$6.5\pm0.04^{\text{a}}$	2 ± 0.16^{i}	$0.61\pm0.04^{\rm a}$

Table 1. Optimization of peanut and water ration for milk extraction

Mean values with different superscripts on the same column differ significantly (Duncan's LSD test, P < 0.05)

Table 2. Effect of different salt concentration on percentage recovery of protein, solid and WRA

Salt [%]	Protein recovery [%]	Solid recovery [%]	WRA [%]
0.1	$59.78 {\pm} 0.02^{a}$	38.16 ± 0.03^{a}	55.7 ± 0.03^{a}
0.2	$61.20{\pm}0.04^{\mathrm{b}}$	40.25 ± 0.01^{b}	57.15 ± 0.05^{b}
0.3	$63.24{\pm}0.02^{ m c}$	$45.91 \pm 0.02^{\circ}$	$60.82{\pm}0.02^{ m c}$
0.4	$67.09{\pm}0.02^{\rm d}$	$49.18{\pm}0.02^{\rm d}$	$64.31{\pm}0.01^{\rm d}$
0.5	$69.21 \pm 0.01^{ m e}$	$51.7 \pm 0.04^{ m e}$	67.39 ± 0.03^{e}
0.6	$69.20 {\pm} 0.05^{ m e}$	$51.73 \pm 0.03^{ m e}$	$67.4 \pm 0.02^{ m e}$

Mean values with different superscripts on the same column differ significantly (Duncan's LSD test, P < 0.05)



Fig. 1. Growth curve of probiotic culture in MRS-Cysteine medium

spread, while smaller salt concentrations led to discontinuous fragments and large holes.

Growth profile of probiotic strain

The growth profile of probiotic bacteria clearly indicated that the exponential phase started from the 4^{th} hour and remained till the 20th hour of incubation. Afterward, the growth of probiotic bacteria entered the decline phase (Fig. 1). It was also observed that absorbance at 600 nm (0.38 to 0.72) doubled between the 10th and the 12th hour. For *Lactobacillus rhamnosus* NCDC18 at 12 h interval, an absorbance of 0.7245 was obtained, indicating a cell density of about 9.00 log CFU/ml according to the Mc-farland scale. Therefore, 12 h of fermentation was considered as the optimum fermentation time and was used for analyses. Sharma et al. (2017) observed similar results during growth profiling of a mixed *Lactobacillus acidophilus* NCDC 291 and *Lactobacillus bulgaricus* NCDC 304 culture in MRS-Cysteine medium.

Preparation of probiotic cheese spread

With the aim to obtain the required levels of viable cells per the probiotic requirement $(10^6-10^7 \text{ CFU/ml})$, seed culture of *Lactobacillus rhanmosus* (5 ml) with cell density of $3.75 \times 10^{11} \text{ CFU/ml}$ was added to the pressed

curd (100 g). After 12 and 18 h of fermentation, the pH value of pressed curd changed from 5.3 ± 0.02 to 5.1 ± 0.03 and 4.8 ± 0.02 , respectively, which indicated a slow growth of microorganisms in the pressed peanut curd. After 24 h of fermentation, the pH value decreased further to 4.2 ± 0.26 , which confirmed the active growth of probiotic microorganisms and production of lactic acid. Thereafter, the fermentation had been stopped as a lower pH can be detrimental to probiotic microorganisms. The results are consistent with those of an early study reported by Angelov et al. (2006) for the fermentation of oat-based mash using Lactobacillus plantarum B25 and Sharma et al. (2017) during whey protein fermentation by a mixed culture of Lactobacillus acidophilus NCDC 291 and Lactobacillus bulgaricus NCDC 304. Thenceforward, the fermented cheese was mixed with flavoring agents (1% salt and 1% black pepper solutions), under aseptic conditions. Neysens et al. (2003) reported that a high concentration (30 g/l) of salt can lead to detrimental effect on bacterial growth due to the presence of specific ions and water binding properties. After adding a flavoring agent, the fermented cheese was stored at 4°C and analyzed for physico-chemical analysis in 5 days' interval during 15 days of storage.

Comparative analysis of physio-chemical properties of coagulated peanut proteins before and after fermentation

Table 3 gives the results of a comparative analysis of physio-chemical properties of coagulated peanut proteins before and after fermentation. No significant $(P \le 0.05)$ change in the moisture, ash, fat, antioxidant, and vitamin C content before and after fermentation with Lactobacillus rhamnosus was observed. The amount of carbohydrates (%) decreased significantly after fermentation (from 7.72 ± 0.03 to 5.34 ± 0.04) due to the utilization by microorganisms or production of organic acids by microorganisms. Kourkoutas et al. (2006) observed similar results during the fermentation of cheese by Lactobacillus casei. Due to the proteolytic activity of the probiotic strain, a significant change in the protein content after fermentation (from 23.38 ± 0.05 to 20.25 ± 0.11) was observed. The pH of the fermented cheese analog also decreased significantly (from 5.4 ± 0.08 to 4.26 ± 0.28) due to the production of organic acids by the probiotic strain, and accordingly, the titratable acidity was found to increase (from 0.88 ± 0.02 to 0.95 ± 003). These results are consistent with those which Panghal et al. (2017) obtained during the fermentation of beet-root juice by *Lactobacillus rhamnosus*.

Proteolytic activity of fermented cheese analog

Proteolytic activity of fermented cheese analog was expressed as OPA (o-phthaldialdehyde) value (µg leucine released per gm of fermented sample relative to the non-fermented sample). OPA value was found to increase from 45 µg to 61 µg leucine per mg of fermented sample after 12 h of fermentation. The low proteolytic activity during fermentation can be related to the consumption of free amino acids by microorganisms (Pescuma et al., 2010) and a slow growth of the probiotic strain in coagulated protein solution. The slow growth of the probiotic strain may be due to the decreased water activity in the fermentation medium. These results are consistent with the data obtained by Sharma et al. (2017) during whey protein fermentation by mixed culture of Lactobacillus acidophilus NCDC 291 and Lactobacillus bulgaricus NCDC 304. During that study, OPA value was found to change from 67.1 to 98.8 µg/ml leucine. Pescuma et al. (2010) reported a similar study during whey-based functional beverage preparation using lactic acid bacteria.

Storage study of fermented cheese analog

During 15 days of 4°C storage of the peanut cheese analog, the viable count of probiotic bacteria decreased (from 2.5×10^{10} to 2.78×10^{6} CFU/ml). These results are in accordance with the results of the research conducted by Sharma et al. (2017) who also reported a decline in the total viable count (from 8.5×10^{10} to 9.5×10^{8}) of Lactobacillus acidophilus and Lactobacillus bulgaricus of whey- and oat-based probiotic beverage stored at $4 \pm 1^{\circ}C$ for 15 days. Due to the production of organic acids by probiotic microorganisms, a significant decrease in pH $(5.98\pm0.08$ to 3.99 ± 0.06 , Table 4) was also observed for fermented samples as storage period increased (0 to 15 days). Similarly, a significant ($P \le 0.05$) increase in titratable acidity $(0.96 \pm 0.03 \text{ to } 0.99 \pm 0.01)$ was observed for fermented samples as the storage period increased (0 to 15 days). The results of our study are consistent with those by Angelov et al. (2015) where the number of viable cells of probiotic bacteria also decreased from 7.3×10^{10} to 9.3×10^{9} in oat-based fermented product

Parameters [%]	Unfermented coagulated protein	Fermented coagulated protein	
Ash	$1.55\pm0.01^{\rm a}$	$1.57\pm0.01^{\texttt{a}}$	
Moisture	65.74 ± 0.03^{a}	$65.73\pm0.03^{\text{a}}$	
Fat	$22.3\pm0.17^{\rm a}$	$22.4\pm0.42^{\texttt{a}}$	
Protein	23.38 ± 0.05^{a}	$20.25\pm0.11^{\text{b}}$	
Carbohydrate	$7.72\pm0.03^{\rm a}$	$5.34\pm0.04^{\text{b}}$	
Antioxidant	$6.47\pm0.05^{\rm a}$	6.46 ± 0.04^{a}	
Vitamin C	$0.27\pm0.02^{\text{a}}$	$0.28\pm0.03^{\text{a}}$	
Titrable acidity	$0.88\pm0.02^{\rm a}$	$0.95\pm0.03^{\rm b}$	
pН	$5.9 \pm 0.08^{\mathtt{a}}$	$4.26\pm0.28^{\rm b}$	

Table 3. Physico-chemical analysis of unfermented and fermented coagulated protein

Table 4. Effect of refrigerated storage on pH, TA and Viable count

	Sample A	Sample B	Sample C	Sample D	Sample E
pН	$5.98\pm0.08^{\text{a}}$	$4.26\pm0.28^{\text{b}}$	$4.24\pm0.04^{\rm c}$	$4.13\pm0.02^{\rm \ d}$	$\boldsymbol{3.99\pm0.06}^{e}$
Titrable acidity [°SH]	$0.88\pm0.02^{\text{a}}$	$0.95\pm0.03^{\text{b}}$	$0.96\pm0.03^{\rm b}$	$0.97\pm0.07^{\text{d}}$	$0.9\pm0.01^{\rm e}$
Viable count	null	$2.5 imes 10^{10}$	$2.34 imes10^{10}$	$3.2 imes 10^7$	$2.78 imes 10^6$

Mean values with different superscripts on the same row differ significantly (Duncan's LSD test, P < 0.05); where, Sample A – coagulated protein, Sample B – fermented sample without flavouring agents, Sample C – fermented sample with 1% salt and 1% black pepper, Sample D – sample C after 7 day refrigerated storage, Sample E – sample C after 15 days storage

after 21 days' storage. A reduction in the viable count of microorganisms during the storage period can be connected with lower pH values of the solution as pH smaller than 4.2 can be detrimental to the probiotic growth.

Conclusions

The results obtained in the present study have shown that cheese spread analog prepared from peanuts can be an alternative to the dairy products. The fermentation with probiotic strain *Lactobacillus rhamnosus* improved the functionality of the product. The peanut and water ratio of 1:6 was found optimum for the extraction of milk for cheese preparation from peanuts. Based on WRA and solid and protein recovery, optimum magnesium chloride concentration for peanut protein coagulation was 0.5%. During the storage period of 15 days, the viable count of probiotic bacteria in fermented cheese analog was found to decrease from 2.34×10^{10} to 2.78×10^{6} , which shows that product functionality decreased with storage period.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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