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Application of Bacillus toyonensis as a Novel Starter Culture on Nutritional Quality and Probiotic Viability of Fermented Carrot Pickles During Refrigerated Storage

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Abstract

This study evaluated the impact of inoculated fermentation (IF) using a novel *Bacillus* strain and natural fermentation (NF) on the physicochemical, microbial, antioxidant, and sensory characteristics of carrot pickle during 13 days of refrigerated storage (4°C). The isolated *Bacillus* strain was identified *Bacillus toyonensis* by 16s rRNA sequencing. The IF sample showed decreased pH, total sugar, and total soluble solids (TSS), and increased titratable acidity compared to the NF sample. Color (L* and b*) values of both samples decreased during the storage period. The viable count of *Bacillus* in the IF sample remained above 10^6 CFU/g, whereas the NF sample lower viable count end of 8 days of storage. The total phenolic content (TPC), carotenoid content and antioxidant capacity (DPPH and FRAP) increased in the IF sample, while decreased the carotenoid and antioxidant capacity and increased the TPC in the NF sample at end of storage period. Sensory evaluation further revealed that inoculated fermentation was significantly superior in terms of appearance, fermented flavor, spiciness, texture, and overall acceptability compared to natural fermentation. These findings indicate that using *Bacillus toyonensis* strain HSTU FPP-1 serves as an effective starter culture to enhance the quality, antioxidant potential, and probiotic functionality of carrot pickles.

1. Introduction

Fermentation is an ancient preservation and processing technique that has been practiced for thousands of years [1]. It has been widely used to extend the availability of seasonal vegetables because of enhancing their nutritional value and sensory qualities. Moreover, fermented foods containing live microorganisms are gaining recognition for their benefits to gut health. Most fermentation includes lactic acid bacteria (LAB) as major fermentation microbes but recent trends the growing application of *Bacillus* spp in fermented food. *Bacillus* species, including *Bacillus toyonensis*, exhibits probiotic potential through its endospore-forming ability, which confer resistance to harsh environmental conditions and ensure survival during storage and passage through the gastrointestinal tract [2]. *Bacillus toyonensis*, a member of the *Bacillus cereus* group, is characterized by its non-toxigenic and non-pathogenic properties, making it a promising candidate for functional food development. Traditionally, dairy-based products such as yogurt, butter, cheese and fermented milk drinks have been the most common carriers of probiotic microorganism. However, the increase of lactose intolerance, dyslipidemia, and allergies to milk proteins have become significant barriers to the development of dairy based probiotic products [3]. The demand for non-dairy probiotic products derived from fruits and vegetables is increasing, driven by lifestyle changes such as vegetarianism, veganism, and dairy allergies, as well as a growing focus on nutrition-based therapeutic diets [4]. Fermented fruits and vegetables have grown in popularity due to their ability to regulate gut microbiota, inhibit pathogens, and provide immunomodulatory, antidiabetic, anti-obesity, and anticancer benefits [5]. A variety of vegetable such as cabbage, radish, cucumber, broccoli, carrot are commonly fermented into products like Kimchi, Paocai, Khalpi, Sunki, and Kanji, have been used as a potential source of probiotic [6]. Among the different vegetables, Carrots (*Daucus carota*)

a member of the Apiaceae family, one of the most consumed and well-known root vegetables in the world [7].

Widely consumed as a vegetable, carrots are recognized for their high carotenoid content, key vitamins (B1, B2, B6, B9, C, and K), dietary fiber, and antioxidant properties [8]. These compounds are associated with multiple biological and medicinal benefits, notably immune system modulation, enhancement of blood circulation, and improved cardiovascular performance [9].

On the other hand, the rich flavor profiles and potential health benefits of spontaneous vegetable fermentations are driving renewed interest in this practice [10]. Currently, spontaneous fermentation remains a widely practiced method for vegetable preservation due to its simplicity and low cost. However, this method depends on the microorganism naturally present on the surface of raw materials. As drawbacks, spontaneous fermentation requires a long duration, which can lead to inconsistent organoleptic characteristics. [11]. It also increases the risk of spoilage, pathogenic bacteria, and formation of harmful biogenic amines and polyamines. Drašković Berger et al. [12] and Torres et al. [13] report that the use of specific starter culture keeps the microbiological stability, sensory qualities, and product quality constant, helps control fermentation, and keeps vegetables from deteriorating.

Several studies carried out the production and functional characteristics of fermented carrot juice, mixed carrot juice, and carrot pulp by the starter culture of *Lactobacillus.* Yue Xu et al. [16] and Mauro et al. [17] investigated the fermentation of carrot and mixed carrot juice using *Lactobacillus gasseri* and *Lactobacillus reuteri*, demonstrating improved microbiological stability and elevated antioxidant capacity during storage. Similarly Zhuo Liu et al. [18] found that fermentation of carrot pulp with various strains effectively enhances sensory qualities, increases antioxidants and bioactive content, and beneficially modulates metabolic pathways, providing a strong foundation for health-promoting fermented carrot products. However, most existing studies have focused on producing of fermented carrots juice, mixed carrot juice, and carrot pulp using various LAB strains. There is growing interest in the application of *Bacillus* species for plant-based fermentation. Bacillus species have been successfully employed in fermented food such as Natto, Cheonggukjang, Sufu, Hawaijar, [19].There is a significant gap in the development of fermented carrots pickle using starter culture of *Bacillus* species. Therefore, the current study is to evaluate the impact of both natural and starter culture fermentation on the microbial viability, antioxidant activity and sensory attributes of fermented carrots pickle using a species of *Bacillus* after fermentation during refrigerated storage.

2. Materials and Methods

2.1 Preparation of carrot pickle and isolation of *Bacillus* spp

Fresh carrots (*Daucus carota*) and spice (Mustard seeds, garlic, ginger, chili flakes, black pepper, and cloves) were purchased from a local market in Dinajpur, Bangladesh. Carrots were washed with potable

water containing 2% (w/v) sodium bicarbonate ($NaHCO_3$) and soaked for 30 minutes to reduce surface microbial load. Afterwards, the carrots were peeled and blanched in boiling water for 5 minutes and shredded using a mechanical grater. The shredded carrots were then mixed with 2% (w/w) coarse salt and left to rest for 1 hour at ambient temperature to facilitate moisture reduction through osmotic action. A spice blend comprising mustard seed 2% (w/w), garlic 2% (w/w), ginger 2% (w/w), chili flakes 0.5% (w/w), black pepper 0.12% (w/w) and clove (two pinches) were thoroughly mixed into the 100g shredded carrots. After that, 200 g sample was transferred into sterilized glass jars. Then sample was incubated at 37°C under anaerobic conditions for 48 hours. After incubation,10 g of the sample was blended in 90 mL of sterile 0.85% (w/v) peptone water using a bag mixer (stomacher). The sample serially diluted up to 10^{-6} and spread onto de Man, Rogosa, and Sharpe (MRS) agar (Hi-Media, Mumbai, India) and incubated at 37°C for 48 hours. Colony was preliminary selected based on Gram staining, catalase testing, and evaluation of colony characteristics and growth behavior.

2.2 Identification of isolated bacteria by PCR amplification of 16s rRNA Gene

The isolated bacteria were molecularly identified by PCR amplification of the 16S rRNA gene from extracted genomic DNA.PCR amplification (Gene Atlas, model: G2, Astec, Japan) was carried out using universal 16S rRNA primers 27F and 1492R in a PCR master mix system. The amplification products were verified through 1% agarose gel electrophoresis. Sequencing of the purified PCR amplicons was carried out by Apical Scientific Institute, Malaysia. The nucleotide sequences obtained were submitted to the NCBI (National Centre for Biotechnology Information) GenBank database, and accession numbers were assigned PZ049891 (https://www.ncbi.nlm.nih.gov/nuccore/3199960019). Sequence similarity analysis was performed using the BLAST tool, which revealed the highest homology with *Bacillus Toyonensis*. Furthermore, the MEGA 11 software was used to build a phylogenetic tree (Fig. 2A) with 1,000 bootstraps.

2.3 Fermentation process of carrot pickle

2.3.1 Sarter culture preparation

Starter culture prepared for fermentation of carrot pickle, following the method of [20] with slight modifications. The selected *Bacillus* strain was cultivated in MRS Borth and incubated for 18 hours at 37°C. The culture was centrifuged at 2743×g for 15 minutes at 4°C using a refrigerated centrifuge. The resultant pellets were resuspended in an equivalent volume of sterile saline followed by twice washed with sterile 0.85% (w/v) saline solution. To standardize the inoculum, the optical density (OD_{600}) of the suspension was determined using a UV-Vis spectrophotometer (Wincom UV-1720, China) and set to 1.00, or approximately to 10^5-10^7 CFU/mL.

2.3.2 Fermentation of carrot pickle

The carrot pickle was prepared using the procedure outlined in Section 2.1. For the fermentation, two experimental groups were prepared: Group 1: Naturally Fermented carrot pickles (NF), without added starter culture, Group 2: Inoculated Fermented carrot pickles (IF), supplemented with suspension of *Bacillus* (OD_{600} ≈ 1.0 ± 0.01). The fermentation was performed under anaerobic conditions for 48 hours at 37°C. Following fermentation, amples were stored at 4°C under refrigeration until subsequent analyses were carried out.

2.4 Determination of physiochemical properties of fermented carrot pickle

The pH of the fermented carrot pickle was measured using a digital pH meter (Jenco 6177M USA). A refractometer (HI-96801, Italy) was used to measure total soluble solids (TSS) and results reported in degrees Brix (°Brix).Titratable acidity (as lactic acid) was determined according to the method described by [21] and titratable acidity was calculated using the following formula:

$$\%TA= \frac{0.1\times\ Volume\ (mL)\ NaOH\times\ 90\times\ 100}{Sample\ weight\ (g)\times\ 1000}$$

Where, Normality of NaOH = 0.1N, Acid factor (Equivalent weight of lactic acid) = 90 g/mol

2.4.1 Total sugar content

Following the method of all [22] with minor modifications, total sugar content was measured using the phenol–sulfuric acid assay. Briefly, 5g fermented carrot pickle was blended and extracted with 30 mL of 80% ethanol (solid to solvent ratio 1:6, w/v) by agitated at 120 rpm for 12 hours at 25°C. The extract was centrifuged at 1935×g for 10 min and filtered through Whatman Filter No. 41. The sample extract was mixed with 5% (w/v) phenol solution in a 1:1 ratio. Subsequently, 5 mL of concentrated sulfuric acid was carefully added to the mixture. The mixture was vortexed, then incubated for 20 min at 25°C in a water bath before absorbance was read at 490 nm on a UV-Vis spectrophotometer (Wincom UV-1720, China). The total sugar content was calculated from a standard curve using glucose and expressed as grams of glucose equivalent per 100 grams (GE/100g) of sample

2.4.2 Color properties of fermented carrot pickle

Color attributes of the fermented carrot pickle including L* (lightness), a* (red to green), and b* (yellow to blue) were analyzed using a colorimeter (BIOBASE BCM-200, China). The sample was uniformly spread as a 5 mm thick layer in a transparent glass Petri dish and color measured.

2.5 Viable cell count of *Bacillus*

The method described by [23] with slight modifications to determine the viable count of *Bacillus*. A 10 g of fermented carrot sample was homogenized with 90 mL of sterile 0.1% (w/v) peptone water in a stomacher bag using a bag mixer. Serial dilutions were prepared up to 10^{-7}, and aliquots were spread-

plated onto de Man, Rogosa and Sharpe (MRS) agar. Following incubation of the plates at 37°C for 48 hours, CFU values were determined using the equation below:

Log CFU/g= $log\frac{No\ of\ colonies \times Dilution\ fractor}{sample\ weight}$

2.6 Bioactive compounds of fermented carrot pickle

2.6.1 Extraction of bioactive compounds

Bioactive compounds were extracted using a solvent extraction technique following the method [17] with slight modifications. Briefly, 5 g of fermented carrot pickle was first blended, then mixed with 30 mL of 80% ethanol (solid to solvent ratio 1:6, w/v) and agitated at 120 rpm for 12 hours at 25°C. Then the sample was centrifuged at 1935×g for 10 min and filtered through Whatman Filter No. 41. The resultant extract was collected for further analysis.

2.6.2 Total phenol content

Total phenolic content (TPC) of the fermented carrot pickle was determined using the Folin-Ciocalteu assay, following the protocol of [24] with slight modifications. The extract (0.5 mL) was mixed with 0.5 mL of Folin-Ciocalteu's reagent, then 1 mL of 7.5% sodium carbonate and 8 mL of distilled water were added. The mixture was vortexed and left to incubate in the dark at room temperature for 35 minutes, followed by centrifugation at 4000×g for 10 minutes. Supernatant absorbance was recorded at 725 nm with a microplate reader (Microplate Reader 282014006, China), and total phenol content was determined from a gallic acid standard curve, expressed as milligrams gallic acid equivalent per gram (mg GAE/g) of sample

2.6.3 DPPH radical scavenging activity

Free radical scavenging activity of fermented carrot sample was determined following the method of [25] with minor modification. Briefly, 1 mL of the fermented carrot extract was mixed with 1.9 mL of DPPH• solution (0.0118 g/100 mL in 80% MeOH). After a 30-minute incubation in the dark at room temperature, the absorbance of the mixture was read at 517 nm on a microplate reader (Microplate Reader 282014006, China). Radical scavenging capacity was derived from a Trolox calibration curve and reported as micromoles of Trolox equivalent per gram (μmol TE/g) of sample.

2.6.4 Ferric reducing antioxidant power

The reducing power of fermented carrot pickles was determined according to [26] method with slight modifications. A 0.5 mL portion of the sample extract was mixed with 2.5 mL of 1% (w/v) potassium ferricyanide [$K_3Fe(CN)_6$], followed by incubation in a water bath at 50°C for 20 minutes. The solution was then mixed with 2.5 mL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 2790×g for 10 minutes. A volume of 2.5 mL of the resulting supernatant was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride ($FeCl_3$). After thorough mixing, the absorbance value was determined at 700 nm using a microplate spectrophotometer (Microplate Reader 282014006, China).

The results were represented as micromoles of Trolox equivalents per gram (μmol TE/g) of sample following calculated using a Trolox standard calibration curve.

2.6.5 Total carotenoid content

The total carotenoid content of fermented carrot pickle samples was determined spectrophotometrically following the method of [27] with slight modifications. In brief, 1 g of fermented carrot pickle taken and made the paste using mortar and pestle with 20 mL of acetone and incubated in a water bath at an ambient temperature for 12 hours with 120 rpm. The sample Then filtered through Whatman No. 41 filter paper, and the filtrate was transferred into a separatory funnel. Subsequently, 30 mL of petroleum ether and 5 mL of distilled water were added, followed by a pinch of anhydrous sodium sulfite (Na_2SO_3). After mixing and allowing the phases to separate, the upper organic layer (petroleum ether phase) containing carotenoids was collected, and the final volume was recorded. The absorbance of the colored extract was measured at 450 nm using a UV-Vis spectrophotometer (Wincom UV-1720, China). The following formula was used to calculate the total carotenoid content [28]:

Carotenoids content(μg/g) = $$\frac{A\times\ v(ml)\times\ 10^4}{A_{1cm}^{1\%}\times\ W}$$

Where A= Absorbance, V = volume of the petroleum ether extract (mL), $A_{1cm}^{1\%}$ = specific extinction coefficient (2592 for carotenoids in petroleum ether), W = sample weight (g).

2.7 Sensory evaluation

A sensory evaluation was conducted after the completion of carrot pickle fermentation. Thirty semi-trained panelists aged 20–35 years were recruited from the Hajee Mohammad Danesh Science and Technology University community (HSTU). The fermented carrot pickle sample was randomly presented to the panelists. Panelists were given drinking water to rinse their mouths between evaluations of each sample. Each sample was rated using a 9-point hedonic scale (1 = extremely dislike, 9 = extremely like) across five sensory attributes: appearance, fermented flavor, spiciness, texture, and overall acceptability.

2.8 Statistical analysis

Values are presented as means ± standard deviation (SD), and all statistical analyses were carried out using IBM SPSS Statistics (version 26.0). Significant differences among groups were evaluated using one-way analysis of variance (ANOVA). Duncan's multiple range test was used for post hoc comparisons when ANOVA revealed significant differences to identify specific group differences. Additionally, paired t-tests were conducted to evaluate differences between the IF and NF samples. For every test, $P < 0.05$ was used as the significance level.

3. Results and Discussion

3.1 Physiochemical properties of fermented carrot pickles during refrigerated storage

Table 1 shows the changes in pH, titratable acidity, TSS, and total sugar during the refrigerated storage of fermented carrot pickle. The pH of the fermented carrot pickle decreased from 5.94 to 3.63 over the storage period. The pH of the inoculated fermented (IF) carrot pickle was significantly lower (3.54) compared to the naturally fermented (NF) carrot pickle (3.79) after fermentation (0 days). The lower pH of the IF sample could be due to the higher lactic acid produced by the greater presence of *Bacillus* in the IF sample (Fig. 1). The pH value sometimes decreased and increased of fermented food by the probiotic bacteria [29] and its directly corresponding to the lactic acid content produced during the growth of probiotic [30]. The pH values increased significantly, ranging from 3.54 to 3.69 for the IF sample and from 3.79 to 3.95 for the NF sample during storage (days 0–8). This may be a fermentation process that had reached the stationary phase. pH decreased significantly to 3.63 in IF samples and 3.87 in NF samples despite lower *Bacillus* counts (Fig. 1) at the end of storage.

Table 1
Changes in physicochemical properties of fermented carrot pickles during refrigerated storage

Storage	Samples	pH	Titratable acidity (%)	Total sugar (g/100 g)	TSS (ºBrix)
Before fermentation	Carrot pickle	5.94 ± 0.02^{a}	0.17 ± 0.01^{a}	9.69 ± 0.27^{a}	7.07 ± 0.12^{a}
After fermentation (0 day)	IF	$^{A}3.54 \pm 0.03^{d}$	$^{A}0.91 \pm 0.02^{b}$	$^{A}6.20 \pm 0.11^{b}$	$^{A}5.43 \pm 0.06^{b}$
	NF	$^{B}3.79 \pm 0.02^{d}$	$^{B}0.83 \pm 0.01^{c}$	$^{A}6.43 \pm 0.36^{b}$	$^{A}5.63 \pm 0.06^{b}$
4 days	IF	$^{A}3.65 \pm 0.01^{bc}$	$^{A}0.89 \pm 0.01^{b}$	$^{A}0.85 \pm 0.34^{e}$	$^{A}4.03 \pm 0.06^{d}$
	NF	$^{B}3.88 \pm 0.01^{c}$	$^{B}0.75 \pm 0.01^{b}$	$^{B}1.67 \pm 0.26^{d}$	$^{B}4.33 \pm 0.06^{d}$
8 days	IF	$^{A}3.69 \pm 0.03^{b}$	$^{A}0.90 \pm 0.02^{b}$	$^{A}3.49 \pm 0.71^{d}$	$^{A}3.93 \pm 0.06^{d}$
	NF	$^{B}3.95 \pm 0.02^{b}$	$^{A}0.85 \pm 0.01^{cd}$	$^{A}2.90 \pm 0.14^{c}$	$^{B}4.83 \pm 0.06^{c}$
13 days	IF	$^{A}3.63 \pm 0.01^{c}$	$^{A}0.89 \pm 0.04^{b}$	$^{A}5.26 \pm 0.28^{c}$	$^{A}4.90 \pm 0.17^{c}$
	NF	$^{B}3.87 \pm 0.01^{c}$	$^{A}0.87 \pm 0.03^{d}$	$^{A}3.77 \pm 1.39^{c}$	$^{A}4.83 \pm 0.15^{c}$

Where.

IF = Inoculated fermented carrot pickle.

NF = Naturally fermented carrot pickle.

All results expressed in mean ± standard deviation.

$^{a–e}$ different superscript letter within a column indicates significant differences ($p < 0.05$) among the same samples during storage.

$^{A–B}$ different superscript letter within a column indicates significant differences ($p < 0.05$) between samples at the specific storage time.

The titratable acidity of fermented carrot pickle ranged from 0.17% to 0.91% throughout storage period. The IF sample exhibited higher acidity (0.91%) compared to the NF sample (0.83%) after fermentation.

Similar acidity ranges (0.14% to 0.98%) were observed by [31] in fermented carrot juice. The higher acidity of the IF sample could be attributed to the inoculated starter culture, which produces more lactic acid compared to the NF sample. The acidity of NF samples (0.75%) significantly decreased, possibly due to an increase in pH after 4 days of storage. There were no significant differences in the acidity of both samples at the end of the storage period. This could be due to the bacteria transitioning into the stationary phase, where growth and lactic acid production slow down.

The total sugar and TSS of both IF and NF samples declined significantly after fermentation at 0 day. Total sugar decreased from 9.69 g/100 g to 0.85 g/100 g and TSS from 7.07°Brix to 4.03°Brix, respectively, in the IF sample, while total sugar decreased from 9.69 g/100 g to 1.67 g/100 g and TSS from 7.07°Brix to 4.33°Brix, respectively, in the NF sample. The higher reduction in the IF sample may be attributed to conversion of sugar into lactic acid and consumption of sugar for the growth of *Bacillus* [32]. However, during the rest of the storage period, the total sugar content (0.85 to 5.26 g/100 g for IF and 1.67 to 3.77 g/100 g for NF) and TSS values (4.03 to 4.90°Brix for IF and 4.33 to 4.83°Brix for NF) exhibited a significant increase, The increased in total sugar might be due to degradation of carrot polysaccharides [33]. Similarly, TSS increased likely due to the increased sugar content.

3.2 Color properties of fermented carrot pickles during refrigerated storage

Color properties of fermented fruits and vegetables significantly affect consumer acceptance. The color of the fermented carrot pickle was evaluated using the L*, a*, and b* values as presented in Table 2. Both IF and NF carrot pickles exhibited significant decreases in L* (IF: 54.61–48.90; NF: 54.61–49.48), a* (IF: 22.46–19.96; NF: 22.46–20.49) and b* (IF: 40.01–32.46; NF: 40.01–33.98) up to 8 days of storage as compared to the before fermentation, then increased all values rest of storage period. The reduction in L* (luminosity) could be due to oxidative activity, which may initiate the enzymatic breakdown of color compounds [34]. The low pH in the fermentation and storage environment can enhance the chemical oxidation of phytochemicals, which reduces the a* value (redness) in carrot pickle.[35]. The changes of b* (yellowness) value of carrot pickle could be due to degradation of carotenoids, which aligns with results reported in an earlier study on fermented carrot pulp [36].

Table 2
Changes in color parameters (L*, a*, b*) of fermented carrot pickles during refrigerated storage

Storage	Samples	L*	a*	b*
Before fermentation	Carrot pickles	54.61 ± 1.50^{a}	22.46 ± 0.49^{ab}	40.01 ± 0.40^{ab}
After fermentation (0 day)	IF	$^{NS}50.82 \pm 0.74^{bc}$	$^{NS}23.37 \pm 1.58^{a}$	$^{NS}38.83 \pm 0.41^{b}$
	NF	53.20 ± 0.94^{ab}	24.16 ± 0.24^{a}	42.02 ± 1.06^{a}
4 days	IF	49.19 ± 0.53^{c}	21.18 ± 2.41^{ab}	36.68 ± 1.02^{b}
	NF	49.48 ± 0.82^{c}	22.54 ± 2.48^{ab}	37.52 ± 2.25^{c}
8 days	IF	48.90 ± 0.74^{c}	19.96 ± 0.85^{b}	32.46 ± 0.86^{c}
	NF	51.54 ± 1.02^{b}	20.49 ± 0.61^{b}	33.98 ± 0.95^{d}
13 days	IF	51.64 ± 1.67^{b}	22.01 ± 0.93^{ab}	36.97 ± 1.27^{b}
	NF	52.39 ± 1.17^{b}	23.98 ± 1.07^{a}	38.77 ± 2.45^{bc}
Where.				
IF = Inoculated fermented carrot pickle.				
NF = Naturally fermented carrot pickle.				
All results expressed in mean ± standard deviation.				
$^{a–c}$ different superscript letter within a column indicates significant differences ($p < 0.05$) among the same samples during storage.				
^{NS} superscript letter within a column indicates not significant differences ($p < 0.05$) between samples at the specific storage time.				
(A)				

However, The L*, a* and b* value of both IF and NF sample increased might be due to increase in carotenoid content (Table 3), possibly due to decline *Bacillus* population at the end of storage period.

Table 3
Changes in antioxidant and bioactive compounds in fermented carrot pickles during refrigerated storage

Storage	Samples	TPC (mg GAE/g)	Total carotenoid content (μg/g)	DPPH• scavenging activity (μmol TE/kg)	FRAP (μmol TE/kg)
Before fermentation	Carrot pickles	2.01 ± 0.05^{b}	66.40 ± 0.23^{c}	1159.88 ± 25.53^{ab}	640.50 ± 52.85^{a}
After fermentation (0 day)	IF	$^{A}1.84 \pm 0.05^{ab}$	$^{A}57.64 \pm 0.20^{a}$	$^{A}1008.90 \pm 23.00^{c}$	$^{A}245.90 \pm 92.42^{c}$
	NF	$^{B}2.12 \pm 0.06^{c}$	$^{B}74.46 \pm 0.67^{a}$	$^{B}1220.67 \pm 11.43^{b}$	$^{A}283.90 \pm 39.95^{c}$
4 days	IF	$^{A}2.61 \pm 0.37^{c}$	$^{A}66.24 \pm 0.58^{c}$	$^{A}1335.57 \pm 126.5^{c}$	$^{A}365.90 \pm 10.82^{bc}$
	NF	$^{A}2.12 \pm 0.04^{c}$	$^{B}63.66 \pm 0.44^{d}$	$^{A}1377.53 \pm 16.48^{c}$	$^{A}382.90 \pm 45.83^{b}$
8 days	IF	$^{A}1.66 \pm 0.12^{a}$	$^{A}58.45 \pm 0.55^{b}$	$^{A}1035.96 \pm 75.35^{ab}$	$^{A}257.90 \pm 91.31^{c}$
	NF	$^{A}1.62 \pm 0.07^{a}$	$^{B}74.31 \pm 0.29^{a}$	$^{A}1230.08 \pm 53.71^{b}$	$^{A}274.90 \pm 55.51^{c}$
13 days	IF	$^{A}2.48 \pm 0.08^{c}$	$^{A}68.75 \pm 0.18^{d}$	$^{A}1106.16 \pm 21.60^{ab}$	$^{A}464.90 \pm 74.36^{b}$
	NF	$^{A}2.50 \pm 0.02^{d}$	$^{B}71.84 \pm 0.29^{b}$	$^{A}1132.82 \pm 39.38^{a}$	$^{B}269.90 \pm 54.03^{c}$

Where.

IF = Inoculated fermented carrot pickle.

NF = Naturally fermented carrot pickle.

All results expressed in mean ± standard deviation.

$^{a–d}$ different superscript letter within a column indicates significant differences ($p < 0.05$) among the same samples during storage.

$^{A–B}$ different superscript letter within a column indicates significant differences ($p < 0.05$) between samples at the specific storage time.

3.3 Variation of *Bacillus* population in fermented carrot pickles during refrigerated storage

Figure 1 displays the changes of *Bacillus* bacteria populations in inoculated and naturally fermented carrot pickle samples monitored over 13 days refrigerated storage period. At the before fermentation, the carrot pickles by inoculated had a significantly higher *Bacillus* count (6.55 log CFU/g) compared to the raw carrot pickles sample (4.53 log CFU/g). The higher number of *Bacillus* initially could be due to starter culture being added to the carrot pickles sample. Conversely, the lower population in the raw carrot pickles sample was possibly due to dependence on natural microorganisms. After fermentation, *Bacillus* populations significantly higher in IF sample (7.71 log CFU/g) compared to NF sample (5.84 log CFU/g). This result align with [37] indicating that inoculation facilitated the rapid colonization of *Bacillus*, whereas the NF sample showed slower LAB population growth due to natural succession of indigenous microorganisms. However, both NF and IF samples showed significant declines (NF: 5.50–4.88 log CFU/g, IF: 6.09–5.86 log CFU/g) during storage 4 to 13 days. The reduction in *Bacillus* counts during storage depends on time, food matrix, and culture type [38]. Throughout the storage period, the inoculated fermented carrot pickle sustains viable *Bacillus* counts over 10^6 CFU/g, indicating its potential as a probiotic product. Conversely the naturally fermented carrot pickle sample did not sustain counts above this threshold.

3.4 Changes bioactive and antioxidant properties of fermented carrot pickle during refrigerated storage

Table 3 depicts the changes in total phenolic content (TPC), total carotenoid content, DPPH• scavenging activity, and FRAP values of inoculated fermented (IF) and naturally fermented (NF) carrot pickles during refrigerated storage. The total phenolic content (TPC) of the IF sample (1.84 mg GAE/g) was significantly lower than the NF sample (2.12 mg GAE/g) after fermentation. The lower TPC could be due to higher *Bacillus* population, which likely facilitated enzymatic metabolism and structural conversion of phenolic compounds [39]. The total phenol content of both IF and NF samples increased up to 4 days of storage then decreased up to 8 days and increased thereafter again by the end of the storage period. This increase may be attributed to greater bio accessibility of free phenolic compound by the *Bacillus*. The free phenolic compound bind with other substances of food and degraded by enzymes and hydrolysis by specific microorganisms might be responsible for decrease of phenolic content [40]. There was good relation observed between TPC and total carotenoid content in both IF and NF samples.

The total carotenoid content of the IF sample (57.64 μg/g) showed a notable decreased compared to the NF sample (74.46 μg/g) after fermentation. During the growth of *Bacillus*, the formation of reactive oxygen species facilitates the oxidation of carotenoids, thereby contributing for the declined carotenoid content found in the IF sample [41]. Notably, the total carotenoid content of IF samples increased up to 4 days of storage, then decreased up to 8 days and subsequently increased again by the end of the storage period. This increase could be associated with the slowdown in enzymatic degradation and

reduced *Bacillus* activity due to the refrigeration storage. The reduced carotenoid content at the end of the storage periods may be attributed to a corresponding decrease in phenolic content.

After fermentation, the DPPH• scavenging activity of IF sample (1008.90 µmol TE/g)

significantly declined compared to the NF sample (1220.67 µmol TE/g). This may be uses of *Bacillus* culture altering compounds like phenols that contribute to antioxidant activity, reducing DPPH• scavenging activity. Similarly [42] observed the uses of some specific strain of LAB reduces phenol, consequently leading to decreased DPPH• activity. During 4 days of storage both IF (1335.57 µmol TE/g) and NF (1377.53 µmol TE/g) sample significantly increased then decreased of storage period (8 to 13 days). The composition of polyphenols and improves the solubilization and availability of bioactive compounds, thereby increasing their antioxidant potential [43]. The decreases may be due to changes in total phenolic content (TPC), which appears to be directly related to DPPH• scavenging activity.

The reducing antioxidant power (FRAP) of both IF (245.90 µmol TE/g) and NF (283.90 µmol TE/g) samples significantly decreased after fermentation, compared to the before fermentation. The decrease in FRAP values may be due to utilizing antioxidant compounds as nutrients and converting them into organic acids with antioxidant properties [44]. The FRAP value of the IF sample (464.90 µmol TE/g) significantly increased over the 13-day storage period, compared to 0 day. This increase in reducing power may be attributed to metabolic intermediates being able to move from inside cells to the product during late exponential to stationary transitions [45, 46].

3.5 Sensory characteristics of fermented carrot pickles

The sensory evaluation of the fermented carrot pickles revealed significant differences between the IF and NF samples are presented in Fig. 3.

The growth of microbial flora and metabolic activity are the primary sources of taste in fermented pickles. The fermented flavor score in IF samples (7.27) were significantly higher than NF samples (4.83). A higher score of flavor means that the flavor was better and appropriate (the intensity was neither too strong nor too weak) for the good quality of the IF sample investigated, according to the preference of consumers. The higher score of the IF sample may be attributed to the control fermentation by specific microorganism responsible for production of organic acids, esters, and other volatile chemicals that enhance the fermented flavor [47].

In terms of appearance, the IF samples (7.40) scored significantly higher than NF samples (6.97). This might be attributed to the controlled fermentation facilitated by *Bacillus* inoculation, which provides consistent microbial activity, resulting in enhanced color consistency and visual appeal [48]

Food texture significantly affects consumer sensory perception and serves as a critical factor influencing food selection. The texture of the IF samples (7.10) was also rated significantly higher relative to the NF samples (6.27), indicating that the inoculated fermentation process resulted in a more favorable textural profile.

As for spiciness, The IF samples (6.85) were significantly higher scored for spiciness than the NF samples (5.92). This may be due to the enhanced fermentation process, which breaks down compounds in chili peppers, thereby intensifying the perceived spicy flavor and pungency.

Finally, the overall acceptability of the IF samples was notably higher than the NF samples, which can be attributed to the combined improvements in appearance, fermented flavor, texture, and spiciness. As a result, compared to natural fermentation, inoculation with *Bacillus* strains leads to carrot pickles with superior and more consistent sensory qualities.

4. Conclusions

This study showed impact the inoculated fermentation by *Bacillus* strain of carrot pickles on physicochemical, microbial viability, antioxidant activity and sensory properties. The IF sample exhibited lower pH, total sugar, TSS and higher titratable acidity compared to NF sample. Microbial viability in IF samples consistently remained within the probiotic range throughout the storage period, whereas NF samples showed lower viability. Elevated levels of carotenoids and phenolic compounds in the IF samples contributed to enhanced antioxidant activity throughout the storage period. Additionally, the IF sample exhibited superior sensory qualities, including fermented flavor, appearance, texture, spiciness and overall acceptability.

The outcomes of this study support the use of *Bacillus toyonensis* as a fermentation starter culture to develop functional, probiotic enriched carrot-based foods.

However, the study also highlighted the challenge of maintaining the long-term probiotic potential of carrot pickles throughout the storage period. Future research should explore the effects of different *Bacillus* strains on the fermentation of carrot pickles and assess the influence of long-term storage on microbial stability to optimize their shelf life as probiotic foods

Declarations

Ethics and Consent to participate

The proposed sensory evaluation involving human participants was reviewed by the ethics committee through the Institute of Research and Training (IRT) at Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh (PAS/2026/05). The study was conducted in accordance with the principles of the Declaration of Helsinki and the applicable ethical guidelines and regulations of Hajee Mohammad Danesh Science and Technology University (HSTU). Informed consent to participate in the study was obtained from all participants before the sensory evaluation.

Consent to Publish

Not Applicable

Declaration of Competing Interest

The authors confirm that they have no conflicts of interest with respect to the work described in the manuscript.

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Author Contribution

Konok Kumar: conceptualization, methodology, Data curation, formal analysis, Visualization, investigation and writing-original draft; Mst. Hasnat Jahan Maya: Formal analysis, investigation, Data curation, Visualization; Md Raihanul Haque: writing-review and editing; Maruf Ahmed: supervision, investigation, Validation, writing-review and editing, Funding acquisition.

Data Availability

The nucleotide sequences obtained were submitted to the NCBI (National Centre for Biotechnology Information) GenBank database, and accession numbers were assigned PZ049891. (https://www.ncbi.nlm.nih.gov/nuccore/3199960019).

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Figures

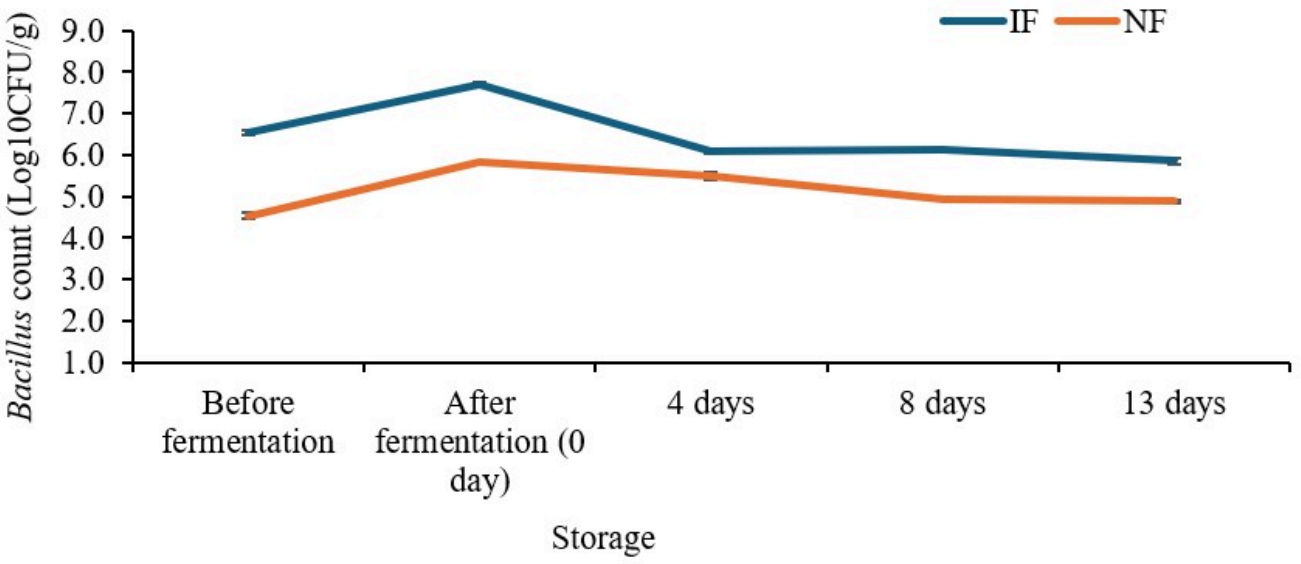


Figure 1

Changes in *Bacillus* population of fermented carrot pickles during refrigerated storage

IF = Inoculated fermented carrot pickle.

NF = Naturally fermented carrot pickle.

$^{a-c}$ different superscript letter indicates significant differences ($p < 0.05$) among the same samples during storage.

$^{A-B}$ different superscript letter indicates significant differences ($p < 0.05$) between samples at the specific storage time.

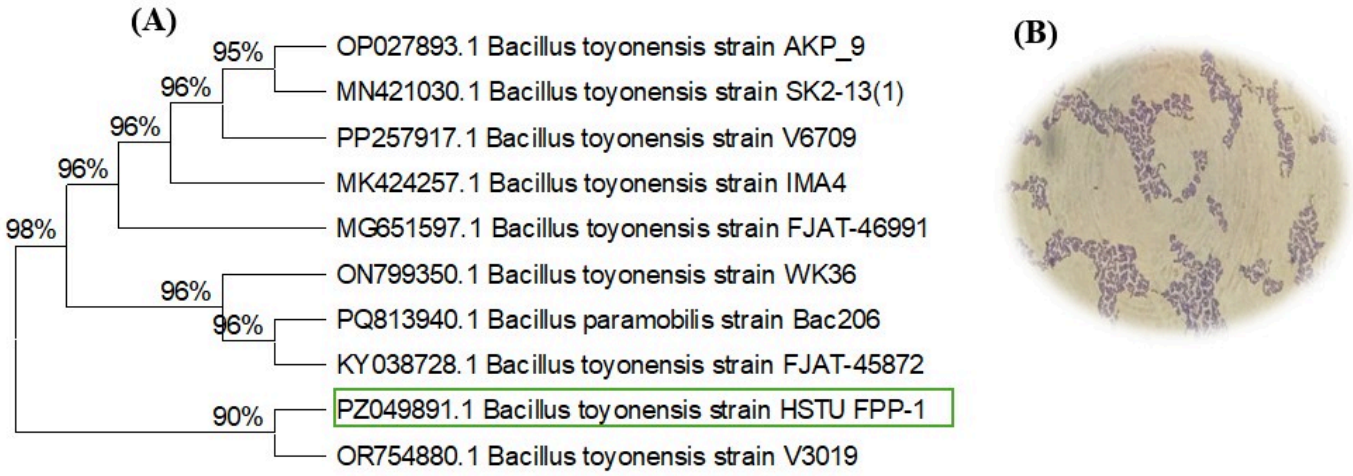


Figure 2

(A) Phylogenetic tree of *Bacillus toyonensis* strain HSTU FPP-1 (B) Morphology of the strain.

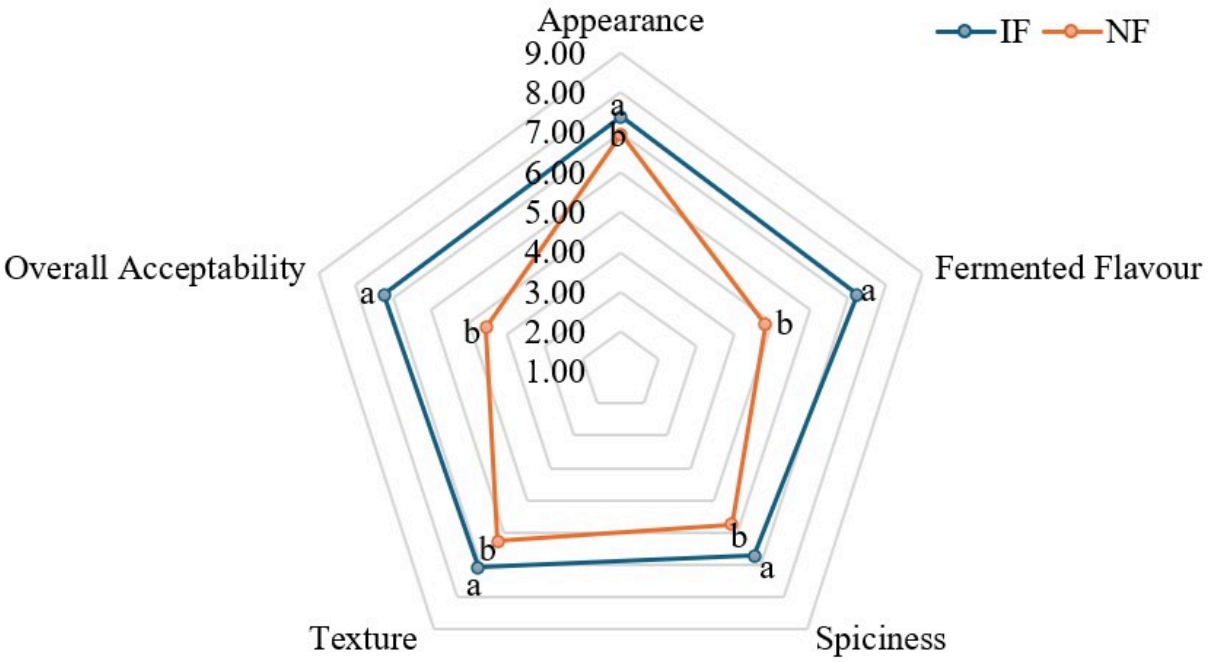


Figure 3

Sensory characteristics of fermented carrot pickles

IF = Inoculated fermented carrot pickle.

NF = Naturally fermented carrot pickle.

$^{a-b}$ different superscript letter indicates significant differences ($p < 0.05$) between samples at the specific storage time.