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Research Paper

Evaluation of *Bacillus* spp. as dough starters for *Adhirasam* - A traditional rice based fermented food of Southern India

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Abstract

Adhirasam is a cereal based, doughnut shaped, deep fried dessert consumed in the southern regions of India. The dough used to prepare *adhirasam* is fermented and contains rice flour and jaggery. The aim of the present study was to characterize the cultivable bacteria associated with this fermented dough and to identify a suitable starter culture for the production of quality *adhirasam*. In total, one hundred and seventy bacterial isolates were recovered from de Man Rogosa Sharp (MRS) agar, nutrient agar, lysogeny agar and tryptic soy agar media. Out of the 170 bacterial isolates, sixteen isolates were selected based on their ability to tolerate glucose and sucrose. All the bacterial isolates tolerated 15% glucose and 30% sucrose. Analyses of 16S rDNA gene sequences of the bacterial isolates showed that the dominant cultivable bacteria were members of the genus *Bacillus*. These strains were further used as starters and tested for their ability to ferment rice flour with jaggery to produce *adhirasam* dough. Organoleptic evaluation was carried out to choose the best starter strain. *Adhirasam* prepared from *Bacillus subtilis* isolates S4-P11, S2-G2-A1 and S1-G15, *Bacillus tequilensis* isolates S2-H16, S3-P9, S3-G10 and *Bacillus siamensis* isolate S2-G13 were highly acceptable to consumers. *Adhirasam* prepared using these starter cultures had superior product characteristics such as softness in texture, flavor and enhanced aroma and sweet taste.

Key words: *Adhirasam*, fermented cereal product, *Bacillus*, South Indian dessert.

Introduction

Cereals are staple foods for billions of people across the globe, especially in the Indian sub-continent, Asia and Africa. Cereals are important substrates in fermented foods and it is reported that fermented foods comprise one third of the diets in the world (Marshall and Mejia, 2011). Several fermented cereal products have been documented including *sourdough* from America, Australia, and Europe (Brandt, 2007), *Idli* and *dosa* from India (Soni *et al.*, 1985; 1991; Agaliya and Jeevaratnam, 2013), and *Puto* from South East Asia (Kelly *et al.*, 1995). The presence of obligate heterofermentative lactic acid bacteria (*Leuconostoc mesenteroides*) and yeast species (*Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Pichia anomala* and *Trichosporon pullulans*) has been documented during the fermentation of *idli* batter (Aidoo *et al.*, 2006). Park *et al.* (2010) have reported that *Bacillus amyloliquefaciens, B. subtilis*, and *Bacillus vallismortis* were dominant in the traditionally fermented Korean soybean paste, *eoyukjang*. *Hong et al. (*2012) have documented an increase in the aroma characteristics of the fermented Korean soybean paste, *Doenjang*, upon inoculation with *B. amyloliquefaciens*. Previously, Lee *et al.* (2013) have isolated the probiotic, *B. subtilis* KU201 from kimchi, which has antifungal and antimicrobial properties. Choi *et al.*

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(2010) have developed a fermented soymilk product with *Bacillus subtilis* as the starter culture and have found that this fermented soymilk exhibited high antioxidant activity. Cheonggukjang, produced with the co-inoculation of the probiotics *B. subtilis* W42 and *B. amyloliquefaciens* MJ1-4, exhibited high antioxidant and fibrinolytic activities (Cho *et al.*, 2014).

Adhirasam is an ethnic fermented cereal-based food of South India, especially Tamil Nadu state. It is a doughnut-shaped, spongy, deep fried food that is consumed during festivals and special occasions. People may also occasionally consume the *adhirasam* dough without deep frying. To the best of our knowledge, there is no literature describing the microbiology of the fermented *adhirasam* dough. Therefore, this study was formulated to investigate the cultivable bacteria involved in the fermentation of *adhirasam* dough and to identify a promising starter culture.

Materials and Methods

Sample collection and isolation of microorganisms

Thirty samples of 3-day fermented *adhirasam* dough were collected from *adhirasam* producers in Madurai district, Tamil Nadu state, India. The samples were packed in polyethylene pouches and stored in the refrigerator at 4 °C for subsequent analysis. Ten grams of *adhirasam* dough from each sample was aseptically transferred into a 250 mL flask containing 90 mL sterile saline solution (0.85 g/L NaCl) and the contents were mixed thoroughly for 30 min at 150 rpm. Serial dilutions (10^{-1} to 10^{-8}) were made for each sample, and 100 µL of the each dilution was spread onto each de Man Rogosa Sharpe agar (MRSA), Nutrient agar (NA), Lysogeny agar (LA), trypticase soy agar media (TSA) (Himedia, India). After two days of incubation at 30 °C, bacterial colonies were isolated and purified. Fungi (yeasts and molds) were enumerated on Rose Bengal choramphenicol agar (Himedia, India) as described previously (Zheng *et al.*, 2013).

Identification of bacterial isolates

Cell morphology, Gram staining, catalase and oxidase activity, curdling, spore formation, sugar fermentation (glucose, sucrose and lactose) and glucose and sucrose tolerance tests were performed to characterize the isolates (Gerhardt *et al.*, 1994). Glucose tolerance was tested in a nutrient broth with 5%, 10%, 15% or 20% glucose after 2 days of fermentation at 30 °C. Sucrose tolerance of bacteria was tested in nutrient broth (devoid of glucose) having sucrose concentrations ranging from 10% to 80% with 10 unit increments, by incubation for 2 days at 30 °C. Curdling activity was tested in toned milk medium prepared from bacteria free toned milk (Terzic-Vidojevic *et al.*, 2009). The hemolytic property of *Bacillus* spp. isolated from *adhirasam* dough was determined according to Benson (2002). Molecular characterization was performed according to Kim *et al.* (2011). Briefly, the gene encoding bacterial 16S rRNA was amplified through Polymerase Chain Reaction (PCR) with forward primer 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1492R: 5'-GGTTACCTTGTTACGACTT-3'. The 16S rRNA nucleotide sequences were obtained by PCR direct sequencing using the fluorescent dye terminator method (ABI Prism™ Bigdye™ Terminator cycle sequencing ready reaction kit v.3.1) and the products were purified using the Millipore-Montage dye removal kit. Finally, the products were run in an ABI3730XL capillary DNA sequencer (50 cm capillary). Nearly complete 16S rRNA gene sequences from the automatic sequencer were aligned and bacterial identities were deduced from the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) to ascertain their closest relatives. The sequences obtained from this study were submitted to NCBI with accession numbers KC851825 to KC851840.

Determination of antimicrobial activity

To examine the antimicrobial activity, 14 *Bacillus* spp. isolated from *adhirasam* dough were used. *Bacillus* spp. isolates were grown in 100 mL of nutrient broth (Himedia, India) at 30 °C, and 120 rpm for 24 h. The antimicrobial activity was investigated against human pathogens *viz., Escherichia coli* MTCC 2622, *Staphylococcus aureus* MTCC 1144, *Listeria monocytogens* MTCC 1143, *Saccharomyces cerevisiae* MTCC 36 and *Bacillus cereus* MTCC 1272 as described previously (Zheng *et al.*, 2013). These pathogenic bacteria were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India.

Preparation of *adhirasam* dough, inoculation with starter cultures and estimation of population dynamics

Bacillus spp. isolates were grown in 25 mL of nutrient broth, in an incubator shaker at 120 rpm for 2 days at 30 °C. The bacterial cells were harvested by centrifugation at 8,000 *g* at 4 °C for 15 min. The bacterial cells were washed 2-3 times with 0.85% NaCl and resuspended in physiological saline.

Rice (*Oryza sativa* L.), local variety 'IR20', and jaggery were purchased from the local market. *Adhirasam* dough was prepared in the laboratory following the traditional method. One kilogram of rice was sorted, washed and soaked at ambient temperature (28-30 °C) for 1 h. Water was decanted, and the soaked rice was pounded and sieved to get rice flour. Jaggery syrup was prepared separately by mixing 750 g of jaggery in 300 mL distilled water and heating to 80 °C to get desired consistency. Immediately, one kilogram of rice flour was mixed with this 300 mL of hot jaggery syrup and kneaded into soft dough under aseptic conditions. After the dough reached room

temperature, equal quantities (100 g) of dough were distributed in 500 mL bottles and sterilized. The dough was then inoculated with 1% saline suspended bacterial inoculum (~ 1×10^6 cfu/mL) and incubated at 30 °C for 3 days. For controls, the dough was not sterilized and inoculated with 1 mL of 0.85% NaCl instead of the bacterial inoculum and incubated at 30 °C for 3 days. This experiment was conducted three times and the replicates were arranged in a completely randomized block design.

The dough (10 g) was withdrawn from each treatment on day 1, 2, and 3. Bacterial survivability and the changes in the inoculated bacterial population in the dough were evaluated in NA after 2 days of incubation at 30 °C. Simultaneously, the pH of the dough was measured after mixing with distilled water (1:5, solid: water). This mixture was allowed to stand for 15 min with intermittent stirring before readings were taken.

Sensory evaluation of dough and the *adhirasam* prepared using different starter culture

Fermented dough, both naturally fermented and fermented with the starter culture, was organoleptically evaluated for color, aroma, perforations and texture by a panel of 10 trained judges using a 9 point hedonic scale as described by Larmond (1977). The judges were provided with a prescribed format to record their observations. The following are the scores of the hedonic scale used: 9 = Like extremely; 8 = Like very much; 7 = Like moderately; 6 = Like slightly; 5 = Neither like nor dislike; 4 = Dislike slightly; 3 = Dislike moderately; 2 = Dislike very much; 1 = Dislike extremely. The panelists were asked to expectorate the dough and rinse their mouth using distilled water between samples. Sensory testing was made in a panel room that was completely free of food/chemical odor, unnecessary sound and mixing of daylight.

All the fermented dough samples, both naturally fermented (control) and starter culture fermented, were portioned, flattened and made in to doughnut shapes and deep fried in hot edible oil until golden brown in color. The color, flavor, texture, taste and overall acceptability of the prepared product was also organoleptically evaluated by a panel of 10 trained judges using the 9 point hedonic scale as described above.

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) with general linear model (ver 9.1; SAS institute Inc, Cary, NC, USA). Means were compared using least significant difference (LSD). The significance levels were within confidence limits of 0.05 or less.

Results and Discussion

Characterization of bacteria isolated from *adhirasam* dough

Fermentation with a starter culture generally reduces fermentation time, imparts volatility during fermentation and improves product characteristics such as flavor, color, texture and taste. Fermented products have both longer shelf life and better value. In the present study, 170 bacterial isolates were obtained from thirty different *adhirasam* dough samples; of these, 40, 52, 40 and 38 bacterial isolates were recovered from MRSA, NA, LA and TSA media, respectively. From the 170 isolates, only 64 were selected for further biochemical characterization based on colony size, color, and morphology. Morphologically 97% of bacterial isolates were Gram positive, spore forming rods, while 75% were oxidase positive and 87.5% were catalase positive. In the curdling test, 42.2% of the isolates produced thick curd with a pleasant curd smell while 40.6% of the isolates produced curd after 24-36 h of incubation. All isolates were able to ferment glucose and 16% of the isolates produced gas. Seventy-five percent of the isolates fermented sucrose and 14% produced gas. Thirty percent of the isolates fermented lactose and 13% produced gas. All isolates were able to grow in 15% of glucose, and 96% of the isolates could tolerate 20% glucose. All the isolates were able to grow in 30% sucrose, 22% of the isolates grew in 70% sucrose. Yeast and molds were not recovered from any of the samples tested. Traditionally, jaggery is prepared by the concentration of a sugarcane juice extract, made from locally cultivated sugarcane, without the use of any chemicals. Jaggery is the major ingredient in the *adhirasam* dough, and can constitute up to 75% of the product. Sucrose is one of the major sugars present in jaggery, and its concentration ranges between 66-77% (Chand *et al.*, 2012). Hence, the 16 bacterial isolates that could tolerate 70% sucrose were alone selected for molecular characterization and further studies.

A summary of the morphological and biochemical characteristics of the selected bacterial isolates is presented in Table 1. The results of the 16S rDNA gene sequence analysis revealed that 14 of the 16 isolates belonged to the *Bacillus* spp. and the remaining 2 isolates were identified as *Enterobacter mori* (Table 2). The Food and Drug Administration (FDA) recognizes some substances derived from *B. subtilis* as GRAS, and this species is also used as a probiotic. Fermented soybean, *natto,* which is commonly consumed in Japan, contains 10^8 viable *B. subtilis* per gram of food; it also has anti-cancer properties and can stimulate the immune system (Hosoi and Kiuchi, 2004). Sarkar *et al.* (1994) isolated *B. subtilis* as the functional bacterium from *kinema*, a fermented soybean food. Out of the16 isolates, 80% of them were able to produce curd from bacteria-free milk. Our results are similar to previous reports of *Bacillus* spp. causing sweet curdling of milk due to the production of

Table 1 - Morphological and biochemical characterization of bacterial isolates from *adhirasam* dough.

Bacterial isolates	Colony morphology	Cell morphology	Gram's reaction	Oxidase	Catalase	Curdling	Spore formation	Sugar fermentation test						Sucrose tolerance test			
								Glucose		Sucrose		Lactose		40%	50%	60%	70%
								Cc	G	Cc	G	Cc	G				
S2-H14	Irregular, creamy	Rod	+ve	-	+	++	+	+	-	+	+	-	-	+	+	+	+
S2-H16	Round, Smooth, Yellowish	Rod	+ve	+	+	++	+	+	-	+	+	+	-	+	+	+	+
S4-P4	Moist, Grey, Smooth	Rod	-ve	-	-	++	-	+	+	-	-	-	-	-	-	-	-
S3-P9	Round, Smooth, Yellowish	Rod	+ve	+	+	+	+	+	-	+	-	+	+	+	+	+	+
S4-P11	Round, Irregular, Wrinkled	Rod	+ve	+	+	++	+	+	-	-	-	+	-	+	+	+	+
S4-P13	Moist, Grey, Smooth	Rod	-ve	-	-	+	-	+	+	+	+	+	+	+	-	-	-
S1-F1	White, Smooth	Rod	+ve	+	+	-	+	+	-	+	-	-	-	+	+	+	+
S4-F3	Round, Irregular, Wrinkled	Rod	+ve	+	+	+	+	+	-	+	-	-	-	+	+	+	+
S2-F10	Irregular, Creamy	Rod	+ve	-	+	++	+	+	-	+	-	-	-	+	+	+	+
S2-F11	Irregular	Rod	+ve	+	+	++	+	+	-	+	-	-	-	+	+	+	+
S2-F14	Slimy, Pink-Red Pigment	Rod	+ve	+	+	++	+	+	-	+	-	-	-	+	+	+	+
S2-G2-A1	Round, Irregular, Wrinkled	Rod	+ve	+	+	++	+	+	-	+	-	-	-	+	+	+	+
S3-G10	Round, Smooth, Yellowish	Rod	+ve	+	+	++	+	+	-	+	+	-	-	+	+	+	+
S2-G12	Creamy White, Mucoid, Raised	Rod	+ve	+	+	++	+	+	-	+	-	-	-	+	+	+	+
S2-G13	Round, Smooth, Yellowish	Rod	+ve	+	+	-	+	+	-	+	-	-	-	+	+	+	+
S1-G15	Round, Irregular, Wrinkled	Rod	+ve	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+, Positive; -, negative; In curdling test ++, curd formation with pleasant curd smell; +, curd formation; -, no curdling; Cc, colour change; G, gas production. All the tested bacterial isolates could grow in 5, 10, 15 and 20% of glucose. All the tested bacterial isolates tolerated upto 30% of sucrose.

Table 2 - Molecular characterization of bacterial isolates obtained from *adhirasam* dough.

S. No.	Bacterial isolates	Closest relative	Identity match (%)	NCBI accession number	Sequenced length (bp)
1	S2-H14	*Bacillus subtilis* subsp. *spizizenii*	99.86	KC851825	1481
2	S2-H16	*Bacillus tequilensis*	99.93	KC851826	1486
3	S4-P4	*Enterobacter mori*	99.32	KC851827	1474
4	S3-P9	*Bacillus tequilensis*	99.93	KC851828	1489
5	S4-P11	*Bacillus subtilis* subsp. *subtilis*	99.93	KC851829	1481
6	S4-P13	*Enterobacter mori*	99.46	KC851830	1473
7	S1-F1	*Bacillus safensis*	100	KC851831	1481
8	S4-F3	*Bacillus subtilis* subsp. *inaquosorum*	99.83	KC851832	1478
9	S2-F10	*Bacillus subtilis* subsp. *spizizenii*	99.86	KC851833	1475
10	S2-F11	*Bacillus aerophilus*	100	KC851834	1487
11	S2-F14	*Bacillus endophyticus*	99.66	KC851835	1481
12	S2-G2-A1	*Bacillus subtilis* subsp. *subtilis*	99.93	KC851836	1477
13	S3-G10	*Bacillus tequilensis*	99.93	KC851837	1487
14	S2-G12	*Bacillus siamensis*	99.93	KC851838	1480
15	S2-G13	*Bacillus tequilensis*	99.93	KC851839	1480
16	S1-G15	*Bacillus subtilis* subsp. *subtilis*	99.93	KC851840	1481

enzymes proteinase, lipase and phospholipase (Sarkar *et al.*, 1994; Meer *et al.*, 1991). As none of the *Bacillus* spp. isolated from this study exhibited any hemolytic activity, they were used for developing starter inocula. The *E. mori* isolates were reported to be plant pathogens and hence were not used for further testing. *Bacillus* spp. isolates S2-H14, S4-P11, S4-F3, S2-F10, S2-G2-A1 and S1-G15 inhibited the growth of E. *coli* MTCC 262 and *S. aureus* MTCC 1144. However, none of the *Bacillus* isolates inhibited the growth of L. *monocytogens* MTCC 1143, *S. cerevisiae* MTCC 36 and *B. cereus* MTCC 1272 (Table 3).

Sensory properties of fermented *adhirasam* dough

On the first day of fermentation, the dough was found to be compact, firm and without perforations. However, on the third day of fermentation, the dough lost some of its texture, and perforations were seen on the surface of the dough (Figure 1). Dough fermented with *B. subtilis* isolates S1-G15, S2-G2-A1, S4-P11 and with the *B. safensis* S2-G12 isolate exhibited greater perforation compared to other isolates, and hence were given maximum scores. The aroma of the dough fermented with these bacterial isolates was also superior to control. Individual inoculation of *B. safensis* S2-G12, *B. subtilis* subsp. *subtilis* S1-G15, S2-G2-A1, S4-P11 and *B. tequilensis* isolates S3-G10, S2-G13, S3-P9, S2-H16 were also scored maximum for aroma enhancement during fermentation. A previous study has documented the aroma producing properties of *B. subtilis*; specifically, acetoin produced leads to a pleasant and buttery odor, and metabolic engineering has further improved its prospective production (Chen *et al.*, 2013). Thus it is possible that the observed aroma enhancement during fermentation was due to the acetoin produced by the isolates. There was not much difference in color among dough fermented with the various isolates, but were all better than control. The texture of the dough improved as fermentation progressed and dough with more perforations attained better texture. The texture scores of all the treatments were higher compared to control, and the overall acceptability improved due to inoculation with the various isolates as starter cultures (Table 4).

B. subtilis is also associated with many other fermented products like *meju,* a Japanese traditional soybean food, *thua-nao*, a Northern Thailand fermented soybeans, fermented soybeans of North East India such as *kinema, hawaijar, tungrymbai, tungtoh, aakhone*/*axone, bekang, peruyaan, bemerthu,* and *maseura,* a black gram fermented food of North East India (Tamang *et al.*, 1999, 2012; Chantawannakul *et al.*, 2002; Terzic-Vidojevic *et al.*, 2009; Kim *et al.*, 2011). Similarly, the *Bacillus* spp. also dominate

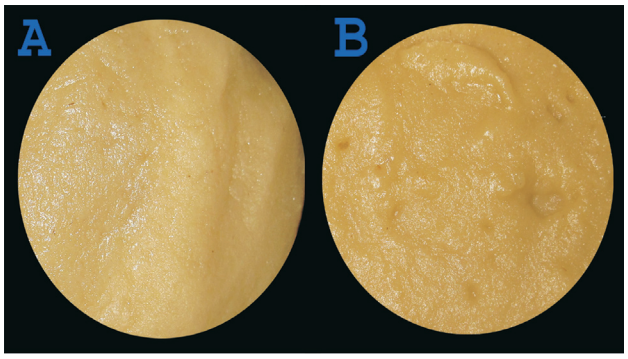

Figure 1 - Perforations in *adhirasam* dough after three days of fermentation; Uninoculated control (a); Dough fermented with inoculation of *B. subtilis* subsp. *subtilis* S1-G15 (b).

in *Daqu*- a traditional fermentation starter used to produce flavored vinegar and Chinese liquor (Zheng *et al.*, 2013). During the preparation of *adhirasam*, jaggery syrup temperature was raised to 80 °C. Such temperature should have a selective effect on the microbiota, favoring the thermotolerant, and aerobic endospore forming bacteria. As the *Bacillus* spp. possess all these characteristics, they can persist and outcompete the fungi and yeast. The antimicrobial activity of *Bacillus* may be another reason why *Bacillus* spp. become dominant in the *adhirasam* dough. The principle requirements of starter culture strains are rapid production of CO_2 from sugars and the generation

Table 3 - Antimicrobial activity of cell free supernatants of *Bacillus* spp.

Antagonistic bacteria	Pathogens				
	Escherchia *coli* MTCC 2622	*Listeria monocytogens* MTCC 1143	*Saccharomyces cerevisiae* MTCC 36	*Bacillus cereus* MTCC 1272	*Staphylococcus aureus* MTCC 1144
Bacillus subtilis subsp. *spizizenii* S2-H14	++	-	-	-	+
Bacillus tequilensis S2-H16	-	-	-	-	-
Bacillus tequilensis S3-P9	-	-	-	-	-
Bacillus subtilis subsp. *subtilis* S4-P11	++	-	-	-	+
Bacillus safensis S1-F1	++	-	-	-	+
Bacillus subtilis subsp. *inaquosorum* S4-F3	++	-	-	-	+
Bacillus subtilis subsp. *Spizizenii* S2-F10	++	-	-	-	+
Bacillus aerophilus S2-F11	-	-	-	-	-
Bacillus endophyticus S2-F14	-	-	-	-	-
Bacillus subtilis subsp. *subtilis* S2-G2-A1	++	-	-	-	+
Bacillus tequilensis S3-G10	-	-	-	-	-
Bacillus siamensis S2-G12	-	-	-	-	-
Bacillus siamensis S2-G13	-	-	-	-	-
Bacillus subtilis subsp. *subtilis* S1-G15	++	-	-	-	+

-, Not detectable; ++ diameter of inhibition zone between 10-15 mm; +, diameter of inhibition zone less than 5 mm.

Table 4 - Organoleptic evaluation of *adhirasam* dough fermented with different bacterial isolates.

Treatments	Aroma	Perforations	Color	Texture	Overall acceptability
Bacillus subtilis subsp. *spizizenii* S2-H14	8.5 ± 0.53^{a}	8.4 ± 0.52^{a}	7.5 ± 0.53^{a}	8.6 ± 0.52^{a}	8.2 ± 0.42^{c}
Bacillus tequilensis S2-H16	8.5 ± 0.53^{a}	6.6 ± 0.52^{c}	7.5 ± 0.53^{a}	7.8 ± 0.42^{b}	8.8 ± 0.42^{a}
Bacillus tequilensis S3-P9	8.6 ± 0.52^{a}	6.7 ± 0.48^{c}	7.3 ± 0.48^{a}	8.6 ± 0.52^{a}	8.8 ± 0.42^{a}
Bacillus subtilis subsp. *subtilis* S4-P11	8.6 ± 0.52^{a}	8.7 ± 0.48^{a}	7.4 ± 0.52^{a}	8.7 ± 0.48^{a}	8.8 ± 0.42^{a}
Bacillus safensis S1-F1	7.6 ± 0.52^{b}	6.7 ± 0.48^{c}	7.4 ± 0.52^{a}	8.5 ± 0.53^{a}	$8.6 \pm 0.52^{a\,b\,c}$
Bacillus subtilis subsp. *inaquosorum* S4-F3	8.6 ± 0.52^{a}	7.6 ± 0.52^{b}	7.3 ± 0.48^{a}	8.5 ± 0.53^{a}	$8.4 \pm 0.52^{a\,b\,c}$
Bacillus subtilis subsp. *Spizizenii* S2-F10	8.6 ± 0.52^{a}	7.8 ± 0.42^{b}	7.3 ± 0.48^{a}	8.7 ± 0.48^{a}	$8.4 \pm 0.52^{a\,b\,c}$
Bacillus aerophilus S2-F11	8.5 ± 0.53^{a}	6.8 ± 0.42^{c}	7.3 ± 0.48^{a}	8.5 ± 0.53^{a}	$8.4 \pm 0.52^{a\,b\,c}$
Bacillus endophyticus S2-F14	8.5 ± 0.53^{a}	6.7 ± 0.48^{c}	7.3 ± 0.48^{a}	8.5 ± 0.53^{a}	$8.3 \pm 0.48^{b\,c}$
Bacillus subtilis subsp. *subtilis* S2-G2-A1	8.8 ± 0.42^{a}	8.8 ± 0.42^{a}	7.3 ± 0.48^{a}	8.8 ± 0.42^{a}	8.8 ± 0.42^{a}
Bacillus tequilensis S3-G10	8.8 ± 0.42^{a}	7.9 ± 0.32^{b}	7.3 ± 0.48^{a}	8.6 ± 0.52^{a}	8.8 ± 0.42^{a}
Bacillus siamensis S2-G12	8.7 ± 0.48^{a}	8.6 ± 0.52^{a}	7.3 ± 0.48^{a}	8.4 ± 0.52^{a}	$8.7 \pm 0.48^{a\,b}$
Bacillus siamensis S2-G13	8.6 ± 0.52^{a}	6.7 ± 0.48^{c}	7.2 ± 0.42^{a}	8.6 ± 0.52^{a}	8.8 ± 0.42^{a}
Bacillus subtilis subsp. *subtilis* S1-G15	8.8 ± 0.42^{a}	8.8 ± 0.42^{a}	7.4 ± 0.52^{a}	8.8 ± 0.42^{a}	8.8 ± 0.42^{a}
LSD ($p \leq 0.05$)	0.44	0.42	0.46	0.44	0.42

Data represents the mean scores (± Standard deviations) of ten judges. Values bearing different superscripts in each column differ significantly ($p < 0.05$). Naturally fermented dough was used as control (7 Like moderately), score 1, dislike extremely; score 2, dislike very much; score 3, dislike moderately; score 4, dislike slightly; score 5, neither like nor dislike; score 6, like slightly; score 8, like very much; score 9, like extremely.

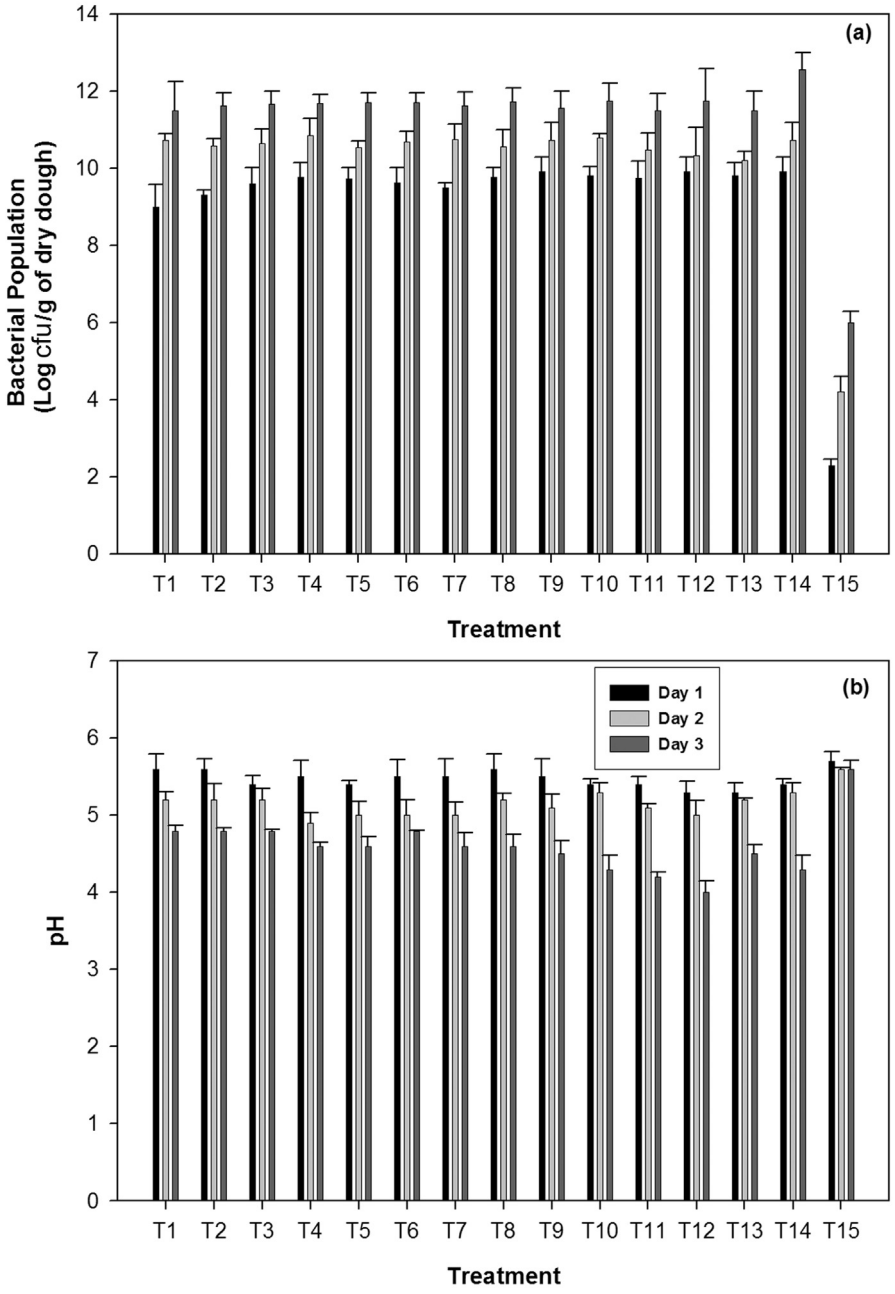

Figure 2 - Changes in bacterial population (a) and pH (b) during fermentation of *adhirasam* dough. Initial pH of dough was 6.0. The treatment details were as follows; T1- *B. subtilis* subsp. *spizizenii* S2-H14; T2-*B. tequilensis* S2-H16; T3-*B. tequilensis* S3-P9; T4- *B. subtilis* subsp. *subtilis* S4-P11; T5-*Bacillus safensis* S1-F1; T6- *B. subtilis* subsp. *inaquosorum* S4-F3; T7-*B. subtilis* subsp. *spizizenii* S2-F10; T8- *Bacillus aerophilus* S2-F11; T9- *Bacillus endophyticus* S2-F14; T10- *B. subtilis* subsp. *subtilis* S2-G2-A1; T11- *B. tequilensis* S3-G10; T12- *B. siamensis* S2-G12; T13-*B. tequilensis* S2-G13; T14- *B. subtilis* subsp. *subtilis* S1-G15; T15- Control.

of good bread flavor (Decock and Cappelle, 2005). In the present study, the inoculated *Bacillus* spp. probably produced thermostable enzymes that degraded cell walls and other polysaccharides that might be implicated in the development of flavor precursors such as pyrazines (Zheng *et al.*, 2013). Yonzan and Tamang (2013) have tested lactic acid bacteria and yeast for their ability to ferment rice flour for making *selroti*, and have also evaluated its organoleptic properties using a 5 point hedonic scale.

Population changes in bacterial species during fermentation

The dynamics of the viable bacterial counts during *adhirasam* fermentation are presented in Figure 2a. Among the different treatments, the lowest total bacterial

count was registered in controls (2 log cfu / g of dry dough) on day 1. As incubation progressed the total number of bacteria also increased. A maximum population of 12 log cfu /g of dry dough was observed in dough inoculated with *B. subtilis* subsp. *subtilis* S1-G15. On day 3 of fermentation, dough inoculated with *Bacillus siamensis* S2-G12 reduced dough pH from 6.0 to 4.0 (Figure 2b). In a previous study, Zheng *et al.* (2013) have reported that *Bacillus* spp. were continuously present throughout the fermentation of *Daqu*. Thus it is possible that the *Bacillus* spp. also similarly persisted throughout the fermentation of the *adhirasam* dough.

Sensory properties of adhirasam prepared from fermented dough

Adhirasam prepared from inoculum fermented dough had better appearance, color, flavor, texture and taste compared to that prepared using control dough. The appearance and color of the product were all equally acceptable, irrespective of the treatment. The flavor and texture of *adhirasam* prepared from various treatments were enhanced due to fermentation (Figure 3). It is generally known that a soft texture and sweet taste with golden brown color of the fried *adhirasam* is considered to be the best by the consumers. The *adhirasam* prepared from dough fermented with *B. subtilis* isolates S2-G2-A1, S4-P11, S1-G15 showed superior flavor and texture retention even after frying in hot edible oil, and were followed by *adhirasam* prepared from dough fermented by *B. tequilensis* isolates S3-G10, S2-G13, S3-P9 and S2-H16. The taste of *adhirasam* prepared using dough fermented with bacterial starter culture(s) was significantly better compared to control (Table 5).

Figure 3 - *Adhirasam* product prepared from dough fermented with *B. subtilis* subsp. *subtilis* S1-G15.

Table 5 - Organoleptic evaluation of *adhirasam* prepared from fermented dough.

Treatment	Appearance	Color	Flavor	Texture	Taste	Overall acceptability
Bacillus subtilis subsp. *spizizenii* S2-H14	8.1 ± 0.32^{e}	8.2 ± 0.42^{d}	8.2 ± 0.42^{c}	8.2 ± 0.42^{c}	8.2 ± 0.42^{d}	8.2 ± 0.42^{c}
Bacillus tequilensis S2-H16	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}
Bacillus tequilensis S3-P9	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}
Bacillus subtilis subsp. *subtilis* S4-P11	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}
Bacillus safensis S1-F1	8.1 ± 0.32^{e}	8.2 ± 0.42^{d}	8.2 ± 0.42^{c}	8.2 ± 0.42^{c}	8.2 ± 0.42^{d}	8.2 ± 0.42^{c}
Bacillus subtilis subsp. *inaquosorum* S4-F3	8.4 ± 0.52^{cde}	8.4 ± 0.52^{cd}	8.5 ± 0.53^{bc}	8.4 ± 0.52^{c}	8.7 ± 0.48^{abc}	8.6 ± 0.52^{ab}
Bacillus subtilis subsp. *spizizenii* S2-F10	8.2 ± 0.42^{de}	8.2 ± 0.42^{d}	8.2 ± 0.42^{c}	8.2 ± 0.42^{c}	8.2 ± 0.42^{d}	8.2 ± 0.42^{c}
Bacillus aerophilus S2-F11	8.6 ± 0.52^{abc}	8.6 ± 0.52^{abc}	8.3 ± 0.48^{c}	8.5 ± 0.53^{bc}	8.4 ± 0.52^{cd}	8.5 ± 0.53^{bc}
Bacillus endophyticus S2-F14	8.4 ± 0.52^{cde}	8.5 ± 0.53^{bcd}	8.7 ± 0.48^{ab}	8.4 ± 0.52^{c}	8.5 ± 0.53^{bcd}	8.5 ± 0.53^{bc}
Bacillus subtilis subsp. *subtilis* S2-G2-A1	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}
Bacillus tequilensis S3-G10	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}
Bacillus siamensis S2-G12	8.5 ± 0.53^{bcd}	8.5 ± 0.53^{bcd}	8.5 ± 0.53^{bc}	8.5 ± 0.53^{bc}	8.5 ± 0.53^{bcd}	8.5 ± 0.53^{bc}
Bacillus siamensis S2-G13	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}	8.8 ± 0.42^{ab}
Bacillus subtilis subsp. *subtilis* S1-G15	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}	8.9 ± 0.32^{a}
LSD ($p \leq 0.05$)	0.38	0.39	0.38	0.39	0.39	0.39

Data represents the mean scores (± standard deviations) of ten judges. Values bearing different superscripts in each column differ significantly ($p < 0.05$). Naturally fermented dough was used as control (7 Like moderately), score 1, dislike extremely; score 2, dislike very much; score 3, dislike moderately; score 4, dislike slightly; score 5, neither like nor dislike; score 6, like slightly; score 8, like very much; score 9, like extremely.

Conclusion

In conclusion, the *Bacillus* spp. were the dominant and active species in *adhirasam* dough and imparted both structure and flavor to *adhirasam*. This study shows that the individual inoculation of the isolates of *B. subtilis* subsp. *subtilis*, *B. tequilensis* and *B. siamensis* as a starter culture in the preparation of *adhirasam* was acceptable to consumers. Thus these isolates can be recognized as suitable starter cultures for fermentation of *adhirasam* dough. In future, the impact of the inoculation of *Bacillus* spp. on the stabilization and quality characteristics, especially accumulation of antioxidants and bacteriocins, of *adhirasam* should be investigated.

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