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Short communication

Bacillus species isolated from *tungrymbai* and *bekang*, naturally fermented soybean foods of India

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ABSTRACT

Tungrymbai and *bekang* are naturally fermented soybean foods commonly consumed in Meghalaya and Mizoram states of India. A total of 39 samples of *tungrymbai* and 43 samples of *bekang* were collected from different villages and markets of Meghalaya and Mizoram, respectively and were analysed for microbial load. In both *tungrymbai* and *bekang*, the average population of *Bacillus* spp. was 8.2 ± 0.1 log cfu/g. A total of 428 isolates of *Bacillus* were isolated from *tungrymbai* (211) and *bekang* (217) for detailed identification. On the basis of a combination of phenotypic and molecular characterisation using ARDRA, ITS-PCR and RAPD-PCR techniques, species of *Bacillus* isolated from *tungrymbai* were identified as *Bacillus licheniformis* (25.5%), *Bacillus pumilus* (19.5%) and *Bacillus subtilis* (55%), and species of *Bacillus* from *bekang* were *Bacillus brevis* (2%), *Bacillus circulans* (7.5%), *Bacillus coagulans* (6.5%), *B. licheniformis* (16.5%), *B. pumilus* (9.1%), *Bacillus sphaericus* (4.6%), *B. subtilis* (51.8%), and *Lysinibacillus fusiformis* (2%). The most dominant bacterium in both products was *B. subtilis*.

1. Introduction

Consumption of naturally fermented soybeans is common among the different ethnic groups of people in the Oriental countries including the Himalayan regions of India, Nepal and Bhutan (Tamang, 2010). *Bacillus*-fermented sticky soybean foods of Asia are *natto* of Japan (Kubo et al., 2011), *chungkokjang* of Korea (Shin et al., 2012), *kinema* of India, Nepal and Bhutan (Tamang, 2003), *aakhone*, *hawaijar* and *peruyaan* of India (Tamang et al., 2012), *thua nao* of Thailand (Inatsu et al., 2006), *pepok* of Myanmar, and *sieng* of Cambodia and Laos (Nagai and Tamang, 2010).

Tungrymbai is a naturally fermented soybean food eaten as a side-dish in Meghalaya state of India (Tamang et al., 2009). During its preparation, local varieties of soybean seeds are washed, soaked for about 4–6 h, the outer skin is removed by rubbing gently between the palms and is cooked (1–2 h) until all the water is absorbed and the soybeans can be pressed easily. The cooked beans are allowed to cool, and are packed with leaves of *Clinogyne dichotoma* or *Phrynium pubinerve* lined in the bamboo basket and covered by a thick cloth or jute bag. The covered basket is kept near the fireplace (25–40 °C) for natural fermentation for 3–5 days to get a sticky product.

Bekang is a naturally fermented, sticky soybean food of Mizoram state in India (Tamang et al., 2009). Small sized, yellow variety of soybeans are soaked overnight, and boiled for 1–2 h until the soybeans are cooked. The cooked soybeans are then spread on a bamboo basket/tray lined with the leaves of *Callicarpa arborea* or leaves of *P. pubinerve*. Little amount of firewood ash is added. The bamboo basket/tray containing the cooked soybeans is then covered with the same leaves, again covered with a soft cloth, and kept near the fireplace (25–40 °C) to ferment naturally for 3–4 days.

Bacillus is the dominant bacterium in many sticky fermented foods of Asia (Meerak et al., 2007; Tamang et al., 2002). Species of *Bacillus* isolated from *kinema* include *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus sphaericus*, *Bacillus subtilis* and *Bacillus thuringiensis* (Sarkar et al., 1994, 2002; Tamang, 2003), however, *B. subtilis* is the dominant functional bacterium in *kinema* (Sarkar and Tamang, 1994; Tamang and Nikkuni, 1996). *Bacillus cereus*, *B. licheniformis* and *B. subtilis* were isolated from *hawaijar* of Manipur state of India (Jeyaram et al., 2008). *Bacillus amyloliquefaciens*, *B. licheniformis*, *Bacillus megaterium*, and *B. subtilis* subsp. *chungkokjang* were isolated from *chungkokjang*, naturally fermented soybean food of Korea (Kwon et al., 2009; Nam et al., 2012; Tamang et al., 2002). Japanese *natto* is the only *Bacillus*-fermented soybean food which is now produced by commercial mono-culture starter *B. subtilis* var. *natto* (Nishito et al., 2010). *B. subtilis* is a functional bacterium in Thai *thua nao* (Inatsu et al., 2006). *Bacillus* species have also been isolated from ethnic non-salted fermented locust bean (*Parkia biglobosa*) foods of Africa such as *dawadawa*, *iru* and *soumbala* which include *B. amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus badius*, *B. cereus*, *Bacillus firmus*, *Bacillus fumus*, *B. licheniformis*, *B. megaterium*, *Bacillus mojavensis*, *Bacillus mycoides*, *Bacillus pumilus*, *B. subtilis*, *B. sphaericus*, and *B. thuringiensis* (Ahaotu et al., 2013; Azokpota et al., 2006; Meerak

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Table 1
pH and microbial load of naturally fermented soybean foods of India.

Food	pH	Log cfu/g sample			
		Bacillus	LAB	Yeast	TVC
Tungrymbai of Meghalaya (n = 39)	7.4 (6.7–8.1) ± 0.2	8.2 (7.8–8.9) ± 0.1	4.1 (3.5–4.8) ± 0.1	2.8 (1.9–3.3) ± 0.2	8.9 (8.6–9.3) ± 0.1
Bekang of Mizoram (n = 43)	7.1 (6.8–7.5) ± 0.1	8.4 (7.8–8.7) ± 0.1	4.0 (3.2–5.4) ± 0.1	3.1 (2.3–3.6) ± 0.1	8.9 (8.5–9.4) ± 0.1

n, number of samples collected from different places of Meghalaya and Mizoram in India.
LAB, lactic acid bacteria; TVC, total viable count. Filamentous mould was not detected.
Data represents the means (±SD) of number of samples collected. Mean pH (±SD) of each sample is shown in parenthesis.

et al., 2008; Ouoba et al., 2010). The aim of this paper is to isolate and identify the predominant species of *Bacillus* in naturally fermented *tungrymbai* and *bekang* of India. These species were characterised using the molecular techniques such as amplified ribosomal DNA restriction analysis (ARDRA), internal transcribed spacer (ITS)-PCR and random amplified polymorphic DNA (RAPD)-PCR.

2. Materials and methods

2.1. Samples

A total of thirty-nine samples of *tungrymbai* and forty-three samples of *bekang* were collected from different villages and markets of

Table 2
Grouping of *Bacillus* based on the restriction profiles of ARDRA.

Sample	Strain	Putative species[a]	Restriction profile			ARDRA group	Species[b]
			*Hinf*I	*Rsa*I	*Cfo*I		
Bekang	BAV:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BD1:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK1:B15	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK1:B24	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK2:B6	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BME:B10	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BME:B20	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BME:B23	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B3	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B9	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B17	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B20	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB1:B10	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB1:B16	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB1:B17	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B8a	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B8b	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B11	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B13	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TM1:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TP1:B4a	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TP1:B4b	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TM1:B5	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TM1:B12	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TP1:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TS1:B6	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TS2:B13	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TSA:B15	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK1:B13	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Bekang	BT:B11	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP1:B2	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP1:B15	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP1:B5	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP2:B10	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Bekang	BAV:B12	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BK2:B8	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BK2:B12	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BME:B6	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TM2:B6	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TS1:B25	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TS2:B24	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TSA:B4	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TSB:B17	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BK1:B18	*B. cereus*	I	IV	V	Group IV	*B. cereus*
Bekang	BAV:B3	*B. subtilis*	I	IV	VII	Group V	*L. fusiformis*
Bekang	BAV2:B6	*L. fusiformis*	I	IV	VII	Group V	*L. fusiformis*
Bekang	BT2:B18	*L. fusiformis*	I	IV	VI	Group VI	*L. fusiformis*
Bekang	BAV:B15	*B. coagulans*	I	III	VI	Group VII	*B. subtilis*

[a] Identified by using the API-CHB kit.
[b] Species determined based on the homology identities of 16S rDNA sequences.