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BACILLUS SPP. FROM FERMENTED *TEF* DOUGH AND *KOCHO*: IDENTITY AND ROLE IN THE TWO ETHIOPIAN FERMENTED FOODS

Ayele Nigatu[1], Berhanu A. Gashe[2] and Tarekegn Ayele[3]

[1] Biodiversity Institute, PO Box 80928, Addis Ababa, Ethiopia

[2] University of Botswana, Private Bag 0022, Gaborone, Botswana

[3] PO Box 33944, Addis Ababa, Ethiopia

ABSTRACT: A significant population of Gram-positive, endospore-forming rods were isolated from fermenting *tef* dough and *kocho*. A taxonomic study showed that *Bacillus circulans*, *B. firmus* and *B. larvae* were common to both foods. The other species were, however, limited to one food or the other. *B. licheniformis* (56%) was the dominant species amongst the *tef* isolates while *B. larvae* (39.3%) was dominant in *kocho*. Most of these strains exhibited a wide spectrum of enzymatic activities and some showed antimicrobial effects against certain food-associated bacterial pathogens. Biochemical features of these bacilli led to the suggestion that they may play active metabolic roles and enrich the substrates for the succession and dominance of the lactic acid bacteria (LAB) which are essential for the characteristic fermentation of the two foods. Further studies are recommended to establish the effect of this group of bacteria and their metabolic products on human health.

Key words/phrases: *Enset*, *injera*, *kocho*, *tef* dough, traditional lactic acid fermentations

INTRODUCTION

In many traditional plant food fermentation processes *Bacillus* spp. breakdown starch into simple sugars which subsequently serve as substrates to initiate growth of organisms involved in fermentation, such as the lactic acid bacteria (LAB), as is the case in cassava (*Manihot esculenta*) (Adegoke and Babalola, 1988). Fermented cassava products are a major diet in Africa and Latin America (Okafor, 1992; Parada *et al.*, 1996) where strains of *B. subtilis* usually

set preconditions by degrading the starch so as to give way to the LAB and yeasts which predominate in the latter periods of fermentation. In this process, *Bacillus* spp. are known to change the texture, taste, flavour, aroma and appearance of the raw cassava to a detoxified product where the enzyme linamarase breaks down the cyanogenic glucosides (linamarin and latomarin) (Oyewolle, 1992).

Bacillus spp. are also known to be actively involved in the production of other foods such as, Japanese *natto* (a side dish with rice from soya beans, fermented by *B. natto/ B. subtilis*) and Sudanese *kawal, sigda* and *furundu* fermented by *B. subtilis* from *Cassia obtusifolia* leaves, *Sesame indicium* seed cake and *Hibiscus sabdoriffa* crushed seeds, respectively (Harper and Collins, 1992). In foods, such as the Equadorian fermented rice products-*arroz fermentado, arroz amarillo* and *arroz requmando*-bacilli, particularly *B. subtilis*, play prominent role in bringing about a golden cinnamon brown colour of the rice (Pederson, 1979).

In Ethiopia, amongst the various fermented plant foods, *injera* from *tef* (*Eragrostis tef*) and bread from *kocho*, pit fermented product of *enset* (*Ensete ventricosum*), are widely consumed dietary sources (ICNND, 1959). The processes of *tef* dough and *enset* fermentations were well described as lactic acid fermentations (Berhanu A. Gashe *et al.*, 1982; Berhanu A. Gashe, 1987). In addition to the major fermenting lactic acid bacterial genera (Berhanu A. Gashe *et al.*, 1982; Berhanu A. Gashe, 1987; Ayele Nigatu, 1992), the presence of members of the family *Enterobacteriaceae*, sporeformers and yeasts in the fermenting foods is also reported (Berhanu A. Gashe *et al.*, 1982; Chaltu Gifawossen and Abraham Besrat, 1982; Berhanu A. Gashe, 1985; Meaza Girma and Berhanu A. Gashe, 1985; Berhanu A. Gashe, 1987).

However, the sporeformers in general and the species of *Bacillus* in particular, associated with these two widely consumed Ethiopian diets have not yet been described. The present study was, therefore, aimed at identifying the common species of *Bacillus* in fermented *tef* dough and *kocho*, and assessing their metabolic and fermentative roles and the possible health implications to consumers.

MATERIALS AND METHODS

Samples

Tef (*Eragrostis tef*) grain was purchased from a market in Addis Ababa and ground into flour using a local flour mill. The flour was thoroughly mixed, in a glass jar, with tap water (flour:water, 1 kg:1.8 l, w:v ratio), mouth covered with lid and allowed to ferment at room temperature (18-22° C). Fermented *kocho* dough (*Ensete ventricosum*) was also purchased from a market in Addis Ababa.

Isolation and maintenance of gram-positive endospore-forming rods, yeasts and molds

Twenty-five ml portions of fermented *tef* dough (72 h) or 25 g portions of *kocho* dough were diluted with 225 ml sterile 0.1% peptone water diluent in screw capped brown bottles and heat-treated at 80° C for 10 min to enrich spore formers. The bottles were then cooled to room temperature. From appropriate serial dilutions, aliquots of sample were seeded in tryptone/soya/agar (TSA) (OXOID) in duplicate plates and incubated at 32° C for 2-5 days. Yeasts, molds and *Actinomycetes* were isolated from non heat-treated fermented *tef* dough and *kocho* samples and maintained using potato/dextrose/agar (OXOID); plates were incubated at 32° C for 2-5 days. Using morphological characteristics of well grown colonies on TSA and microscopic appearance of portions of 24 h culture in nutrient broth for Gram's reaction and possession of endospore, yeasts, molds and *Actinomycetes* were separated from the bacteria. Representative colonies of all Gram-positive, spore-forming, rod-shaped bacteria from TSA were then picked, purified and stock cultures maintained on agar slants for further identification. TSA also served for total count.

Identification of Bacillus *isolates*

Stock cultures from agar slants were subcultured into sterile nutrient broth (OXOID) and incubated at 30° C for 24 h. All isolates were further characterized using the dichotomous key of Norris *et al.* (1981).

Phenotypic tests and culture media

The following major physical and biochemical tests were employed to assign the aerobic endospore-forming rods into different species of *Bacillus*. These included Gram's reaction, size of cell, presence and position of spore, oxygen

requirement for growth using anaerobic medium, growth at 50 and 65° C, growth in 7% sodium chloride, production of catalase, reduction of nitrate to nitrite, pH and reaction in Voges-Proskaur test, decomposition of casein, hydrolysis of starch, and production of acid and/or gas from glucose. All of these tests were performed in duplicate and average results of three experiments were recorded. Corning Model 140 pH meter (USA) was used to measure pH.

Media employed in phenotypic tests included: nutrient broth (OXOID) for culture maintenance and for testing growth at different temperatures and salt tolerance; MRVP medium (OXOID) for acid and Acetyl methyl carbinol production. After 3, 5, and 7 days of incubation, pH of the medium was monitored. Acetyl methyl carbinol (VP) production was detected as recommended by Collins and Lyne (1976) and Norris *et al.* (1981). Milk/agar (OXOID) was utilized for testing casein hydrolysis (Collins and Lyne, 1976). Starch/agar (DIFCO) was prepared by adding 0.5% soluble potato/starch into nutrient agar (DIFCO) and employed for testing hydrolysis of starch. Thioglycollate medium (OXOID) was used to determine anaerobic growth.

***Isolation and identification of non-sporing microflora from* tef *dough and* kocho**

Twenty-five ml portions of fermented *tef* dough (72 h) or 25 g of *kocho* dough were diluted in 0.1% sterile peptone water from which serially diluted aliquots were seeded in plates of appropriate media. Incubation of plates for members of the family *Enterobacteriaceae* and LAB was carried out, at 37 and 32° C, for 1 and 2–5 days, respectively.

Culture media utilized for asporogenous bacteria

Culture media used for isolation, purification and maintenance of non-sporeforming bacteria from fermented *tef* or *kocho* were the following: Mac Conkey agar (OXOID) for members of the family *Enterobacteriaceae*, Rogosa agar (OXOID) for lactobacilli; dextrose/tryptone/agar (OXOID) for pediococci; sucrose/gelatin/agar (prepared as follows, $g(l)^{-1}$ of distilled water: sucrose, 10; gelatin, 20; nutrient broth, 13; agar, 15; all chemicals were from BDH, England) for *Leuconostoc* species and Slanetz and Bartley medium (OXOID) for streptococci. The LAB were isolated following procedures described earlier (Berhanu A. Gashe, 1985; 1987).

Determination of moisture content

The method described by Welcher (1975) was modified and employed as follows. Food samples (10 g) were placed in an oven desiccator at 95° C for 24 h. Moisture content was calculated based on differences in weight before and after drying.

Determination of reducing sugars

Reducing sugars were determined after Miller (1959) using dinitrosalicylic acid reagent (DNS), prepared by dissolving 8.0 g of dinitrosalicylic acid, 1.6 g of phenol, 0.4 g of sodium sulfite, 160.0 g of potassium/sodium/tartarate and 8.0 g of sodium hydroxide in 800 ml of distilled water. The reagent was kept in brown bottles until use.

To each 1 ml portion of the supernatant liquids from *tef* or *kocho* dough, 1 ml of DNS reagent was added and mixed thoroughly. This was placed in boiling water bath for 5 minutes. After cooling, 5 ml of distilled water was added and the absorbance read at 540 nm using a spectrophotometer (Spectronic 21, Bausch and Lomb, USA). The amount of reducing sugars present in the samples was extrapolated from a curve prepared using glucose as a standard.

Determination of free amino acids

Free amino acids were quantified using a modification of the method outlined by Riemeredes and Klostermayer (1976). Ninhydrin solution was prepared by dissolving 5 g of Ninhydrin in 188 ml of methyl cellosolve. This was then combined with 62 ml of 0.1% $SnCl_2$ in sodium/acetate buffer, pH 5.5. The buffer was prepared by combining 5.44 g of sodium/acetate, trihydrated, in 100 ml of distilled water and 100 ml of glacial acetic acid. The final volume was raised to one liter after pH adjustment.

To one ml aliquots of the clear supernatants (food samples) obtained by centrifuging, 1 ml of the ninhydrin solution and 1 ml of the acetate buffer were thoroughly mixed and the tube was immersed in boiling water for 10 minutes. The intensity of the colour that developed, quantified using a spectrophotometer at 570 nm, was used to estimate free amino acids using aspartic acid as a standard. In all cases mean values of three experiments were recorded.

RESULTS AND DISCUSSION

Fermented *tef* dough (72 h) and *kocho* when purchased had pH values of 4.0 and 4.3, respectively. Their nutrient composition is shown in Table 1. The predominant and important fermentative microflora isolated from these acidic foods are presented in Table 2. In this study members of the family *Enterobacteriaceae* were hardly isolated from *kocho*. The overall microbial load per gram of adequately fermented *tef* dough (fermented for 72 h) was higher than the same weight of fully fermented *kocho*. *Bacillus* spp. in both foods had lower counts than the other fermentative microflora.

A total of 25 and 28 strains of *Bacillus* were isolated from fermented *tef* dough and from *kocho* samples, respectively (Tables 3 and 4). The different strains isolated from both foods were further tested for growth under different temperatures, conditions of oxygen, concentrations of salt and utilization of various biochemicals. The isolates from *tef* were accordingly assigned into six species of *Bacillus: Bacillus licheniformis* (56%), *B. circulans* (12%) and *B. laterosporus*, *B. firmus*, *B. alvei* and *B. larvae* (each comprising 8%). On the other hand, isolates from *kocho* belonged to the following 10 species: *B. larvae* (39.3%), *B. popilliae* (14.29%), *B. sphaericus* and *B. coagulans* (each, 10.71%), *B. firmus* (7.14%) and *B. polymyxa*, *B. circulans*, *B. megaterium*, *B. subtilis* and *B. thuringensis* (each, 3.57%).

Table 1. Nutrient composition of fermented *tef* and kocho. The values are mean results of four food samples in triplicate experiments.

Type of food	Fermentation time	pH	Content per g dry wt of *tef* flour or *kocho*		
			Moisture (%)	Reducing sugars (mg)	Free amino acids (mg)
Tef dough	72 h	4.0	66.4±2.2	34.2±1.0	4.3±0.1
Kocho dough	Purchased fermented	4.3	44.0±3.5	0.73±0.06	5.6±0.3

Table 2. Microbial composition of fermented *tef* and *kocho*. Each value is a mean of four food samples from triplicate experiments. Populations of molds and other spore-formers were variable and not presented here as they are not involved as fermentative microflora.

Food type	pH	Estimated number of organisms per g dry wt of *tef* flour or *kocho*							
		Enterobacteriaceae	*Lactobacillus* spp.	*Pediococcus* spp.	*Leuconostoc* spp.	*Streptococcus* spp.	*Bacillus* spp.	Yeasts	Total count
Tef dough (72 h)	4.0	$1x10^4$	$3.5x10^7$	$3.3x10^8$	$3.7x10^6$	$1x10^5$	$2x10^5$	$1.4x10^5$	$3.7x10^8$
Kocho dough (as purchased)	4.3	—	$5.2x10^6$	$2.3x10^6$	$1.9x10^4$	<10	$1.4x10^4$	$2.8x10^5$	$7.8x10^6$

—, No growth.

The isolates from *kocho* showed larger number and greater diversity than those from *tef*. *B. larvae*, *B. circulans* and *B. firmus* were common to both of these plant-based fermented foods. Species, such as *B. licheniformis*, *B. laterosporus* and *B. alvei* were restricted to *tef* whereas *B. subtilis*, *B. sphaericus*, *B. polymyxa*, *B. popilliae*, *B. megaterium*, *B. coagulans* and *B. thuringensis* were limited only to *kocho* samples.

As shown in Table 1, fermented *tef* dough (72 h, pH 4.0) and fermented *kocho* (pH 4.3) are acidic foods mainly fermented for improved qualities. The pH values dropped arising from accumulation of non-volatile organic acids, including lactic acid (Melaku Umeta and Faulks, 1989). During the onset of fermentation, the raw foods had near-neutral pH values, wherein the case of *tef* flour it was 6.6 and in *kocho* 6.7. The final pH values of completely fermented products from both foods, not only contributed to the characteristic aroma and taste of the foods, but also to the inhibitory properties against most spoilage and food-borne bacterial pathogens (Meaza Girma *et al.*, 1989; Ayele Nigatu, 1992; Ayele Nigatu and Berhanu A. Gashe, 1994a,b).

As shown in Tables 3 and 4, however, the spectrum of the species as well as the composition and number of strains in the two foods were not the same. In whatever way they differ, both foods also shared same species of *Bacillus*, such as *B. circulans*, *B. firmus* and *B. larvae*.

Threshing of *tef* seeds is usually carried out on a dry ground plastered with cow dung, where domestic animals, such as oxen and cows, are used for trampling, to shake-off the seeds (Berhanu A. Gashe *et al.* 1982; Berhanu A. Gashe, 1987). *Kocho* on the other hand remains entirely moist throughout its processing. The fermentation conditions of the two foods and their environments also differ. *Kocho* is fermented outdoors in underground pits covered by the remaining litter from decorticated *enset* plants while *tef* is fermented indoors in partially-clean, earthen, plastic, wooden or metallic vessels. In both foods, however, the microbial constituents come mainly from starters used to initiate the fermentations, and/or the processing ground, plants and animals (Berhanu A. Gashe, 1987; Ayele *et al.*, Unpublished data).

Table 3. Species composition and characteristics of *Bacillus* isolates from fermented *tef* dough (72h).

-, all strains negative; (-), 78.5% of strains negative; +, all strains positive; ±, 50% of strains positive; (+), 91.4% of strains positive; A(F), one strain aerobic and the other facultative; F, facultative; R, rod.

	Isolate number and properties					
Characteristics	1,5,7,8,9,11,12,15,16,17,19,22,24,25	2,3,18	4,10	6,21	13,23	14,20
Gram	+	+	+	+	+	+
Shape	R	R	R	R	R	R
Catalase	+	+	+	+	+	-
Voges-Proskaur	+	-	-	-	+	±
Oxygen relationship	F	F	A(F)	A(F)	A(F)	A(F)
Growth at 50° C	+	-	-	±	-	+
Growth at 65° C	-	-	-	-	-	-
Growth in 7% NaCl	+	+	±	-	±	±
Acid & gas from glucose	-	-	-	-	-	+
Nitrate to nitrite	+	+	+	+	-	+
Hydrolysis of starch	±	+	-	+	±	+
Hydrolysis of casein	(-)	+	±	±	+	+
Rod width ≥1.0 μm	-	-	-	-	-	-
pH in V-P<6.0	(+)	+	±	-	+	+
Acid from glucose	+	+	±	+	+	+
Identified as	*B. licheniformis*	*B. circulans*	*B. laterosporus*	*B. firmus*	*B. alvei*	*B. larvae*

Table 4. Species composition and characteristics of *Bacillus* isolates from *kocho*. -, all strains negative; (-), 63.6% of strains negative; (-)*, 66.7% of strains negative; (-)**, 72.7% of strains positive; (-)***, 81.8% of strains negative; +, all strains positive; ±, 50% of strains positive; (+), 66.7% of strains positive; (+)*, 81.8% of strains positive; A, aerobic; AN, anaerobic; F, facultative; F-A, facultative to aerobic; R, rod.

Characteristics	Isolate number and properties									
	1	2,11,15	3,4,5,7, 12,13,19, 23,24,25,26	6	8,9	10,14,21,22	16	17	18,27,28	20
Gram	+	+	+	+	+	+	+	+	+	+
Shape	R	R	R	R	R	R	R	R	R	R
Catalase	+	+	-	+	+	-	+	+	+	+
Voges-Proskaur	-	-	±	-	-	±	-	-	-	+
Oxygen relationship	AN	F-A	F-A	F	A	F	F	A	F	AN
Growth at 50° C	-	-	(-)	-	-	±	-	-	-	+
Growth at 65° C	-	-	-	-	-	-	-	-	-	-
Growth in 7% NaCl	+	(+)	+	-	+	+	-	+	(+)	-
Acid & gas from glucose	-	-	-	+	-	-	-	-	-	-
Nitrate to nitrite	+	-	(-)***	-	+	+	-	-	-	+
Hydrolysis of starch	+	+	+*	+	+	±	+	+	+	-
Hydrolysis of casein	+	(-)*	+	+	-	-	-	+	+	-
Rod width ≥1.0 μm	+	+	+	+	+	+	-	+	+	+
pH in V-P<6.0	-	(-)*	(-)**	-	+	±	-	+	+	+
Acid from glucose	+	-	(-)	+	+	+	-	+	+	-
Identified as	*B. subtilis*	*B. sphaericus*	*B. larvae*	*B. polymyxa*	*B. firmus*	*B. popilliae*	*B. circulans*	*B. megaterium*	*B. coagulans*	*B. thuringensis*

The larger number and higher diversity of bacilli from *kocho* than from *tef* could be attributed to the constant contact of *kocho* with wet surfaces and soil starting from the time of cultivation until termination of fermentation. The dry and closed storage of *tef* seeds in containers, such as sacks, traditional granaries and the like for years, coupled with the thorough sifting and clearing of dust before milling as well as the clean indoor fermentation of the dough, could have minimized the chance for continuous influx and thus to the reduced diversity of these bacilli therein.

The two foods also had distinct species associated with each of them, such as *B. popilliae*, *B. sphaericus* and *B. coagulans* to *kocho* and *B. licheniformis*, *B. alvei*, and *B. laterosporus* to *tef*. This may also relate to the higher chance of *kocho* for contact with diverse groups of insects and worms during processing and fermentation as a source of nourishment as opposed to the dry *tef* seeds which interact differently with microorganisms.

Bacillus spp. can grow on a variety of substrates as the result of their diverse enzymatic activity. Most species of *Bacillus* are known to produce α amylase, (Aderibigbe *et al.*, 1990; Parada *et al.*, 1996) and may hydrolyse starch in *tef* and *kocho* in the early phases of fermentation into simple sugars (Adegoke and Babalola, 1988; Lealem Fikru and Berhanu A. Gashe, 1994).

Most of the isolates had proteolytic enzymes which might degrade the available proteins and also anabolize them into essential amino acids thereby supporting growth of the fastidious LAB. This continuous supply of free amino acid and simple sugars could apparently support growth of microorganisms, especially of those dominating in the latter stages of fermentation (Table 1) (Berhanu A. Gashe, 1985; Ayele Nigatu, 1992). This event also agrees with the fact that organisms such as *B. megaterium* are equipped with the entire enzymatic machinery necessary to make all compounds required for growth (Gottschalk, 1986). This species was common in the starchy food, *kocho*, but not in *tef*, probably enriching the low-protein food, *kocho*, with indispensable nutrients, a likely and beneficial association especially for the LAB.

Species like *B. circulans* are known to produce the antibiotic circulin; *B. subtilis* subtilin, mycobacillin, bacitracin and bacillin; *B. polymyxa* polymyxin and aerosporin (Shoji, 1978). *Tef* and *kocho* doughs may possess these metabolites.

On the other hand, though infrequently, certain species of *Bacillus*, other than the well recognized pathogens such as *B. anthracis* and *B. cereus* are known to be involved in human disease processes. In most cases, however, these bacilli are not known as causative agents and are rather found associated with infected organs (Norris *et al.*, 1981). Therefore, the status of these bacilli in traditional foods remains controversial.

Nevertheless, although no confirmatory experiments were done to assess whether our mesophilic isolates are non-pathogenic to man, the fermentation and baking processes could inactivate these endospore-forming bacilli. The absence of *B. cereus* and clostridia in both of these two traditional fermented foods may also serve as a good clue to their safety and wider consumption by millions of people.

Further work to quantify the amounts and qualify whether or not their secondary metabolites are toxic to consumers in the monotonous diet would be essential for there are indications that antibiotics produced by species like *B. licheniformis* to have a nephrotoxic effect on animals including man (Thimann, 1963).

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