Chapter 8
Other Fermentations

Christèle Humblot and Jean-Pierre Guyot

Abstract Fermented foods are staples for numerous consumers in many countries, especially in developing and emerging countries (DEC) where fermentation is often the only way to preserve food from microbial contaminations. Fermented foods from DEC are characterized by their wide diversity: they differ in the starting material (cereals, pulses, roots, vegetables, etc.), technology of production and the main microorganisms implicated in the fermentation process (lactic or acetic acid bacteria, yeasts, etc.). For cash crops like cocoa and coffee, fermentation is also an important step in the processing of cocoa beans and coffee cherries.

Compared to their large diversity, very few DEC fermentations were investigated using culture-independent methods. Mexican pozol and Korean kimchi can be considered model fermented foods for cereals and vegetables, respectively, to which culture-independent methods were applied for the first time. These techniques were also applied to other starchy foods, such as cassava dough, and more recently to investigate the microbial ecology of "rice black-vinegar" in Japan, cocoa and coffee fermentations. Such novel approaches are expected to improve the knowledge of the microbiology associated to these particular fermented foods.

1 Introduction

Fermented foods are essential components of the diet in many countries. Traditional know-how was transmitted and sometimes improved over centuries, contributing to the uniqueness of these foods and to the socio-cultural identity of their consumers. The fermentation is realized by microorganisms that play a key role in the physical, nutritional and organoleptic modification of the raw material. Researchers and the agro-food industry have paid close attention to these microorganisms for many years. Nevertheless, most of the studies focused on the fermented foods consumed in Western countries, mainly dairy products but also some vegetable-based foods and beverages like sauerkraut and wine, casting aside the numerous other fermented products that are staples or important economical resources for many consumers in developing and emerging countries (DEC). In Western countries food fermentations are often integrated in marketing strategies to construct nutritional claims, in

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response to the increased attention paid by consumers to a healthy way of life, and to address specific organoleptic characteristics. This is, of course, a shortcut to emphasize the gap between the expectations of consumers from these countries and those of most developing countries and rural areas of emerging countries where, before other considerations, fermentation is still the unique and cheapest way to preserve food. The necessity of bringing safe food to DEC populations could be illustrated by the recurrent data from international organizations. It is estimated that 1.5 billion episodes of diarrhea occur annually in children under the age of 5, resulting in some 1.8 million deaths. Up to 70 percent of diarrheal episodes in DEC may be caused by food-borne contaminants (WHO 1997; Käferstein 2003). These public health considerations are very important, however, the economical importance of some raw materials must also be taken into account. For instance, important cash crops like cocoa and coffee are produced in DEC and a fermentation step is an important stage of the processing of cocoa beans and coffee cherries, which contributes to the organoleptic characteristics and facilitates the removal of the mucilage.

Without a doubt, culture-dependent methods brought to light important knowledge about those foods. However, our level of understanding must be deepened by using molecular approaches, mainly those that use culture-independent methods, for getting complementary or new information on previously studied fermentations, and to investigate other foods not yet or seldom studied. Molecular methods are essential to investigate the microbiota of fermented foods through community analysis, detection and characterization of yet-uncultured microorganisms, and to investigate the relationships between microbial diversity and functional properties. In this chapter such molecular approaches will be described for some fermented foods from DEC that differ in their raw materials and processing conditions.

2 Fermented Foods from Developing and Emerging Countries: A Brief Overview

The traditional fermented foods from DEC are very diverse. Table 8.1 lists some of the most common products prepared and consumed in various countries. A large variety of raw materials is used: cereals such as millet, maize, rice or sorghum; roots such as cassava or taro; pulses such as bean, chickpea or soybean, but also cocoa beans and coffee cherries. Fermented foods are prepared mainly in Africa and Asia, but also in Latin America and the Pacific Islands. They are consumed as beverages, gruels, porridges, soups, etc. and designated by specific names (Beuchat 1997). Sometimes different vernacular names can be used for the same or similar foods, generating some confusion in their identification. For example, ogi is also called akamu or agidi; moreover, it can be made of maize, millet or sorghum using similar processes (FAO 1993). Technologies used to make these foods are very diverse, resulting in complex changes of biochemical, sensory and nutritional characteristics. There can be one or several fermentation steps lasting from a few hours to several months, depending on the food. Fermentation is mostly natural (spontaneous) and,

therefore, noncontrolled but sometimes starter cultures are employed; for example, *Bacillus subtilis* causes the viscous appearance and texture of natto, a Japanese fermented food (Beuchat 1997). The microorganisms involved in the natural fermentations are more often lactic acid bacteria (LAB) and yeasts. They are responsible of the modification of the raw materials by their numerous lytic activities (proteolytic, amylolytic, lipasic, phytasic, etc.), and in the case of LAB, they allow the preservation of foods by inhibiting the development of spoilage and food-borne pathogens through acidification and bacteriocin production. However, the recognized importance of LAB and yeast should not dim the role of some fungi and *Bacillus* in producing some fermented foods or condiments. Fungi are responsible for soybean fermentation to produce tempeh and sufu (Kiers, et al. 2000; Han, et al. 2001), two important Asian fermented foods, whereas *Bacillus* species are responsible for the fermentation of African locust beans and soybean to produce soumbala and kinema, respectively (Sarkar, et al. 2002 ; Ouoba, et al. 2004). Soumbala is used as a flavoring agent in Burkina Faso, whereas kinema serves as a major source of protein in Nepal.

Most of the fermented foods presented here have been studied using a classical culture-dependent approach that selects a fraction of the existing microbial population. Microorganisms with important functional properties (e.g., production of antimicrobial compound, enzymes able to hydrolyze anti-nutritional factors, etc.) could, therefore, be underestimated by those which thrive on classical cultivation media or are numerically dominant in their natural environment. Moreover, the studies on fermented foods from DEC often describe the microbiota from only a few samples and do not take into account the possible variability between different production units and geographical areas. It is, therefore, sometimes difficult to draw general conclusions since the representativeness of results could be questioned. Classical microbiological methods are too heavy to implement when the microbiota of many samples coming from different production units and areas have to be analyzed. Food microbial ecology has known new developments since the advent of molecular methods which allowed a global approach without bias due to cultivation techniques (Ercolini 2004). To date, only a limited number of foods from DEC were studied by using such methods. The first works using culture-independent methods to investigate the microbiota of fermented foods from DEC were on maize-based foods, mainly pozol, a Mexican beverage made of a fermented maize dough, and kimchi, a Korean fermentation of vegetables. A few works present interesting results on cassava-, sorghum-, rice- and soybean-based fermented foods, and on cocoa and coffee.

3 Application of Molecular Methods to Cereal, Cassava and Soybean Fermentations

3.1 Maize-based Fermented Foods

Pozol (Table 8.1) is the first fermented food to which a culture-independent approach was applied to study the food microbiota in combination with the use of

Table 8.1 Examples of Traditional Fermented Foods Consumed in Various Countries

Raw material	Product	Country	Main microorganisms	Nature of product	Product use
African locust bean	Soumbala	Burkina Faso	*Bacillus spp.*	Solid	Condiment
African locust bean	Dawadawa	Nigeria	*Bacillus subtilis, Bacillus licheniformis*	Solid	Side dish with rice
Cabbage/ vegetables	Kimchi	Korea	LAB	Solid	Eaten with rice
Cassava	Chickwangue	Congo	LAB	Paste	Staple
Cassava	Gari	West Africa	LAB, yeasts	Flour	Staple
Cassava	Lafun	West Africa	LAB	Paste	Staple
Cassava	Cassava sour starch	Colombia, Brazil	LAB	Wet flour	Bread making
Chickpea and wheat	Khaman	India	LAB	Solid, cake-like	Breakfast
Chickpea and wheat	Dhokla	India	LAB	Spongy	Staple
Fish	Fish sauce	Asia	Bacteria	Liquid	Seasoning
Maize	Poto-poto	Congo	LAB	Paste	Breakfast, mush
Maize	Kenkey	Ghana	LAB, yeasts	Dough	Staple, stiff mush
Maize	Pozol	Mexico, Guatemala	LAB	Dough	"Food-beverage"
Maize, millet, sorghum	Ogi, mawè	West Africa	LAB, yeasts	Paste, porridge	Staple, mush
Maize, cassava	Banku	Ghana	LAB, yeasts	Dough	Staple
Maize, cassava	Mahewu	South Africa	LAB (*Lactobacillus delbrueckii*)	Liquid	Drink
Maize and finger millet malt	Togwa	East Africa	LAB	Liquid	Drink
Pearl millet	Bensaalga, koko	Burkina Faso, Ghana	LAB	Liquid	Mush
Rice	Puto	Philippines	LAB, other bacteria, *Saccharomyces*	Solid	Snack
Rice	Black rice vinegar	Japan	LAB, Acetic acid bacteria, yeasts, fungi	Liquid	Seasoning
Rice and bean	Dosai	India	*Lc. mesenteroides*, yeasts	Spongy, pancake-like	Breakfast
Rice and bean	Idli	India	LAB (*Lc. mesenteroides*), yeasts	Spongy, bread	Bread substitute

(continued)

Table 8.1 Examples of Traditional Fermented Foods Consumed in Various Countries (continued)

Raw material	Product	Country	Main microorganisms	Nature of product	Product use
Rice and soybeans or other cereals	Miso	Japan, China	LAB, yeasts	Paste	Soup base, seasoning
Sorghum	Hussuwa	Sudan	LAB	Dough	Staple
Sorghum and cassava	Burukutu	Nigeria	LAB, *Candida* spp., *Saccharomyces cerevisiae*	Liquid	Drink
Sorghum, maize	Pito	Nigeria	LAB, yeasts	Liquid	Drink
Soybean	Kinema	Nepal	*B. subtilis*, *Enterococcus faecium*	Solid	Staple
Soybean	Natto	Japan	*B. subtilis*	Moist, mucilaginous	Meat substitute
Soybean	Tempeh	Indonesia, Malaysia	*Rhizopus* spp.,	Solid	Meat substitute
Soybean	Sufu	China	*Rhizopus*, *Mucor*, *Actinomucor*	Creamy cheese-type	Like cheese
Soybean and wheat	Soy sauce	Asia	LAB, *Aspergillus oryzae*, *Zygosaccharomyces rouxii*	Liquid	Seasoning
Taro	Poi	Hawaii	LAB, *Candida vini*, *G. candidum*	Semi-solid	Side dish with fish, meat

culture-dependent methods in a polyphasic approach. Pozol is a traditional fermented maize dough prepared by Indians and Mestizos in Mexico and Guatemala. Nixtamalization is the first step during which kernels of white maize are cooked in the presence of lime. Thereafter, the grains are washed to remove the pericarps, coarsely ground, shaped into balls, wrapped in banana leaves and allowed to ferment at ambient temperature for two to seven or more days. The resulting fermented dough is suspended in water and drunk as a refreshing beverage. A wide variety of microorganisms, including yeasts, fungi and bacteria, were isolated from this spontaneous fermentation (Wacher, et al. 1993). LAB are by far the dominant microflora (Wacher, et al. 1993; Ampe, et al. 1999a; Ampe, et al. 1999b; ben Omar, et al. 2000a; Escalante, et al. 2001).

The abundance of the active microbial populations was evaluated by using a quantitative RNA hybridization method employing rRNA-targeted taxon-specific oligonucleotide probes (Ampe, et al. 1999a). The authors found that eukaryotes represented less than 6 percent of the microorganisms in pozol and, within bacteria, LAB were the most abundant microorganisms. Among LAB, there was a dominance of

Lactobacillus (20 percent to 65 percent) and *Streptococcus* (25 percent to 75 percent) while *Lactococcus* and *Leuconostoc* represented only a minor fraction of the bacterial community (Ampe, et al. 1999a; Ampe, et al. 1999b; ben Omar, et al. 2000a). The authors also investigated the spatial and temporal distribution of the microorganisms in pozol. They found more bacteria in the center of the ball than in the periphery, except for *Lactobacillus*, bifidobacteria and enterobacteria. Using this method, it was also possible to determine the succession of microflora. During pozol fermentation, the relative abundance of bifidobacteria, *Streptococcus* and *Lactobacillus* increased while it decreased for *Lactococcus* and *Leuconostoc* (Ampe, et al. 1999a; ben Omar, et al. 2000a). As for *Lb. fermentum*, it increased until 48 hours of fermentation and decreased at the end of the process (ben Omar, et al. 2000a). The results of rRNA quantification were compared to plate counts. LAB represented more than 90 percent of the active population, based on rRNA quantification, but only 10 percent to 50 percent by plate counts (Ampe, et al. 1999a, 1999b).

In parallel to the hybridization method, Ampe, et al. (1999b) used PCR-DGGE for community analysis. The PCR-DGGE fingerprints showed the presence of 18 bands for bacteria without major differences between the periphery and the center of the dough, and 20 faint bands at the periphery of the dough for eukaryotes (ben Omar, et al. 2000a). The major shift during fermentation of maize took place between 24 and 72 hours. The majority of the bands in the DGGE fingerprints of the bacterial community of pozol was sequenced and identified as *Acetobacter* sp., *B. subtilis, Lb. delbrueckii, Exiguobacterium acetylicum, Enterococcus saccharolyticus, Bifidobacterium minimum, Lb. casei* subgroup, *Streptococcus* sp., *Weissella-Leuconostoc* group and *Lb. plantarum-pentosus* group (Ampe, et al. 1999b; ben Omar, et al. 2000a). DNA extracted from each bacterial isolate from pozol was submitted to PCR-DGGE. They found that seven out of the 136 LAB strains isolated did not co-migrate with any of the bands identified in pozol samples, but conversely eight bands did not correspond to any of the isolates, in particular they failed to isolate the *Streptococcus* strain. These results illustrated that cultivation methods have limitations and molecular methods can fail in giving an exact picture of reality. This might be explained, in part, by biases due to PCR conditions and to preferential amplification of dominant and subdominant bacteria (Ercolini 2004). Another method, also based on a metagenomic approach, was used to study the pozol microbiota (Escalante, et al. 2001). Following the extraction of total DNA from the pozol, the 16S rRNA gene was amplified and cloned, and then sequenced and the corresponding bacteria were identified. The authors identified the species *L. lactis, St. suis, Lb. plantarum, Lb. casei, Lb. alimentarius, Lb. delbruekii* and *Clostridium* sp. Consistently with other results (Ampe, et al. 1999a, 1999b; ben Omar, et al. 2000a) these species, except for *Clostridium* sp., were dominant as determined by 16S rRNA hybridization and sequencing of amplicons from DGGE bands. The pozol samples also contained *Bifidobacterium, Enterococcus* and enterobacteria, suggesting a fecal origin of some microorganisms (Ampe, et al. 1999b; ben Omar, et al. 2000a; Escalante, et al. 2001).

Different fermented maize-based foods – namely pozol, poto-poto and ogi from Mexico, Congo and Benin, respectively – (Ampe, et al. 2000) were compared on the basis of their DGGE profiles. To obtain poto-poto, maize is soaked (12 to 96 hours),

ground and sieved; then, water is added and the mixture is decanted (10 hours to 5 days) before putting the slurry in a bag and fermented for 10 hours to 7 days. Ogi is obtained after adding boiling water to the maize, soaking (24 hours), draining, grinding and sieving; then, water is added and the mix is fermented for two to seven days. Cluster analysis of DGGE profiles obtained for each type of food showed that microbial communities grouped in distinct clusters corresponding to a distinct type of foodstuff. Within a cluster similar richness and biodiversity indexes were shared. Interestingly, biodiversity and richness indexes were higher in pozol samples; the authors attributed these results to process conditions, e.g., higher initial pH of the product due to nixtamalization and some limited oxic conditions. Three DGGE bands corresponding to *Lb. plantarum, Lb. delbrueckii* and *Lb. fermentum* species were common to all the foods of different origins, suggesting that these species were particularly well adapted to the fermentation of maize (Ampe, et al. 2000). A similar approach was applied by Abriouel, et al. (2006) to poto poto and dégué (a pearl millet fermented dough from Burkina Faso). The authors performed community analysis by using temporal temperature gel electrophoresis (TTGE) to separate the PCR products obtained by amplification of the V3 region of the 16S rRNA gene from total DNA extracted according to three different methods, and concluded that the performance of DNA extraction largely depends on the food composition.

For the study of kenkey no culture-independent methods were applied, but isolates were characterized by applying molecular methods for identification and typing. Kenkey is a Ghanaian fermented maize dough, obtained by steeping the maize for 24 to 48 hours, followed by a milling step. The milled maize is reconstituted with water to form a stiff dough which is packed in troughs and left to ferment spontaneously for 48 to 72 hours. Hayford, et al. (1999) studied the diversity of *Lactobacillus* isolated from kenkey (Table 8.1) by random amplified polymorphic DNA (RAPD)-PCR. The authors showed that *Lb. fermentum* was dominant among other *Lactobacillus* spp. and had a large intraspecific diversity.

3.2 *Sorghum-based Fermented Foods*

Kunene, et al. (2000) investigated the lactic acid microflora in a sorghum powder and corresponding fermented cooked porridge made in South Africa. They used a similar polyphasic approach to that of Hayford, et al. (1999) for kenkey, except that diversity among the sorghum LAB isolates was analyzed by using both total soluble proteins and amplified fragment length polymorphism (AFLP). The authors found that the majority of the LAB belonged to *Lactobacillus* and *Leuconostoc* genera and, to a less extent, to *Lactococcus* and *Pediococcus* genera. The analysis of diversity by AFLP showed that the dominant *Lb. plantarum* strains were originated from the sorghum powder while the dominant *Lc. mesenteroides* strains were originated from both the sorghum powder and the household environment.

In a similar approach Yousif, et al. (2005) studied the *Enterococcus* population of husuwa, a semi-solid sorghum-based fermented food (Table 8.1). Using RAPD

fingerprinting, 16S rRNA sequencing and RFLP analysis, the authors found that all the *Enterococcus* isolates were *E. faecium* species with a great genetic diversity (Yousif, et al. 2005).

3.3 *Cassava-based Fermented Foods*

Three cassava-based fermented products have been studied, namely the cassava sour starch from Colombia, a fermented cassava dough and gari from the Congo (Table 8.1) using a combination of culture-independent methods and cultivation techniques.

Cassava sour starch processing starts by washing and peeling the roots, that are then grated in rotors with perforated blades. The starch present in the suspension is separated by sieving from the pulp or bran; the slurry from sieving is allowed to settle and particles of fibers and other fine materials that are not removed by sieving are separated from the slurry. The wet starch is then passed through a series of tanks in which it remains for several weeks (ben Omar, et al. 2000b). Gari is prepared by grating the cassava root, followed by dewatering, fermentation for two days at ambient temperature and roasting of the fermented mash (Kostinek, et al. 2005).

The microbiota of cassava sour starch was studied by RAPD-PCR, plasmid profiling, hybridization using rRNA phylogenetic probes and partial 16S rRNA gene sequencing. A large diversity of bacteria and yeasts was described, namely *Lb. manihotivorans, Lb. plantarum, Lb. casei, Lb. hilgardii, Lb. buchneri, Lb. fermentum, Lb. perolans, Lb. brevis, Lc. mesenteroides, Pediococcus* sp., a low number of *B. cereus, Galactomyces geothricum, Issatchenkia* sp. and *Candida ethanolica* (ben Omar, et al. 2000b; Lacerda, et al. 2005). However, the most frequently isolated species were *Lb. plantarum* and *Lb. manihotivorans* with a large molecular diversity as revealed by RAPD analysis (ben Omar, et al. 2000b). Using PCR-DGGE followed by sequencing of the most intense bands, Ampe, et al. (2001) confirmed that LAB were the dominant organisms, mainly close relatives of *L. lactis, Streptococcus* sp., *E. saccharolyticus, Lb. plantarum, Lb. panis, Lc. mesenteroides* and *Lc. citreum* (Ampe, et al. 2001). As the PCR products from *Lb. manihotivorans* and *L. lactis* co-migrate, a complementary analysis using hybridization of 16S rRNA with phylogenetic probes was necessary to detect the presence of the species *Lb. manihotivorans* (Ampe, et al. 2001). The use of molecular methods showed that in sour cassava starch, *Lb. manihotivorans* could represent up to 20 percent of total LAB (Ampe 2000; Ampe, et al. 2001). It is, therefore, not surprising that in a previous study on the amylolytic LAB in this Colombian niche product, *Lb. manihotivorans* strains were isolated for the first time (Morlon-Guyot, et al. 1998). In this case the isolation of a new microorganism preceded its detection by molecular tools which, in turn, enabled scientists to confirm its status as dominant LAB. In contrast, another study using PCR-DGGE and sequencing of 16S rRNA genes allowed detecting the presence of this bacterium in a Congolese fermented cassava dough, whereas cultivation techniques failed in isolating it (Miambi, et al. 2003).

As for the African fermented cassava dough, Miambi, et al. (2003) coupled the two approaches: they compared the results obtained by using PCR- DGGE and classical culture-dependent methods, from samples submitted or not to an enrichment culture step. It appeared that DGGE profiles of total DNA of cassava dough exhibited 10 distinguishable bands. As it could be expected, DGGE fingerprints of bacteria recovered from enrichment cultures of fermented dough gave variable profiles containing fewer bands. Bands corresponding to five bacterial species detected by direct PCR-DGGE of total DNA from cassava dough were also observed in DGGE patterns from enrichment cultures. Eighteen strains were isolated from cultures selected on the basis of their DGGE banding patterns. The sequence of DGGE bands revealed that representative bacteria of fermented cassava dough were *Lactobacillus* and *Pediococcus* species, as well as species of *Clostridium*, *Propionibacterium* and *Bacillus*. Some *Lactobacillus* species detected in dough samples by sequence analysis of DGGE bands were not recovered in any of the five culture media and conditions used. On the contrary, some species recovered as pure cultures from enrichments were not detected by direct DGGE analysis of total bacterial DNA from cassava dough (Miambi, et al. 2003). These results illustrate the same limitations previously described for pozol.

The study of LAB diversity in gari by Kostinek, et al (2005) was made by using phenotypic tests and genotypic methods such as RAPD-PCR, DNA-DNA hybridization or sequencing of the 16S rRNA genes. One-hundred-thirty-nine strains isolated from fermented cassava were identified. *Lb. plantarum* was the most abundant species (54.6 percent of isolates), followed by *Lc. fallax* (22.3 percent) and *Lb. fermentum* (18.0 percent). Moreover, *Lb. brevis*, *Lc. pseudomesenteroides* and *W. paramesenteroides* were sporadically isolated.

3.4 Miscellaneous

Molecular methods were applied to some soybean and rice fermented foods (Haruta, et al. 2006; Inatsu, et al. 2006; Suezawa, et al. 2006).

Inatsu, et al. (2006) investigated the diversity of *B. subtilis* strains isolated from thua nao, a traditional Thai fermented soybean food, by RAPD-PCR fingerprinting. They found that the strains were divided into 19 types, including a type with the same pattern as a Japanese natto-producing strain (Inatsu, et al. 2006). In Japan, Suezawa, et al. (2006) analyzed the sequences of the D1D2 domain of the 26S ribosomal RNA gene, and the region of internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS sequence) of the miso and soy sauce fermentation yeasts, *C. etchellsii* and *C. versatilis*. They found that those molecular methods were rapid and precise compared with the physiological method for the identification and typing of these two species (Suezawa, et al. 2006).

PCR-DGGE based on the 16S rRNA gene was applied to a traditional Japanese fermentation process to produce "rice black vinegar" (kome-kurozu), in which the conversion of rice starch into acetic acid proceeds in a ceramic pot inoculated with

rice koji. Pot vinegar fermentation offers an interesting field of study, since three microbiological processes involving yeast, fungi and bacteria occur simultaneously or sequentially. The fungal DGGE profiles during the pot vinegar fermentation process indicated that the transition from *Aspergillus oryzae* to *Saccharomyces* sp. took place at the initial stage at which alcohol production was observed. The early stage was characterized by the coexistence of *Saccharomyces* sp. and LAB. Most of the bacterial DGGE bands related to LAB were replaced by bands derived from *Lb. acetotolerans* and *Acetobacter pasteurianus* when acetic acid started to accumulate. Similar to other studies, among the bacteria isolated at the early stage, some species differed from those detected by DGGE (Haruta, et al. 2006).

4 Kimchi: A Case Study for Molecular Ecology of Vegetable Fermentation

Kimchi (Table 8.1) is a traditional Korean fermented food prepared by trimming oriental cabbage (or other vegetables), brining, blending with various spices (including garlic, ginger and hot pepper), and fermentation (Cheigh, et al. 1994). Microbial ecology of this food was investigated by both culture-dependent and -independent methods.

First works were based on the use of molecular methods to identify the microorganisms isolated from kimchi, often in parallel with phenotypic characterization. For example, Choi, et al. (2003) have shown through 16S rRNA sequencing that 68 percent of the 120 LAB isolated from kimchi were *Lc. citreum*. The other dominant species were *Lb. sakei, Lb. curvatus, Lb. brevis* and *W. confusa*-like microorganisms. *Lc. citreum* was dominant during the early and mid phase of kimchi fermentations while the other bacteria were found during the later stages (Choi, et al. 2003). In another study on microbial diversity using different methods based on amplified 16S rRNA gene-based restriction enzyme assay, Cho, et al. (2006) determined that the 970 bacteria isolated from kimchi belong to 15 species of the genera *Lactobacillus, Leuconostoc* and *Weissella*. They investigated the influence of fermentation temperature and showed that the *Leuconostoc* species was favored during the preliminary two-day incubation at 15 °C, while *W. koreensis* predominated in the second fermentation phase realized at −1 °C. When the preliminary incubation period was realized at 10 °C for four days, only *W. koreensis* grew rapidly at the beginning of the process (Cho et, al. 2006).

The use of 16S rRNA gene sequencing and DNA-DNA hybridization on LAB isolated from kimchi also enabled the identification of several novel species, namely *Lc. kimchii* (Kim, et al. 2000), *Lb. kimchii* (Yoon, et al. 2000), *W. kimchii* (Choi, et al. 2002), *W. koreensis* (Lee, et al. 2002) and *Lc. inhae* (Kim, et al. 2003).

Further studies on the microbiota of kimchi used culture-independent approaches. The examination of 16S rRNA gene clone libraries using amplified ribosomal DNA restriction analysis (ARDRA) and sequencing showed that *W. koreensis* was the only species found in all kimchi samples from five manufacturers. It was generally the most abundant, followed by *Leuconostoc* and *Lactobacillus* genera (Kim, et al. 2005).

Using PCR-DGGE for microbial community analysis, Lee, et al. (2005) found that kimchi samples fermented at 10 °C or 20 °C for 30 or 20 days, respectively, exhibited up to 12 bands. Their sequencing revealed that the main microorganisms responsible for kimchi fermentation were *W. confusa, Lc. citreum, Lb sakei* and *Lb. curvatus*. Bands corresponding to *W. confusa* and *Lc. citreum* remained present throughout the fermentation process, indicating their importance in kimchi fermentation. *Lb. sakei* and *Lb. curvatus* were also identified as significant components of the bacterial community. The authors found that the microorganisms involved in the initial stage of kimchi fermentation were different from those in the late stage. For example, an uncultured bacterium present initially disappeared during the fermentation and *L. lactis* subsp. *lactis* only appeared after two days of incubation. The fermentation at both temperatures gave some slight differences: *Lc. gelidum* and *Serratia marcescens* were only found in the kimchi fermented at 10 °C (Lee, et al. 2005).

The identification of pathogenic microorganisms was also realized on commercially available kimchi using a multiplex PCR designed for the simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes*. The authors found that one out of four samples were contaminated with *Listeria monocytogenes* and none with the other pathogens (Park, et al. 2006).

The genome-probing microarray (GPM) introduced a genomic technology in the study of microbial ecology of DEC foods. GPM allows the profiling of a microbial community based on whole-genome DNA-DNA hybridization. As a probe the GPM contained genomic DNA isolated from 149 different strains of LAB. When compared to other culture-independent methods its main advantage is independence on PCR amplification (Bae, et al. 2005). The authors found that the number of positive signals in the late phase of commercial kimchi samples (71 to 99 species) was higher than in the early phase samples (28 to 45 species). As the fermentation progressed, the most abundant species belonged to the genus *Weissella* and *Leuconostoc* and, to a lesser extent, to the genus *Lactobacillus, Enterococcus, Pediococcus* and *Lactococcus*. A few *Streptococcus* species were also present and no *Bifidobacterium* were found in kimchi. Most species in the genus *Weissella* were present in the late kimchi phase of fermentation. In the same study, the authors compared the GPM to PCR-DGGE analysis. They found only nine different microorganisms from DGGE analysis whereas GMP analysis revealed the presence of 99 microorganisms, therefore establishing itself as a powerful tool to investigate food microbiota without bias due to limitations of PCR-based methods. The use of GPM confirmed the presence of species usually found in kimchi, such as those belonging to the genera *Weissella* or *Leuconostoc*, and enabled the detection of species not usually described in kimchi fermentation, such as *Pediococcus* (Choi, et al. 2003, 2006).

Different works published on kimchi show some discrepancies regarding population structure and dynamics. These works were based either on kimchi made at laboratory scale or on commercial kimchi purchased from different manufacturers. Furthermore, "kimchi" is a generic term for a group of fermented vegetable foods

in Korea, for which more than 100 types of vegetables can be used. Therefore, the variability in results is not surprising and could be explained by differences in the processing conditions and the vegetables used.

5 Cocoa and Coffee Fermentation

5.1 Cocoa Fermentation

Cocoa beans are derived from the fruit pods of the tree *Theobroma cacao*. Each fruit pod contains 30 to 40 beans embedded in a mucilaginous pulp. Raw cocoa has an astringent, unpleasant taste and flavor and must be fermented, dried and roasted to obtain the characteristic cocoa flavor and taste (Beuchat 1997). Several different fermentation systems are used around the world; of these heap and box fermentations are the most commonly used (Baker, et al. 1994). As for "rice black vinegar," a complex pattern of fermentation involves the succession of yeasts, LAB and acetic acid bacteria. Several studies investigated the cocoa bean fermentations using culture-dependent methods (Ardhana, et al. 2003; Lagunes, et al. 2007). The microbial ecology of cocoa fermentation has been newly investigated using a polyphasic approach combining molecular methods, including PCR-DGGE and chromosome length polymorphism (CLP), with culture-dependent methods (Jespersen, et al. 2005; Nielsen, et al. 2005; Camu, et al. 2007; Nielsen, et al. 2007).

The yeast and bacterial populations from tray and traditional fermentations in Ghana were investigated on samples collected at 12-hour intervals during 96- to 144-hour fermentation. Yeasts, LAB, acetic acid bacteria (AAB) and *Bacillus* spp. were enumerated and identified using phenotypic and molecular methods, and further investigated using PCR-DGGE (Jespersen, et al. 2005; Nielsen, et al. 2005; Camu, et al. 2007; Nielsen, et al. 2007). A microbiological succession was observed during the fermentations. At the onset of the fermentation yeasts were the dominating microorganisms. LAB became dominant after 12 to 24 hours up to the end of the fermentation, and AAB reached high counts in the mid-phase (Nielsen, et al. 2005).

With regard to yeasts, *Hanseniaspora guilliermondii* and *Pichia membranifaciens* were dominant in both types of fermentation (Jespersen, et al. 2005; Nielsen, et al. 2005, 2007). A number of other yeast species – *C. krusei, Pichia kluyveri, Trichosporon asahii* and *S. cerevisiae* – were found depending on the study (Jespersen, et al. 2005; Nielsen, et al. 2005). For dominant yeasts intraspecies variations were examined by CLP using pulsed-field gel electrophoresis, showing that several different strains were involved in the fermentations (Jespersen, et al. 2005). In general, the culture-based findings were confirmed using PCR-DGGE. Nevertheless, the use of PCR-DGGE revealed the presence of *C. zemplinina* that were not found using culture-dependent methods. On the other hand, *T. asahii* yielded only faint bands in DGGE, despite the fact that it was detected using

culture-based methods. This is explained by the fact that the targeted region of the 26S rRNA gene was poorly amplified in *T. asahii*, whereas all other investigated isolates were amplified efficiently (Nielsen, et al. 2005). On one occasion three putatively undescribed yeast species were isolated (Nielsen, et al. 2007). The origin of the yeasts was also researched. Isolates of *C. krusei, Pi. membranifaciens, H. guilliermondii, T. asahii* and *Rhodotorula glutinis* were found on the surface of the cocoa pods and, in some cases, on the production equipment whereas the origin of other yeasts (e.g., *S. cerevisiae*) was not elucidated (Jespersen, et al. 2005). As for bacteria, *Bacillus* spp. were only detected during heap fermentations where they reached high numbers during the later stages of fermentation (Nielsen, et al. 2007). Four main clusters, namely *Lb. plantarum, Lb. fermentum, Lc. pseudomesenteroides*, and *E. casseliflavus*, were identified among the LAB isolated (Camu, et al. 2007). Several other LAB, including *Lc. pseudoficulneum, P. acidilactici* and the genus *Weissella*, were also detected (Nielsen, et al. 2007). Only four clusters were found among the AAB identified: *Acetobacter pasteurianus, A. syzygii*-like bacteria, and two small clusters of *A. tropicalis*-like bacteria (Camu, et al. 2007; Nielsen, et al. 2007). The culture-based findings differed slightly from the DGGE outcomes. For example, DGGE indicated that *Lc. pseudoficulneum* plays a more important role during the fermentation of cocoa than expected from the culture-based findings as it yielded a strong band in most DGGE fingerprints. A newly proposed species of LAB ("*Weissella ghanaensis*") was detected by PCR-DGGE in heap fermentations and only occasionally isolated. Also, two new species of *Acetobacter* – tentatively named "*Acetobacter senegalensis*" (*A. tropicalis*-like) and "*Acetobacter ghanaensis*" (*A. syzygii*-like) – were isolated (Camu, et al. 2007).

The authors concluded that the fermentation of cocoa beans is a very inhomogeneous process with great variations in both yeast counts and species composition. Cluster analysis of the DGGE fingerprints revealed that the variations seem to depend especially on the processing procedure, but also the season and the post-harvest storage are likely to influence the yeast counts and the species composition (Jespersen, et al. 2005; Nielsen, et al. 2005, 2007).

5.2 *Coffee Fermentation*

Commercial coffee beans belong to the species *Coffea arabica* and *Coffea canephora* var. *robusta*. To separate beans from pulp, coffee is processed by dry or wet method. The dry method is mainly used for *robusta* coffee. In wet processing the ripe coffee cherries are pulped, followed by fermentation and drying. Coffee fermentation removes the pectineous mucilage adhering to coffee beans. Yeasts involved in *Coffea arabica* fermentation in Tanzania were characterized by genotyping of the isolates using ITS-PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene. DGGE was performed on PCR-amplified 26S rRNA gene to detect yeasts from coffee samples (Masoud, et al. 2004). This work showed that *Pi. kluyveri* was dominant during fermentation and drying; *H. uvarum* was dominant during fermentation whereas numerous *Pi. anomala* were found during drying.

S. cerevisiae and *C. xestobii* were not isolated, but they were detected by DGGE. The authors found a good agreement between the sequence analysis of the D1/D2 domain of the 26S rRNA gene and sequencing of the DGGE bands.

6 Perspectives

This examples in this survey illustrate what was accomplished by using molecular methods to study traditional fermented foods from DEC. A reasonable combination of molecular methods, through a metagenomic approach, and culture-dependent methods would offer better chances to improve our knowledge of the microbial ecology of such foods. Within the molecular tool box, PCR-DGGE is one of the most popular methods for community analysis. It is considered relatively inexpensive and easy to use, and might easily be applied by researchers in DEC countries where research resources are scarce. Alternatively, TTGE could also be easier to implement since no chemical denaturing gradient is necessary. One of the main benefits of using culture-independent methods is the potential to investigate microbial diversity in numerous samples, thereby eliminating the need for culture-dependent techniques that necessitate huge amounts of Petri dishes and cultivation medium. However, it will still be necessary to isolate pure strains, to select them for their interesting biochemical characteristics and also to develop appropriate starter cultures if the traditional processes are upgraded to a larger and safer scale of production.

In general, investigations into the microbial ecology of foods in the tropical and sub-tropical world reveal that many identified microorganisms are also found in traditional Western fermentations. For instance, among the most common microorganisms, LAB like *Lb. plantarum* and *Lb. fermentum* and yeasts like *S. cerevisiae* are ubiquitous microorganisms, which are repeatedly isolated from fermentations around the world, sometimes sharing the same "fermentation pot" (like in African sorghum or maize-based beers). Why does this happen? This is a difficult question to address. Over centuries, trade between continents, invasions, exchange of seeds or introduction of plants from one continent to another – like cassava and maize introduced by the Portuguese from Latin America to Africa in the 16th century – or dissemination through bird migrations among other reasons could have contributed to spread these food microorganisms throughout the world. What would a phylogeographical study reveal about the migratory scheme of one ubiquitous species? In contrast, investigations on traditional fermented foods, as described here, indicate that some microorganisms could be specific to their food niche, such as *Lb. manihotivorans* (Morlon-Guyot, et al. 1998), *W. kimchii* (Choi, et al. 2002), *W. koreensis* (Lee, et al. 2002), etc., but are they really as specific as they first appear (e.g., only found in those niches) or was their isolation facilitated because they were dominant in their food niche? That question is illustrated by the case of *Lb. manihotivorans* which was dominant in the Colombian cassava sour starch and easily isolated from that product, but never isolated elsewhere or only detectable in a Congolese cassava fermented dough by a culture-independent method. However, questions remain regarding the reason for their dominance in specific areas and foods, and why they

do not disseminate around the world like other ubiquitous microorganisms. Therefore, tracking such "specific" microorganisms in different foods around the world by using molecular approaches is an interesting ecological issue to address.

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