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A culture-dependent and -independent approach for the identification of lactic acid bacteria associated with the production of nem chua, a Vietnamese fermented meat product

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ABSTRACT

In this study, the diversity of the native lactic acid bacteria (LAB) population in nem chua, a popular traditional Vietnamese uncooked fermented meat, was described using a combination of culture-dependent and culture-independent methods. A total of two hundred seventy-three LAB isolates were subjected to a polyphasic identification approach combining $(GTG)_5$-PCR fingerprinting and phenylalanyl-tRNA synthase α subunit (*pheS*) and RNA polymerase α subunit (*rpoA*) gene sequence analysis. LAB associated with nem chua were identified as *Lactobacillus pentosus* (21%), *Lactobacillus plantarum* (29.7%), *Lactobacillus brevis* (5%), *Lactobacillus paracasei* (0.4%), *Lactobacillus fermentum* (0.7%), *Lactobacillus acidipiscis* (0.4%), *Lactobacillus farciminis* (23%), *Lactobacillus rossiae* (0.4%), *Lactobacillus fuchuensis* (0.7%), *Lactobacillus namurensis* (0.4%), *Lactococcus lactis* (0.4%), *Leuconostoc citreum* (9.5%), *Leuconostoc fallax* (1%), *Pediococcus acidilactici* (1%), *Pediococcus pentosaceus* (4%), *Pediococcus stilesii* (1%), *Weissella cibaria* (0.7%) and *Weissella paramesenteroides* (0.7%). Furthermore, PCR-DGGE was also applied as a culture-independent method in this study. Results indicated the presence of species of which no isolates were recovered, i.e. *Lactobacillus helveticus/crispatus*, *Lactococcus garvieae* and *Vagococcus* sp. Conversely, not all isolated bacteria were detected by PCR-DGGE. Principal component and discriminant analysis disclosed correlations between the different production locations and certain isolated LAB species and strains and/or DGGE bands suggesting possible influences of locally prevailing production practices on the nem chua LAB microbiota.

1. Introduction

Nem chua is a traditional fermented meat product of Vietnam. It is made of lean ground pork mixed with boiled pig skin cut into thin strings. The meat paste is shaped into cubes on which some producers put a thin slice of garlic. The cubes are partly wrapped in a leaf 'Oi' of the plant *Psidium guajava* for decoration and flavor. After that, the cubes are wrapped with banana leaves to provide the anaerobic environment for the fermentation process and to inhibit entrance of potentially pathogenic microorganisms. The fermentation takes place without addition of a starter culture or any further cooking or heating and occurs for 2 to 4 days at ambient temperature (Nguyen, Elegado, Librojo-Basilio, Mabesa, & Dizon, 2011; Tran, May, Smooker, Van, & Coloe, 2011).

Nem chua is mainly produced on 'cottage industry' scale. It has a shelf-life of five days when preserved at room temperature. However, the shelf-life of the fermented product can be prolonged up to one month in the refrigerator (Nguyen et al., 2011). Previous research has shown that lactic acid bacteria (LAB), especially members of the genus *Lactobacillus*, and coagulase-negative cocci (CNC), *Staphylococcus* and *Kocuria* spp., are two main groups of bacteria that are considered technologically important in the fermentation and ripening of fermented meat products (Rantsiou & Cocolin, 2008). In high hygienic quality raw meat, LAB are usually present at low numbers (10^2 to 10^3 cfu/g), but due to the anaerobic environment (Rantsiou & Cocolin, 2008) they rapidly dominate the fermentation and their numbers can increase up to levels of around 10^8 cfu/g during the course of fermentation (Adams, 2010). LAB not only produce a range of antimicrobial compounds to suppress growth of spoilage or pathogenic microorganisms, but also are responsible for the unique and typical sensory characteristics of the fermented meat product (Hugas & Monfort, 1997). *Staphylococcus* and *Kocuria* spp. also contribute to the development of color and flavor in fermented meat products, mainly by degrading free amino acids and by inhibiting the oxidation of unsaturated free fatty acids (Leroy, Verluyten, & De Vuyst, 2006). However, although these organisms are

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generally the most active at the beginning of the fermentation, they are less abundant, with numbers in the range of 10^4–10^6 cfu/g as they tend to be inhibited as LAB start to dominate and the pH declines (Adams, 2010).

Interest for describing the diversity and dynamics of LAB populations associated with the production of traditional fermented food products is still high, especially with the advancements in methods in molecular microbiology. For many years, and still now, culture-dependent methods based on conventional culturing followed by phenotypic and/or genotypic (e.g. sequencing, genetic fingerprinting) identification of a (randomly) selected subset of purified isolates have been used for identifying the members of the LAB communities associated with fermented food products (Rantsiou & Cocolin, 2008). Rebecchi, Crivori, Sarra, and Cocconcelli (1998) used a combination of physiological tests and 16S rDNA sequencing to study the development of bacterial communities during sausage fermentation. LAB isolated from Turkish fermented sausages were identified according to their phenotypic properties and BOX-PCR genotypic fingerprints (Adiguzel & Atasever, 2009). Although 16S rRNA gene sequencing is the most frequently used and generally accepted method for the identification of bacteria, it is also well known that the taxonomic resolution of the 16S rRNA gene is often too low to accurately identify at species level, and this is also the case for closely related LAB (Cachat & Priest, 2005). To circumvent this problem inherent to the 16S rRNA gene, the use of housekeeping genes, such as the phenylalanyl-tRNA synthase α subunit (*pheS*), was introduced (Naser et al., 2007). The use of this gene as a taxonomic marker has already proven its potential in several diversity studies of other fermented food ecosystems where it allowed identifying LAB isolates belonging to a broad spectrum of genera including *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella* (De Bruyne et al., 2008; Scheirlinck et al., 2007; Van Hoorde, Verstraete, Vandamme, & Huys, 2008).

In recent years, culture-independent methods have also been introduced to characterize the microbiota from fermented food products without the need for preceding culturing (Cocolin, Manzano, Cantoni, & Comi, 2001). These techniques often allow to analyze multiple samples simultaneously and there is no need for prior knowledge of the ecosystem's diversity. One culture-independent method which proved successful for studying the diversity of microbial communities is the analysis of PCR products by using denaturing gradient gel electrophoresis (DGGE) (Cocolin et al., 2001). This method has been applied for describing the diversity and dynamics of LAB in many kinds of food products such as cheese (Van Hoorde et al., 2008), sourdough (Scheirlinck et al., 2007) and sausages (Fontana, Vignolo, & Cocconcelli, 2005). DGGE allows separation of DNA molecules that differ by a single base (Myers, Maniatis, & Lerman, 1987) and, hence, has the potential to provide information about variation in a bacterial population. Moreover, modern image analysis systems have been of additional value for the analysis of DGGE bands and their associated patterns (Rantsiou & Cocolin, 2008). The possibility to extract and sequence bands from the DGGE gels offers an additional valuable tool for the identification of predominant ecosystem members.

Despite our growing knowledge on the LAB ecology of many fermented meats in general, little is still known on the native LAB populations associated with the production of Vietnamese fermented sausages such as nem chua. Only recently, two attempts have been made to identify LAB species from nem chua, one report using morphological, physiological and biochemical tests in combination with 16S rDNA sequencing (Nguyen et al., 2011), and a second applying repetitive-PCR (rep-PCR) and pulse-field gel electrophoresis (PFGE) fingerprinting (Tran et al., 2011). The objective of the present study was to give a more extensive and detailed description of the LAB diversity of nem chua using a polyphasic approach consisting of $(GTG)_5$-PCR fingerprinting and sequence analysis of the phenylalanyl-tRNA synthase (*pheS*) and/or RNA polymerase α subunit (*rpoA*) genes. As has been previously demonstrated that diversity studies can benefit from a combined culture-dependent and culture-independent approach (Ercolini, Moschetti, Blaiotta, & Coppola, 2001; Rantsiou et al., 2005; Van Hoorde et al., 2008) the above mentioned culture-dependent approach was used in combination with PCR-DGGE in order to obtain an even more complete picture of the nem chua LAB community.

2. Materials and methods

2.1. Samples and strains

Ten samples of nem chua were collected from various households in Hanoi and Thanh Hoa, two cities in Northern Vietnam. In Hanoi, the samples were obtained from seven households spread over two villages, Ve and Phung, i.e. DV4, DV5 and DV6, and DP4, DP5, DP6 and DP7, respectively. In addition, three samples were collected from 3 households in Thanh Hoa, designated as DH5, DH6 and DH7. Nem chua samples were collected at the same stage of fermentation, i.e. after approximately 48 to 72 h of fermentation at ambient temperature. Ambient temperature varied between 30 °C in Hanoi and 32 °C in Thanh Hoa. Each of the ten samples was characterized by different pH in the range of 4.3 to 5.0 (Table 1). LAB reference strains were obtained from the BBCM™/LMG Bacteria Collection Ghent University (http://bccm.belspo.be).

2.2. Isolation of lactic acid bacteria

For microbial analysis, each nem chua sample (25 g) was homogenized in 225 mL Maximum Recovery Diluent (MRD) (Oxoid, CM0733, Erembodegem-Aalst) by using a Stomacher Lab Blender 80 (Seward Medical, London, UK), after which 10-fold serial dilutions were prepared in MRD. MRS agar (Oxoid, CM 0361) was used for the isolation of LAB. MRS plates were incubated for 48 h at 30 °C. Following incubation, a total of 300 colonies were picked up randomly. All isolates were initially screened for the production of catalase. Only catalase negative isolates (n = 273) were considered as LAB and were stored in Microbank™ vials (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at −80 °C until further analysis (Urso, Comi, & Cocolin, 2006).

Table 1
Main characteristics of 10 nem chua samples included in this study.

Samples designation	Village or street — city	Fermentation time (h)	Fermentation temperature (°C)	pH	cfu/g	Number of LAB isolates recovered
DV4	Ve — Hanoi	72	30	5.0	11×10^7	21
DV5		72	30	4.9	45×10^9	44
DV6		72	30	5.0	31×10^8	25
DP4	Phung — Hanoi	72	30	4.7	60×10^9	47
DP5		72	30	4.7	23×10^8	19
DP6		48	30	4.7	7×10^8	22
DP7		48	30	4.7	70×10^8	24
DH5	324 Truong Thi — Thanh Hoa	48	32	4.6	22×10^9	25
DH6	326 Truong Thi — Thanh Hoa	48	32	4.3	19×10^9	24
DH7	Doi Cung — Thanh Hoa	48	32	4.4	42×10^8	22