Diversity of *Acetobacter pasteurianus* Strains Isolated From Solid-State Fermentation of Cereal Vinegars

JiaJia Wu · Maria Gullo · FuSheng Chen · Paolo Giudici

Abstract Vinegar production is based on the acetification process by indigenous acetic acid bacteria (AAB). Among vinegar technologies, solid-state fermentation (SSF) processes are widespread in Asian countries to produce vinegar at small-scale. In this study, 21 AAB strains isolated from Chinese cereal vinegars produced by SSF collected in different regions of China were characterized by enterobacterial repetitive intergenic consensus (ERIC)–PCR fingerprinting. Isolates exhibited high degree of phenotypic variability as well as suitable traits for their uses as selected strains in SSF vinegar production (growth modality by superficial biofilm, no production of cellulose, ability to growth on ethanol media). 16S rRNA gene sequencing analysis of representative strains showed that strains of *Acetobacter pasteurianus* have a close association to cereal vinegars, whereas *Gluconacetobacter europaeus* population is not favoured. Selection of single or multiple strains culture within *A. pasteurianus* species was predicted in view of their application in SSF technology. This seems to be the first report showing phenotypic and genetic variability of AAB strains involved in SSF processes. Results can be exploited for the implementation of large-scale SSF processes by selected strains for vinegar production and other innovative biotechnological applications.

J. Wu · F. Chen
College of Food Science and Technology, Huazhong Agricultural University, 430070 Wuhan, Hubei, China

M. Gullo (✉) · P. Giudici
Department of Agricultural and Food Science, University of Modena and Reggio Emilia, Via Amendola, 2, 42100 Reggio Emilia, Italy
e-mail: maria.gullo@unimore.it

Introduction

Recently, the interest on acetic acid bacteria (AAB) related to vinegar production has been increased and several works on the ecology and functionality of culturable AAB have been published. *Gluconacetobacter europaeus*, *Acetobacter pasteurianus* and *Acetobacter aceti* species were mainly detected in wine and other vinegars produced by static and submerged cultures [7]. In China, Japan and other Asian countries, an age-old and traditional process, known as solid-state fermentation (SSF), is largely used to produce vinegars from cereals at small-scale. The basic process steps of these kinds of vinegar are: (1) crushing and steaming of cereals; (2) addition of water and Qu or Koji (respectively in Chinese and Japanese) that is a specific cereal preparation containing moulds, yeasts and bacteria; (3) alcoholic fermentation; (4) acetic acid oxidation during which wheat bran and rice (or other cereals) hull are mixed with old Pei (acetic acid fermented product from last batch as seed vinegar) [2]. Recently, SSF processes have attracted interest due to its potential not only in vinegar field, but also for the production of food and pharmaceuticals [11]. However, to date, no large-scale application of SSF is achieved mainly due to limited microbiologic knowledge about the ecology, physiology and function of the involved AAB as well as about the genetic variability among strains. Among DNA typing methods, enterobacterial repetitive intergenic consensus–PCR (ERIC/PCR) is based on the occurrence of repetitive element containing a highly conserved central inverted repeat located in non-coding extragenic regions of chromosome. The robustness of ERIC/PCR to discriminate bacterial strains under species level generating highly specific genomic DNA fingerprinting has been extensively stated for individual Eubacteria strains [19].

This technique has been also used to type AAB strains from rice and wine vinegar [6, 9, 14].

The present study was designed to collect representative AAB strains of cereal vinegars produced in SSF and to investigate suitable traits, which can be used as basic information to select strains for starter culture implementation.

Materials and Methods

Samples and Bacterial Strains

Three kinds of vinegar Pei (semi-finished acetic acid fermented product in SSF) samples, labelled as DL, SX and ZJ, were collected from Tianjin Duliu Old Aged Vinegar, Shanxi Old Aged Vinegar and Zhenjiang Aromatic Vinegar factories, respectively, in different regions in China. To avoid stress to cells, samples were diluted in GYE containing (per litre) glucose 10 g, yeast extract 10 g, ethanol 30 ml with the ratio of 1:1 (sample/broth). Cultures were incubated at 28°C for 24 h with shaking at 120 rpm. Enriched cultures were serially diluted in GYE ranging from 1×10^1 to 1×10^7 cell/ml and 500 μl of each dilution was spread onto GYEC plates (glucose 10 g, yeast extract 10 g, ethanol 30 ml, calcium carbonate 15 g, agar 8 g, 1 l distilled H_2O). After incubating at 28°C for 3 days, colonies showing clear zone on GYEC plates from 10^5 to 10^7 dilutions were picked up and purified. Type strain *A. pasteurianus* DSM 3509[T] and reference strains *Gluconacetobacter xylinus* DSM 2004 and *A. pasteurianus* CICC 7009 were cultivated and preserved according to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and China Centre of Industrial Culture Collection (CICC) procedures, respectively.

Detection of Isolates Variability

Bacterial strains diversity was determined by ERIC/PCR. To perform ERIC/PCR, genomic DNA was extracted by enzymatic lyses according to method reported before [8] and quantified by Nanodrop Spectrophotometer (ND-1000). ERIC reactions were carried out with DNA polymerase from Takara Bio, Inc., (Japan) as described previously [9, 19]. PCR amplifications were performed using Hybaid Thermal Cycler (Celbio, Italy) with initial denaturation at 94°C for 5 min, followed by 30 cycles of the denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and polymerization at 65°C for 4 min. The polymerization was completed by a final cycle of 8 min at 65°C for extension. PCR products were resolved on 2% agarose gel, stained with ethidium bromide and visualized by UV illumination. Reproducibility of ERIC/PCR was assessed by the use of

DNAs from different extractions and by repetition in at least three independent assays. Amplicons size was estimated by BioDocAnalyze (BDA) analysis software (Germany).

Strain Identification

Selected strains were identified by 16S rRNA gene sequencing. Genomic DNA was extracted by fresh culture as reported above and PCR reaction was performed on template DNAs using Ex Taq DNA polymerase (Takara Bio, Inc., Japan) according to the manufacturer's conditions and procedures. Primers 16S 616 5′-TACGGGAGGCAG-CAG-3′ (position 342–356 on 16S rRNA gene, *Escherichia coli* numbering) and 16S 1492 5′-GGCTACCTTGT-TACGACTT-3′ (position 1490–1510 on rRNA gene, *Escherichia coli* numbering) were used and PCR reactions performed [7]. The amplified PCR products were purified with Montage® PCR Cleanup Kit, according to the manufacturer's instructions and quantified by Nanodrop. Direct sequencing of 16S rRNA gene was performed by automated sequencing service of Eurofins MWG Operon (Ebersberg, Germany). Sequences contigs were assembled using CHROMASPro (Version 1.41), and similarities searched using the software BLAST (http://blast.ncbi.nlm.nih.gov/Blast).

Nucleotide Sequence Accession Number

The nucleotide sequence data have been deposited into EMBL databases under the accession numbers listed in Table 2.

Phenotypic and Technological Features of Strains

Cell shape was observed after incubating cultures in GYE broth at 28°C for 3 days. Gram-staining and catalase production were tested as described previously [15]. KOH test was performed emulsifying cell cultures on slide containing 3.0% KOH solution. Oxidation of ethanol to acetic acid was tested EYC medium containing (per litre) ethanol 30 ml, yeast extract 10 g, calcium carbonate 20 g, agar 8 g; plates were observed after 3 days incubation at 28°C. Cellulose production was examined boiling pellicles in 4 ml of 5.0% NaOH solution for 2 h and *Ga. xylinus* (DSM 2004) was used as positive control. Growth on 30% D-glucose and tolerance to ethanol (5, 10, 15 and 20%, v/v) were tested according to previously study [16]. Growth on single carbon sources was carried out by a modification of the previous method [18]. The carbon sources were sterilized by filtration (0.2 μm) and added to the sterile basal medium (yeast extract, 0.5 g; vitamin-free casamino acid, 3 g) to a final concentration of 0.3%.

Results

Strains Isolation and Characterization

Strains were obtained from colonies of 1×10^5–1×10^7 cell/ml dilutions of each vinegar sample showing clear zone in an area up to 10 mm from the edge of GYEC plates. All the selected colonies appeared small, circle and pale on GYEC medium after incubation at 28°C for 5 days. To characterize different strains, the isolates were screened by ERIC/PCR. Among all the isolates, 21 of them provided unique ERIC/PCR patterns. The number of ERIC amplicons varied from 3 (strain SX461) to 14 (strain ZJ361B)

and the size from 93 to 4317 bp (Table 1). To verify that the resolution power was restricted to the strain level, *A. pasteurianus* strain CICC 7009 was used as species-specific control (Fig. 1). Amplification of genomic DNA from some strains (SX463, SX861, ZJ172, ZJ25B and ZJ555) provided background smearing also after several attempts were performed on genomic DNA from different extractions.

Strains Identification

At least three isolates from each sample were identified to the species level on the basis of the nearly complete 16S

Table 1 Number and size of amplicons generated from strains typed by ERIC/PCR

Isolate	Amplicons no.	ERIC fingerprinting Amplicons size (bp)[q]
DL13[a]	6	2946-1023-561-427-419-334
DL15[b]	8	3002-1023-835-626-579-427-420-334
DL21A[c]	8	3904-670-544-380-318-287-218-130
SX363[d]	6	3904-544-383-331-291-242
SX461[e]	3	2836-676-437
SX561[f]	10	3054-2501-1830-1375-1125-665-442-383-326-93
SX563[g]	13	2231-1545-1091-1048-976-832-662-440-412-384-328-293-184
SX661[h]	8	2416-920-558-400-343-308-261-230
SX862[i]	10	4138-919-863-770-695-556-404-334-240-170
ZJ153A[j]	6	2792-991-729-403-241-141
ZJ171[k]	12	2730-1024-990-897-712-562-407-368-319-252-236-148
ZJ271[l]	6	2666-918-914-737-334-274
ZJ273[m]	9	4171-802-693-517-447-351-289-244-177
ZJ361B[n]	14	4171-2590-1476-916-860-787-569-490-412-357-316-262-248-192
ZJ362[o]	10	2756-1934-1624-1321-1088-923-751-343-249-157
7009[p]	13	4317-1523-925-919-806-687-600-531-432-355-315-237-169

In brackets ±SD

[a] (2.08)-(3.74)-(3.61)-(2.87)-(4.00)-(6.51)

[b] (7.21)-(5.03)-(6.24)-(5.57)-(6.03)-(6.11)-(6.11)-(4.51)

[c] (4.64)-(4.50)-(4.90)-(8.18)-(7.79)-(6.55)-(6.16)-(7.41)

[d] (5.29)-(4.51)-(6.11)-(4.51)-(4.51)-(5.13)

[e] (6.66)-(4.58)-(7.02)

[f] (5.51)-(3.61)-(9.00)-(6.56)-(6.00)-(6.00)-(3.06)-(5.03)-(5.13)-(4.16)

[g] (4.16)-(5.29)-(5.57)-(7.09)-(4.51)-(7.55)-(4.36)-(5.51)-(4.36)-(4.04)-(4.62)-(5.86)-(4.16)

[h] (4.51)-(2.52)-(4.51)-(2.52)-(4.73)-(3.21)-(3.61)-(4.00)

[i] (2.65)-(4.00)-(4.04)-(3.00)-(6.24)-(6.11)-(5.00)-(5.57)-(2.52)-(2.52)

[j] (4.36)-(4.00)-(6.03)-(5.51)-(4.58)-(3.21)

[k] (4.51)-(5.69)-(2.52)-(3.61)-(8.50)-(4.04)-(6.66)-(6.24)-(4.04)-(4.73)-(5.86)-(3.00)

[l] (6.66)-(8.50)-(5.57)-(6.66)-(5.51)-(5.13)

[m] (4.04)-(3.79)-(6.11)-(6.56)-(5.03)-(3.21)-(3.06)-(5.03)-(6.56)

[n] (6.03)-(6.35)-(5.51)-(6.03)-(5.51)-(6.66)-(1.73)-(5.57)-(4.36)-(4.16)-(6.11)-(3.79)-(6.66)-(3.61)

[o] (4.58)-(5.03)-(5.51)-(3.46)-(2.89)-(4.73)-(4.36)-(4.36)-(3.21)-(6.00)

[p] (6.11)-(7.09)-(4.36)-(4.04)-(5.29)-(6.66)-(5.00)-(3.61)-(4.16)-(4.36)-(4.51)-(5.86)-(4.04)
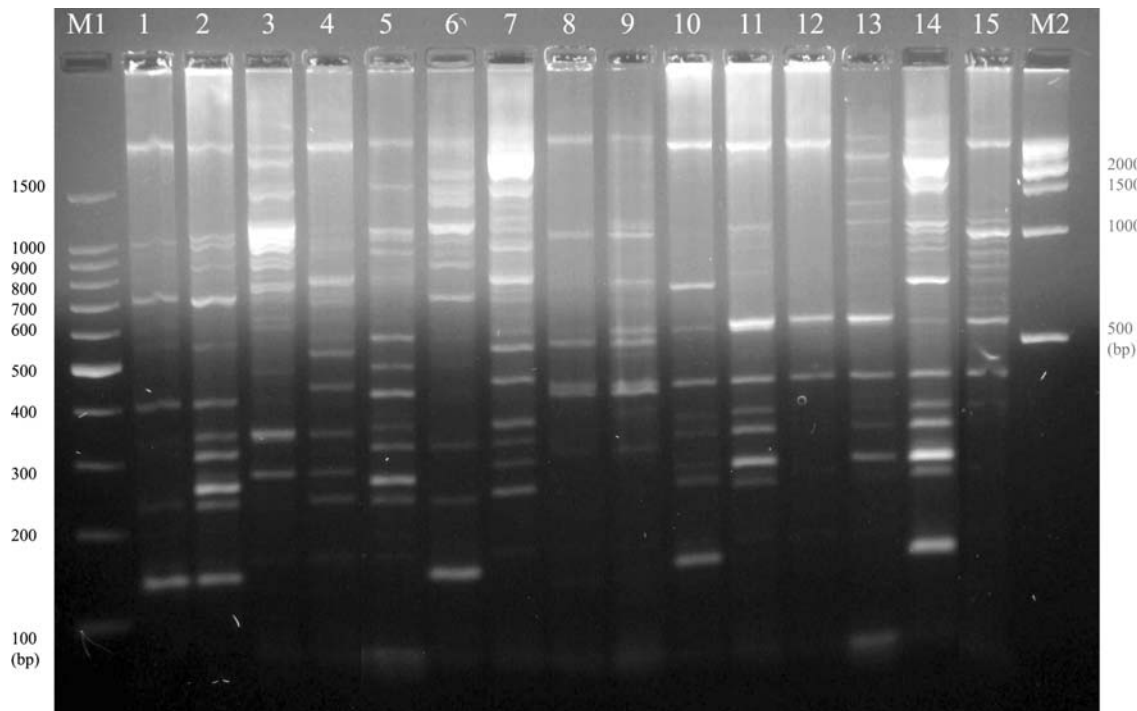
[q] Average of three replica

Fig. 1 ERIC/PCR of representative acetic acid bacteria strains. *M1*. 100 bp DNA ladder (Takara, Japan), 1. ZJ153A, 2. ZJ171, 3. ZJ271, 4. ZJ273, 5. ZJ361B, 6. ZJ362, 7. 7009 (*A. pasteurianus*, reference strain), 8. DL13, 9. DL15, 10. DL21A, 11. SX363, 12. SX461, 13. SX561, 14. SX563, 15. SX862, *M2* 500 bp DNA ladder (Takara, Japan)

rRNA gene sequencing (1374–1404 nucleotides). We identified strains able to grow starting from 5% (v/v) of ethanol because the ability to grow on it is one of main phenotypic traits to select AAB for vinegar production [7]. Moreover, strains DL13 and ZJ362, not growing on ethanol medium, were identified. A total of 12 representative strains sequences of 16S rRNA gene were obtained and compared with those of the recognized *Acetobacter* species and type strains of the other genera in the family *Acetobacteraceae* retrieved from EMBL. On this basis strains DL13, DL15, DL21A, SX363, SX461, SX862, ZJ153A, ZJ25B, ZJ273, ZJ361B and ZJ362 were assigned to *A. pasteurianus* species and strain ZJ555 to *Ga. europaeus* (Table 2).

Phenotypic and Technological Traits

All the strains showed the basic characteristic of AAB: Gram-negative, KOH positive and catalase positive reactions, mainly occurring in pairs or in short chains and all oxidized ethanol to acetic acid. Technological tests showed that among *A. pasteurianus* strains there is a high degree of variability for almost all the considered traits. In particular, the ability to grow at different ethanol concentrations showed that increased concentrations from (5–20% (v/v)) inhibited bacterial growth and 15% was tolerated only by strains SX861, SX862, ZJ25B, ZJ273 and ZJ555. Six

Table 2 16S rRNA gene analysis of representative isolates

Isolate	Sequence length of 16S rRNA gene (bp)	Accession number (this study)	Closest relative organism/acc. no.	% of Similarity
DL13	1381	FN429064	*A. pasteurianus*/ DQ887334	99
DL15	1401	FN429065		99
SX363	1404	FN429067		99
SX862	1383	FN429069		100
ZJ25B	1337	FN429071		99
ZJ273	1395	FN429072		99
ZJ361B	1384	FN429073		100
DL21A	1404	FN429066		99
ZJ362	1403	FN429074		99
ZJ153A	1398	FN429070		99
SX461	1374	FN429068	*A. pasteurianus*/ FJ227313	99
ZJ555	1401	FN429075	*Ga. europaeus*/ AB166742	100

strains grew on 30% D-glucose. Concerning the growth on single carbon sources, except from strains DL13, ZJ153A and SX561 that showed weak or no growth on all carbon sources tested, a heterogeneous behaviour was observed. No strains produced cellulose (Table 3).

Table 3 Phenotypic and technological features of acetic acid bacteria isolates

Isolate[a]	Cell morphology	Growth in liquid GYE	Ethanol (%) 5	10	15	20	D-Glucose (%) 30	Growth on single carbon source (%) D-Fructose	Glycerol	Raffinose	Sucrose	D-Mannitol	Sorbitol
DL13	Small rod-shaped	Clear, pellicle on top	–	–	–	–	w	–	–	–	–	w/–	–
DL15	Rod, in pairs	Cloudy, pellicle on top	+	–	–	–	–	w	–	–	w/–	+	+
DL21A	Rod, in pairs	Slightly cloudy, pellicle on top	+	–	–	–	–	–	–	+	+	–	–
SX363	Rod, in pairs	Cloudy, pellicle on top	+	+	–	–	+	–	w	+	+	+	+
SX461	Rod, in pairs	Cloudy, pellicle on top	+	+	–	–	–	–	w	+	w/–	+	+
SX463	Rod, in pairs	Slightly cloudy, pellicle on top	+	+	–	–	+	w	–	+	–	+	–
SX561	Rod, in pairs	Cloudy, pellicle on top	+	–	–	–	+	–	–	–	w/–	w/–	–
SX563	Rod, in pairs	Cloudy, pellicle on top	+	+	–	–	–	–	–	+	–	+	–
SX661	Rod, in pairs	Cloudy, pellicle on top	+	–	–	–	–	+	+	–	w/–	+	+
SX861	Rod, in pairs	Cloudy, pellicle on top	+	+	+	–	–	w	–	w/–	+	+	+
SX862	Rod, in pairs	Clear, pellicle on top	+	+	+	–	+	+	w	–	+	–	+
ZJ153A	Rod, in pairs	Clear, pellicle on top	+	+	–	–	–	–	–	–	–	–	–
ZJ171	Rod, in pairs	Cloudy, pellicle on top	+	+	–	–	+	w	w	w/–	–	w/–	+
ZJ172	Rod, in pairs and short chain	Cloudy, pellicle on top	+	+	–	–	–	w	w	+	–	–	+
ZJ25B	Rod, in pairs	Cloudy, pellicle on top	+	+	+	–	+	w	–	+	w/–	+	–
ZJ271	Rod, in pairs and long chain	Low turbidity, no pellicle on top	–	–	–	–	–	w	–	+	w/–	–	w/–
ZJ273	Rod, in pairs or in chain	Cloudy, pellicle on top	+	+	+	–	–	w	–	–	w/–	+	+
ZJ361A	Rod, in pairs	Cloudy, pellicle on top	–	–	–	–	–	–	–	+	w/–	+	+
ZJ361B	Rod, in pairs	Cloudy, pellicle on top	+	+	–	–	–	–	–	w/–	w/–	+	+
ZJ362	Small rod-shaped, in pairs	Low turbidity, no pellicle on top	–	–	–	–	–	+	w	+	+	+	+
ZJ555	Small rod-shaped, in pairs	Low turbidity, no pellicle on top	+	+	+	–	–	+	–	–	w/–	w/–	+

[a] All the strains were positive to gram reaction, KOH, catalase reaction and oxidation of ethanol to acetic acid and negative to cellulose production in duplicates assays. *w* weak

Discussion

In this study, AAB from SSF Chinese vinegars were typed by ERIC/PCR. Among highly conserved repetitive DNA elements that are widely distributed in the genome of bacteria, ERIC/PCR has been demonstrated to be effective in detecting intraspecific variability of bacterial strains including AAB [4, 6, 13]. In our study, apart from some variations in band intensity, no differences were observed between the ERIC profiles obtained from each strain's DNA analyzed in different assays. These differences were tested in triplicate assays and all the band patterns proved highly reproducible allowing the direct generation of strain specific genomic fingerprinting. From our data, the most culturable species recovered was *A. pasteurianus*. This evidence was consistent with previously studies showing the dominance of *A. pasteurianus* in cereal vinegars [10, 14]. We also detected one strain belonging to *Ga. europaeus* species, which strains are responsible for high acid (10–14% acetic acid) production in industrial wine vinegar. *Ga. europaeus* has distinctive phenotypic traits such as absolute requirement of acetic acid for growth, low biomass production and short viability out side vinegar environment. The recovery of only one *Ga. europaeus* strain suggests that discontinuous acetic acid availability affects the colonization by *Ga. europaeus.*

Within the species *A. pasteurianus*, we recovered different strains also from the same vinegar sample. This was in agreement with previously studies reporting that high complex strain microflora occurs in vinegars produced by biofilm on mechanical supports and on spoiled wines, whereas single dominant strains in submerged vinegar fermentations [1, 12, 17]. In our study, one of the most probable cause of strains diversity could be due to the effect of raw material composition and/or technological steps as source of variability. According to the producing practice, the addition of wheat bran or hull of cereals to induce the acetification can promote the establishment of less stringent environmental conditions that favourite the occurrence and functionality of different indigenous AAB. Furthermore, the AAB belonging to *A. pasteurianus* species showed a high degree of phenotypic variability such as tolerance to ethanol, growth on 30% of D-glucose and growth on single carbon sources. Some of these features were considered previously as discriminative tools for taxonomic purposes, for instances, growth on D-mannitol to differentiate *Acetobacter* from *Gluconobacter* and partly *Gluconacetobacter* genera and 30% of D-Glucose to discriminate *Acetobacter malorum* from *A. pasteurianus*. However, it is well known that phenotypic features are too controversial for the identification at species level and comparative phenotypic analysis of AAB is not considered a suitable tool [3]. Our data confirmed the evidence that the resolution power of phenotypic information is not reliable to taxonomically differentiated AAB. In contrast, phenotypic characterization still remains of basic relevance to screen strains in selection strategies providing data to cluster strains according to suitable metabolic profiles and allowing the exploitation of selected strains on specific fermentative substrates [5].

The application of strains of this study in selected starter cultures implementation can be predicted since they possess both a number of basic and specific phenotypic suitable traits. In this respect, growth modality by superficial biofilm, joint to no production of cellulose, contribute to avoid oxidation stuck due to oxygen limitation in static conditions, undesired sensorial profile and low acidity of the final product. The ability to grow on ethanol media, as preferred substrate, favourite efficient oxidation followed by a fast production of acetic acid that prevents the restarting of alcoholic fermentation and avoids spoilage by other organisms.

In this study, for the first time, phenotypic and genetic variability of AAB strains involved in SSF processes was stated. Results can be exploited for the implementation of large-scale SSF processes by selected strains for vinegar production and for innovative biotechnological applications.

References

1. Bartowsky EJ, Henschke PA (2008) Acetic acid bacteria spoilage of bottled red wine—a review. Int J Food Microbiol 125:60–70
2. Chen FS, Li L, Qu J, Chen CX (2009) Cereal vinegars made by solid-state fermentation in China. In: Giudici P, Solieri L (eds) Vinegar of the world. Springer, Milan, pp 243–259
3. Cleenwerck I, De Vos P (2008) Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. Int J Food Microbiol 125:2–14
4. De Vuyst L, Camu N, De Winter T, Vandemeulebroeckeb K, Van de Perre V, Vancanneyt M, De Vos P, Cleenwerck I (2008) Validation of the (GTG)$_5$-rep-PCR fingerprinting technique for rapid classification and identification of acetic acid bacteria, with a focus on isolates from Ghanaian fermented cocoa beans. Int J Food Microbiol 125:79–90
5. Giudici P, Solieri L, Pulvirenti AM, Cassanelli S (2005) Strategies and perspectives for genetic improvement of wine yeasts. Appl Microbiol Biotechnol 66:622–628
6. Gonzalez A, Hierro N, Poblet M, Rozes N, Mas A, Guillamon JM (2004) Application of molecular methods for the differentiation of acetic acid bacteria in a red wine fermentation. J Appl Microbiol 96:853–860
7. Gullo M, Giudici P (2008) Acetic acid bacteria in traditional balsamic vinegar: phenotypic traits relevant for starter cultures selection. Int J Food Microbiol 125:46–53

8. Gullo M, Caggia C, De Vero L, Giudici P (2006) Characterization of acetic acid bacteria in "traditional balsamic vinegar". Int J Food Microbiol 106:209–212

9. Gullo M, De Vero L, Giudici P (2009) Succession of selected strains of *Acetobacter pasteurianus* and other acetic acid bacteria in traditional balsamic vinegar. Appl Environ Microbiol 75:2585–2589

10. Haruta S, Ueno S, Egawa I, Hashiguchi K, Fujii A, Nagano M, Ishii M, Igarashi Y (2006) Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. Int J Food Microbiol 109:79–87

11. Liu DR, Zhu Y, Beeftink R, Ooijkaas L, Rinzema A, Chen J (2004) Chinese vinegar and its solid-state fermentation process. Food Rev Int 20:407–424

12. Mariette I, Schwarz E, Vogel RF, Hammes WP (1991) Characterization by plasmid profile analysis of acetic acid bacteria from wine, spirit and cider acetators for industrial vinegar production. J Appl Bacteriol 71:134–138

13. Mondal KK, Mani C (2009) ERIC/PCR-generated genomic fingerprints and their relationship with pathogenic variability of *Xanthomonas campestris* pv. *punicae*, the incitant of bacterial blight of pomegranate. Curr Microbiol. Published online: 25 August, 2009. doi:10.1007/s00284-009-9482-z

14. Nanda K, Taniguchi M, Ujike S, Ishihara N, Mori H, Ono H, Murooka Y (2001) Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (Komesu) and unpolished rice vinegar (Kurosu) produced in Japan. Appl Biochem Biotechnol 67:986–990

15. Navarro RR, Komagata K (1999) Differentiation of *Gluconacetobacter liquefaciens* and *Gluconacetobacter xylinus* on the basis of DNA base composition. J Gen Appl Microbiol 45:7–15

16. Sievers M, Swings J (2005) Family *Acetobacteraceae*, Ed. In: Garrity GM (ed) 2nd Bergey's manual of systematic bacteriology, vol 2. Springer, New York, pp 41–95 (Part C)

17. Sievers M, Teuber M (1995) The microbiology and taxonomy of *Acetobacter europaeus* in commercial vinegar production. J Appl Bacteriol 79:84S–95S

18. Swings J, Gillis M, Kersters K (1992) Phenotypic identification of acetic acid bacteria. In: Board RG, Jones D, Skinner FA (eds) Identification methods in applied and environmental microbiology. Blackwell Scientific, Oxford, pp 103–110

19. Versalovic J, Koeuth T, Lupski RJ (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823–6831