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Recovery of total microbial RNA from lactic acid fermented foods with a high starch content

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F. AMPE, N. BEN OMAR AND J.-P. GUYOT. 1998. An optimized procedure for the recovery of RNA from micro-organisms involved in the fermentation of starchy foods (mainly hard-to-lyse lactic acid bacteria) is reported. Critical steps for the extraction were: cell recovery by differential centrifugation; cell wall digestion with both mutanolysin and lysozyme; and CTAB treatment for the elimination of starch. Digestion of starch with α-amylase did not improve extraction yields. The method yielded high amounts of RNA from pozol, a Mexican maize-based fermented food, and was found to extract total RNA efficiently from all the micro-organisms potentially present in these ecosystems. Both rRNA and mRNA recovered were of high quality and suitable for hybridization studies.

INTRODUCTION

Lactic acid bacteria are widely used for the preparation of fermented foods, among which starch-based foods (e.g. cassava, maize, rice) are of great economic importance in southern countries. Many of these foods are fermented by a complex microflora which develops spontaneously, the lactic acid bacteria generally overcoming the other flora. To date, the microbial ecology of these fermentations has been studied using cultivation-based techniques (e.g. Hounhouigan *et al.* 1993; Wacher *et al.* 1993; Johansson *et al.* 1995; Brauman *et al.* 1996). However, it is well established that such a strategy leads to bias in the estimation of the biodiversity and quantification of the microbial groups (Amann *et al.* 1995). An alternative approach is the use of molecular tools for the characterization of microbial ecosystems (Akkermans *et al.* 1994). Since the pioneering work of Stahl *et al.* (1988) on the ecology of rumen microflora, molecular tools have successfully been used to quantify the microbial groups in different ecosystems but, as far as is known, never in fermented foods.

One of the main hurdles to this molecular approach is the recovery of nucleic acids (DNA and RNA) directly from the environmental samples. The extraction procedure should be non-selective towards the organisms present and should yield high amounts of nucleic acids of sufficient purity to enable subsequent hybridization or amplification (Lin *et al.* 1997).

Preliminary results indicated that the presence of starch in foods not only decreases the performance of classical extraction procedures for both DNA and RNA dramatically, but also induces a retardation in the migration of nucleic acids in agarose gels (data not shown). A procedure has therefore been developed for the extraction of total RNA from lactic acid bacteria communities in starchy foods yielding high quality RNA suitable for slot and Northern blot hybridizations. Pozol, a fermented maize dough from Mexico (Wacher *et al.* 1993), was used to assess the validity of the procedure.

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MATERIALS AND METHODS

Pozol samples

The pozol samples from south-east Mexico were kindly provided by C. Wacher (UNAM, Mexico).

Microbial strains

The following 38 microbial strains were used to check the RNA extraction protocol: *Clostridium acetobutylicum* ATCC 824, *Corynebacterium glutamicum* ATCC 17965, *Enterococcus durans* NCDO 596T, *Ent. faecalis* CIP 103015T, *Escherichia coli* JM109, *Lactococcus lactis* subsp. *cremoris* ATCC 11454T, *L. lactis* subsp. *lactis* ATCC 11454T, *Lactobacillus amylophilus*

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CNCM 102988 T, *Lact. amylovorus* CIP 102989, *Lact. brevis* DSM 1268, *Lact. casei* ATCC 393T, *Lact. cellobiosus* ATCC 9846T, *Lact. crispatus* LC6, *Lact. curvatus* ATCC 25601T, *Lact. delbrueckii* subsp. *bulgaricus* ATCC 1184T, *Lact. delbrueckii* subsp. *delbrueckii* ATCC 9649T, *Lact. delbrueckii* subsp. *lactis* ATCC 12315T, *Lact. fermentum* ATCC 14931T, *Lact. manihotivorans* LMG 18010T and LMG 18011 (Morlon-Guyot *et al.* 1998), *Lact. paracasei* ATCC 25302T, *Lact. plantarum* LMG 18053, *Lact. plantarum* DSM20174 T, *Lact. reuteri* ATCC 23272T, *Lact. rhamnosus* ATCC 7469T, *Lact. sake* ATCC 15521, *Lact. salivarius* ATCC 11741T, *Lact. sharpeae* LMG 9214T, *Leuconostoc dextranicum* INRA 18G, *Leuc. mesenteroides* ATCC 10832, *Oenococcus oeni* ATCC 23277, *Pc. damnosus* DSM 20331, *Pediococcus acidilacti* L, *Saccharomyces cerevisiae* FL100, *Streptococcus salivarius* subsp. *thermophilus* CNCM 10303T, *Weissella confusa* ATCC 10881T, *W. paramesenteroides* ATCC 33313 T and *Xanthomonas campestris* pv. *malvacearum* ORST IA.

All strains were grown to mid-log phase on an appropriate rich medium, and the pellet from a 10 ml culture was used for the extraction of nucleic acids.

Extraction of RNA

A 1 g sample of pozol was resuspended in 10 ml sterile water, homogenized for 30 s at maximum speed in an Ultraturrax T25 (Janke & Kunkel, IKA® Labortechnik), and centrifuged for 1 min at 800 *g*. The recovered pellet was extracted twice more with 10 ml sterile water. The combined supernatant fluids (30 ml) were used for further extraction of the RNA. After centrifugation for 10 min at 12 000 *g*, the cell pellets were kept overnight at −80 °C. Cells were resuspended in 400 μl TS buffer (Tris-Cl 50 mmol l^{-1}, pH 7·5, 10% sucrose), transferred to sterile microcentrifuge tubes and 400 μl 0·2 U μl^{-1} lysozyme (Eurobio) in Tris-Cl 50 mmol l^{-1}, pH 7·5, and 20 μl 1 U μl^{-1} mutanolysin (Sigma), added to each sample. The cell walls were digested by incubation at 37 °C. The optimal incubation time was 30 min; a longer incubation did not lead to a higher recovery of RNA.

To each sample was added 100 μl 5 mol l^{-1} NaCl and 80 μl 10% hexadecyltrimethyl-ammonium bromide (CTAB, Sigma)–0·7 mol l^{-1} NaCl, and the suspension incubated for 10 min at 65 °C. To lyse the cell membranes and separate RNA from cell debris, DNA and protein, 30 μl 20% hot SDS, 800 μl hot (65 °C) water-saturated phenol pH 4·5–5·0 (Eurobio) and 200 μl 2% hot bentone MA (Rheox Inc., UK) were then added to each sample. Tubes were incubated at 65 °C for 6 min and mixed by inversion every minute, followed by microcentrifugation for 10 min at 12 000 *g*. Bentone formed a solid interphase between the organic and the aqueous phases which facilitated the recovery of the latter. This aqueous phase was transferred to a new tube and another extraction was performed with acid phenol, followed by two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and one extraction with chloroform. The resulting aqueous phase was precipitated by the addition of 0·15 volume of sodium acetate (2 mol l^{-1}, pH 5·2) and 2·5 volumes of absolute ethanol. Samples were held at −80 °C for at least 1 h and again centrifuged. RNA pellets were washed twice with 70% ethanol, dried briefly under vacuum, dissolved in 50 μl water and stored at −80 °C. Unless otherwise stated, all manipulations were performed on ice.

The quality of the RNA recovered was routinely checked on formaldehyde-agarose gels and RNA was quantified spectrophotometrically (Sambrook *et al.* 1989).

Slot blot hybridization

RNA blotting was performed as described by Stahl *et al.* (1988). RNA was denatured by the addition of 3 volumes of 2% glutaraldehyde and diluted in 1 μg of poly(A) (Sigma) ml^{-1}. Samples (100 μl; 0·01–20 $\mu g\ ml^{-1}$) were applied to nylon membranes (Hybond-N^+; Amersham, UK) using a slot-blot device (PR648, Hoeffer Scientific Instruments, San Francisco, CA, USA) under slight vacuum. *Escherichia coli* RNA (Boehringer Mannheim) was used as hybridization control. The membrane was air-dried and baked for 2 h at 80 °C before hybridization. The DNA oligonucleotide probe (S-*-Univ-1390-a-A-18, GACGGGCGGTGTGTACAA; Zheng *et al.* 1996) was 3′-end labelled with digoxigenin-ddUTP following the manufacturers' instructions (Boehringer Mannheim). Baked membrane was pre-hybridized in 20 ml hybridization buffer (0·9 mol l^{-1} NaCl, 50 mmol l^{-1} $NaPO_4$, 5 mmol l^{-1} EDTA, 10 × Denhardt solution (Sambrook *et al.* 1989) and 0·5 mg poly(A) ml^{-1} (pH 7)) for 2 h at 40 °C. Hybridization was performed overnight at 40 °C with 10 ml hybridization buffer containing the labelled probe. Filters were washed twice in 100 ml 1 × SSC (0·15 mol l^{-1} NaCl, 0·015 mol l^{-1} sodium citrate)–1% SDS at 44 °C for 30 min. Chemiluminescence was then detected according to the manufacturers' instructions (Boehringer Mannheim).

Northern blot hybridization

Electrophoresis of RNA samples on an agarose-formaldehyde gel and blotting with capillary transfer were carried out as previously described (Ampe and Lindley 1995). A 371 bp DIG-labelled DNA, prepared as described by Giraud and Cuny (1997), was used as a probe targeting α-amylase. Detection was performed as for slot blot. All experiments were repeated three times.

RESULTS AND DISCUSSION

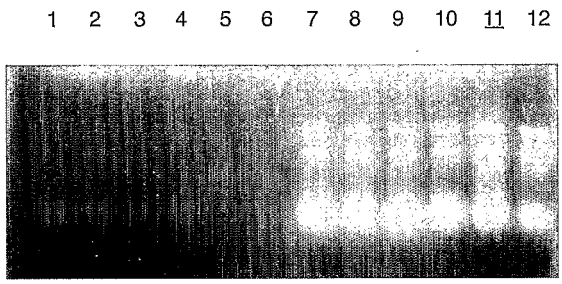
Several protocols for the extraction of RNA from fermented foods with a high starch content were tested with pozol, a

traditional Mexican fermented maize dough (Wacher *et al.* 1993). The main factors tested were: the homogenization and resuspension of the samples; the extraction of the microbial fraction; the elimination of starch by filtration and/or the action of α-amylase by precipitation with CTAB (hexadecyltrimethyl-ammonium bromide); the digestion of cell walls by a combination of lysozyme and mutanolysin; and the storage of the samples overnight at −80 °C. In all cases, an acid phenol extraction was then performed. The most significant results are shown in Fig. 1, and the optimal extraction procedure is described in Materials and Methods.

All the tested protocols designed to extract RNA directly from the pozol samples failed to yield RNA, probably due to a co-precipitation of starch and nucleic acids (Fig. 1, lanes 1–6). Filtration of homogenized samples did not improve these results (Fig. 1 lane 5). Therefore, the microbial fraction was extracted from the pozol samples by differential centrifugation. The resulting cell extract was found to contain more than 90% of the microbial cells as estimated by enumeration on MRS-agar medium (data not shown), and was used for further extraction of the RNA. When the microbial fraction was first extracted, undegraded RNA could be recovered (Fig. 1 lane 7). To improve this extraction, the soluble starch still present in the microbial fraction was tentatively eliminated by using either α-amylase digestion or CTAB precipitation, or a combination of the two. Digestion with α-amylase (2000 U α-amylase (E.C. 3·2.1·1 Sigma)) for 3 or 10 min at room temperature did not improve the extraction yield, either with crude samples before the extraction of the microbial fraction (Fig. 1 lanes 2, 4 and 6) or with extracted cells (Fig. 1 lanes 9 and 10). In contrast, CTAB greatly increased the amount of RNA recovered. The combination of α-amylase and CTAB gave poorer results than those obtained for CTAB alone. With the optimized protocol (Fig. 1 lane 11), the large and small ribosomal RNA bands appeared to be undegraded, the A_{260}/A_{280} of the extract was between 1·8 and 2·0, and yields were routinely around 25 μg microbial RNA g^{-1} pozol.

To confirm the applicability of the method to ecological studies, the optimized protocol was followed to extract total RNA from a wide number of micro-organisms potentially present in starchy fermented foods, mainly the hard-to-lyse lactic acid bacteria (LAB). High RNA yields were recovered from all tested organisms, i.e. 33 LAB strains (including species of the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus* and *Weissella*), as well as other Gram-positive (*Clostridium, Corynebacterium*) and Gram-negative bacteria, and the yeast *Saccharomyces cerevisiae*. In particular, the action of mutanolysin was found to be critical for the lysis of more than 50% of the LAB tested (data not shown). Therefore, this protocol does not appear to be particularly selective towards the various micro-organisms found, and the RNA recovered can be considered as representative of the microbial diversity in pozol.

To evaluate the suitability of extracted rRNA for the quantification of microbial groups, a slot blot hybridization



pre-treatments*	1	2	3	4	5	6	7	8	9	10	11	12
Vortex			x	x								
Ultraturrax					x	x	x	x	x	x	x	x
Filtration					x	x						
Amy. 10 min		x		x		x			x			x
Diff. Centrif.							x	x	x	x	x	x
Stor. at –80 °C								x				
Amy. 3 min										x		
CTAB											x	x

Fig. 1 Effect of various treatments on the extraction of RNA. Formaldehyde-ethidium bromide-agarose (1%) gel electrophoresis of 2 μl samples. The table summarizes the pre-treatments performed before cell lysis and phenol extraction for the corresponding lanes. *Vortex or Ultraturrax: strong homogenization of the sample with vortex (10 min) or ultraturrax; Filtration: filtration of the sample; Amy. 3 min: treatment of the pozol sample with 2000 U of α-amylase for 3 min; diff. centrif.: differential centrifugation to extract the microbial fraction; Amy. 10: treatment of the cells pellet with 2000 U of α-amylase for 10 min; Stor. at −80 °C: storage of the pellet at −80 °C overnight; CTAB: treatment of the digested cells with CTAB. Lane 11 shows the results obtained with the optimized procedure as described in the Material and Methods

was carried out (Fig. 2). Total RNA extracts from two pozol samples were probed with the digoxigenin-labelled universal probe S-*-Univ-1390-a-A-18 targeting 16S rRNA (Zheng *et al.* 1996). Results show that extracted rRNA can be successfully hybridized with a phylogenetic oligonucleotide probe and will therefore be suitable for further molecular ecology studies (Fig. 2).

Finally, to evaluate the quality of the mRNA in the RNA extracts, a Northern hybridization was carried out. As highly homologous *amyA* genes encoding for α-amylase have been found in several species of the genus *Lactobacillus* (Giraud and Cuny 1997), *amyA* was used as an indicator of the presence of related α-amylase genes in the fermentation of starchy products such as sour cassava, cassava retting or pozol. Our target transcript therefore consisted of the *amyA* mRNA of *Lactobacillus manihotivorans*. Using an *amyA*-derivated DNA probe, a single hybridizing band was revealed in *Lact. manihotivorans* grown on MRS-starch, and no transcript was detected in the same strain grown on MRS-glucose (Fig. 3). The proposed protocol therefore preserves the integrity of microbial mRNA.

The data illustrate that the described protocol can be used for the study of the molecular ecology of starchy fermented foods for both quantifying the microbial groups present and following the expression of genes encoding such relevant activities as α-amylase expression.

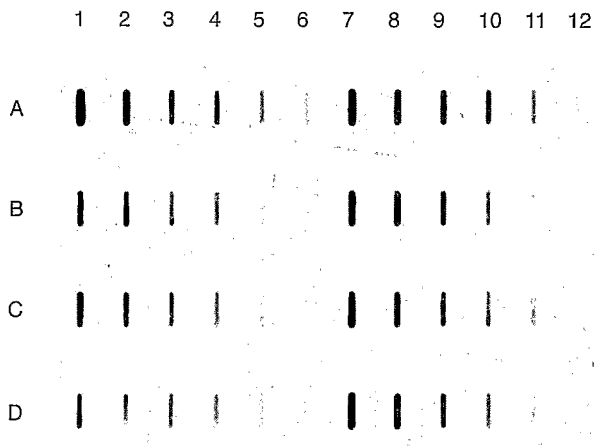


Fig. 2 Quantitative slot blot hybridization analysis of the pozol microflora. Total RNA extracts from two pozol samples were denatured and slotted onto a nylon membrane in triplicate series of six twofold dilutions (from 100 to 3·1 ng), and hybridized with the digoxigenin-labelled universal probe S-*-Univ-1390-a-A-18 targeting 16S rRNA. The arrangement of the RNA extracts on the membrane was as follows (slot numbers): (A1-A12) *Escherichia coli* RNA standard; (B1-D6) pozol sample no. 1; (B7-D12) pozol sample no. 2. The amount of RNA slotted was: (A1-D1 and A7-D7) 100 ng; (A2-D2 and A8-D8) 50 ng; (A3-D3 and A9-D9) 25 ng; (A4-D4 and A10-D10) 12·5 ng; (A5-D5 and A11-D11) 6·3 ng; (A6-D6 and A12-D12) 3·1 ng

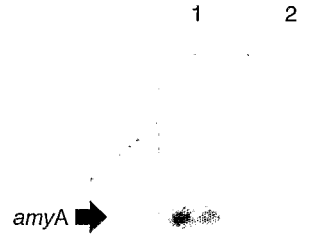


Fig. 3 Northern blot analysis of extracted RNA. Northern filter hybridization of 10 μg of total RNA from *Lactobacillus manihotivorans* containing the *amyA* gene coding for α-amylase, and grown on MRS-starch (Lane 1) or MRS-glucose (Lane 2). Samples were subjected to formaldehyde-agarose gel (1%) electrophoresis followed by transfer to a nylon membrane. The filter was hybridized with a digoxigenin-labelled DNA *amyA* specific probe

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