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Quantification of Yeast and Bacterial Gene Transcripts in Retail Cheeses by Reverse Transcription-Quantitative PCR

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The cheese microbiota contributes to a large extent to the development of the typical color, flavor, and texture of the final product. Its composition is not well defined in most cases and varies from one cheese to another. The aim of the present study was to establish procedures for gene transcript quantification in cheeses by reverse transcription-quantitative PCR. Total RNA was extracted from five smear-ripened cheeses purchased on the retail market, using a method that does not involve prior separation of microbial cells. 16S rRNA and malate:quinone oxidoreductase gene transcripts of *Corynebacterium casei*, *Brevibacterium aurantiacum*, and *Arthrobacter arilaitensis* and 26S rRNA and beta tubulin gene transcripts of *Geotrichum candidum* and *Debaryomyces hansenii* could be detected and quantified in most of the samples. Three types of normalization were applied: against total RNA, against the amount of cheese, and against a reference gene. For the first two types of normalization, differences of reverse transcription efficiencies from one sample to another were taken into account by analysis of exogenous control mRNA. No good correlation was found between the abundances of target mRNA or rRNA transcripts and the viable cell concentration of the corresponding species. However, in most cases, no mRNA transcripts were detected for species that did not belong to the dominant species. The applications of gene expression measurement in cheeses containing an undefined microbiota, as well as issues concerning the strategy of normalization and the assessment of amplification specificity, are discussed.

Reverse transcription-quantitative PCR is the method of choice for measuring gene expression levels (1). In most cases, the relative quantification method is used. It determines changes in mRNA levels of a gene across multiple samples and biological replicates relative to a control sample that is designated as the calibrator. Normalization of gene expression has to be performed to compensate for variations in reverse transcription and RNA extraction efficiencies from one sample to another. For that purpose, the quantity of target sequence is normalized to the quantity of one or several internal reference sequences (1). The corresponding reference genes must be shown to be stable under the existing experimental conditions and are evaluated using software programs such as geNorm and Bestkeeper (2, 3).

In microbiological studies, reverse transcription-quantitative PCR analyses make it possible to compare the expression levels of the same gene (present in the same microbial strain) in different samples, e.g., under different culture conditions. Gene expression studies in food products are an interesting way to improve our understanding of the physiology of microorganisms within food products. For example, it is interesting to supplement genomic data, which give indications of the potential of a given microorganism in terms of metabolic activities, with gene expression data, which reveal the true capabilities of the microorganism inside the food product. Gene expression analyses have been performed with experimental cheeses or experimental fermented milks (4–18). To our knowledge, no study devoted to the quantification of gene transcripts in retail cheeses has been published as of this time. One interesting application of these types of analyses would be to compare different cheese samples for their abundance in transcripts of genes involved in ripening such as those encoding proteases or lipases or of genes involved in the production of undesirable compounds such as biogenic amines. However, several major problems have to be taken into account. First, in retail cheeses, the exact microbial composition is unknown. In general, several microbial species are present together, and each species may comprise several distinct strains, which vary from one product to another. As a consequence, and unlike classical reverse transcription-quantitative PCR analyses, it is not the expressions of the same strain that are being compared in the different samples but the expression of strains that belong to the same species or phylogenetic group, depending on the specificity of the PCR primers that are used. This also means that since the samples do not correspond to different gene expression profiles of the same strain, it is not possible to demonstrate that selected reference genes have a stable expression level. Nonspecific amplifications may also occur, for example, if the target genes have close homologs in the other species present in the cheese sample. In addition, since the cheeses contain a mixture of dead and viable cells, both intact and degraded RNA materials are present in the RNA extracts, even when no degradation occurs during the RNA extraction step. Furthermore, the abundance of RNA transcripts in commercial cheeses may be low, especially after the ripening step, when there is no or only little microbial growth.

The aim of the present study was to propose strategies for quantification of bacterial and yeast gene transcripts in retail smear-ripened cheeses. Three types of normalization were applied: against total RNA, against the amount of cheese, and against

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a reference gene. The possible applications and the limits of such analyses are discussed.

MATERIALS AND METHODS

Strains and growth conditions. *Corynebacterium casei* UCMA 3821 was obtained from UR ABTE (Unité de Recherche Aliments Bioprocédés Toxicologie Environnements, Caen, France), *Brevibacterium aurantiacum* ATCC 9174 and *Geotrichum candidum* ATCC 204307 were obtained from the American Type Culture Collection (Rockville, MD), *Arthrobacter arilaitensis* Re117 (CIP 108037) from the CIP (Collection of the Pasteur Institute, Paris, France), and *Debaryomyces hansenii* 304 from the GMPA culture collection (Joint Research Unit 782, Food Process Engineering and Microbiology, Thiverval-Grignon, France). All these strains were originally isolated from cheeses. The bacteria were routinely grown under aerobic conditions (rotary shaker at 150 rpm) at 25°C in 50-ml conical flasks containing 10 ml of brain heart infusion broth (Biokar Diagnostics, Beauvais, France). The yeasts were grown under the same conditions, except that potato dextrose broth (Biokar Diagnostics) was used as the growth medium.

Cheese sampling. Five French smear-ripened cheeses (C1 to C5, from various manufacturers) sold on the retail market were investigated. After their purchase, they were incubated for 24 h at +7°C before being sampled. Each cheese was divided into three equivalent portions, and the upper and lower parts (rinds) of the cheeses were removed with a knife (thickness, 5 mm), pooled, and rapidly homogenized with a mortar and pestle.

Microbiological analyses. One gram of cheese rind, sampled as described above, was mixed with 9 ml of physiological saline (9 g/liter NaCl). After dispersion with a mechanical blender (Ultra-Turrax model T25; Ika Labortechnik, Staufen, Germany) for 1 min at 14,000 rpm, 10-fold serial dilutions were performed in physiological saline and plated in triplicate onto agar plates. After 3 days of incubation at 25°C, the aerobic ripening bacteria (referring to the group of cultivable bacteria that does not include lactic acid bacteria) were counted on brain heart infusion agar supplemented with 50 mg/liter amphotericin (Biokar Diagnostics), which inhibits the growth of fungi. On this medium and under aerobic conditions, lactic acid bacteria form very small colonies, which can be easily distinguished from those of the other bacteria. The lactic acid bacteria were counted on de Man-Rogosa-Sharpe agar (MRS) (pH 6.5; Biokar Diagnostics) supplemented with 50 mg/liter amphotericin after 3 days of incubation at 30°C under anaerobic conditions. The yeasts were counted on yeast extract-glucose-chloramphenicol agar (YGCA) (Biokar Diagnostics) supplemented with 0.01 g/liter 2,3,5-triphenyltetrazolium chloride (TTC) after 3 days of incubation at 25°C.

Sequencing of the 16S rRNA gene and the variable D1/D2 domain of the 26S rRNA gene from selected colony morphotypes. For each cheese sample, the presence of different colony morphotypes (shape, size, and color of the colonies) was evaluated by analyzing approximately 1,000 colonies on brain heart infusion agar supplemented with 50 mg/liter amphotericin. One representative colony was then selected for each morphotype, and the 16S rRNA gene was amplified using primers pA and pH (19). DNA was extracted from the isolates as described by Mounier et al. (20). Thermostable *Taq* DNA polymerase, buffer, and deoxynucleoside triphosphate (dNTP) mixture were purchased from TaKaRa Biomedicals (Shiga, Japan). The concentrations of primers, *Taq* DNA polymerase, dNTPs, and $MgCl_2$ in the reaction mixture were 0.2 μM, 25 U/ml, 0.2 mM, and 2 mM, respectively. Genomic DNA was added to the PCR mixture at a concentration of 0.1 ng/μl. After an initial denaturation (5 min at 94°C) and 25 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 57°C), and elongation (2 min at 72°C), a final incubation for 5 min at 72°C was performed. The resulting amplicon was sequenced by GATC Biotech (Konstanz, Germany), using the same primers as those used for the PCR amplification. The sequences were then assembled using the CAP3 program (21) and compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the closest known relatives of the 16S rRNA gene sequences. The D1/D2 domain of the 26S rRNA gene from selected yeast colony morphotypes on yeast extract-glucose-chloramphenicol agar supplemented with 0.01 g/liter 2,3,5-triphenyltetrazolium chloride was amplified using primers NL1 and NL4 (22). The same method was used as for 16S rRNA gene sequencing, except that the annealing temperature was set at 54°C. The primer sequences are shown in Table 1.

Extraction of RNA from cheese samples, DNase treatment, and reverse transcription. RNA was extracted from 500-mg rind samples without prior separation of microbial cells, as previously described (11). Three separate extractions were performed for each type of cheese. Purified RNA was quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The quality of the RNA was analyzed with the 2100 Bioanalyzer (Agilent, Palo Alto, CA) using RNA 6000 Nano chips according to the manufacturer's instructions. The concentration of extracted RNA was adjusted to 125 ng/μl by the addition of RNase-free water, and DNase treatment was performed using the Turbo DNA-free kit (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Absence of DNA contamination in RNA samples was confirmed with non-reverse-transcribed samples (minus-RT controls). Reverse transcription was performed using the SuperScript Vilo cDNA Synthesis kit (Invitrogen). The reaction mixture contained 500 ng of DNase-treated RNA and 0.3 ng of luciferase control RNA (Promega, Charbonnières, France) in a final volume of 20 μl. The reverse transcription procedure was performed according to the manufacturer's recommendations.

Extraction of RNA from liquid cultures. Yeast or bacterial liquid cultures (10 ml) were centrifuged for 5 min at 15,000 × *g*. RNA was then extracted from the cell pellets, using the same protocol as for cheese.

Real-time PCR. Oligonucleotide primers were designed using LightCycler probe design software (v1.0; Roche Applied Science, Mannheim, Germany) and synthesized by Eurogentec (Seraing, Belgium). The thermocycling program consisted of an initial denaturation at 95°C for 8 min, followed by 45 cycles of denaturation (95°C/10 s), annealing (60°C/7 s), and extension (72°C/6 s). Fluorescence acquisition (530 nm) was done at the end of each extension. After real-time PCR, a melting-curve analysis was performed by measuring fluorescence during heating from 65 to 95°C at a transition rate of 0.1°C/s. Quantification cycle (C_q) values were determined with LightCycler software (version 3.3), using the second derivative method. RNA samples that were extracted from pure cultures of the three bacteria and two yeasts were reverse transcribed, and standard curves were generated by plotting C_q values as a function of the log cDNA concentrations. PCR efficiency (E) was calculated for each primer pair from the slopes of the standard curves using the following formula: $E = 10^{-1/\text{slope}}$ (24). The genes investigated in the present study and the corresponding primer pairs are presented in Table 1. Real-time PCR measurements of each sample were performed using 1/10 dilutions of the reverse-transcribed RNA.

Real-time PCR data analysis. The abundances of RNA transcripts were normalized against the amount of RNA, the weight of the cheese, and 26S rRNA (for yeast genes) or 16S rRNA (for bacterial genes).

For normalization against the amount of RNA, the *Cq* value for each gene of interest (GOI) was transformed into relative quantities with a calibrator (cal) sample, taking differences of reverse transcription efficiency (RTE) between the sample and the calibrator into account, as determined by analysis of the exogenous RNA (luciferase mRNA) added to the cheese RNA samples before the reverse transcription reaction. The resulting relative abundance value was designated A_{RNA} and calculated as follows:

$$A_{\text{RNA}} = \{E_{\text{GOI}}^{[\text{cal}Cq(\text{GOI})-\text{samp}Cq(\text{GOI})]}\}/\{E_{\text{LUC}}^{[\text{cal}Cq(\text{LUC})-\text{samp}Cq(\text{LUC})]}\}$$

where E_{GOI} and E_{LUC} are the PCR efficiencies for the gene of interest (GOI) and luciferase (LUC) targets, and Cq(GOI) and Cq(LUC) are the corresponding Cq values for the considered sample (samp) or the calibrator sample (cal). This corresponds to normalization against total RNA,

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TABLE 1 Primers used in this study[a]

Primer or primer pair	Target	Application	Sequence (5′→3′)	Reference
pA	*Eubacteria*, 16S rRNA gene	Sequencing	AGAGTTTGATCCTGGCTCAG	19
pH	*Eubacteria*, 16S rRNA gene	Sequencing	AAGGAGGTGATCCAGCCGCA	19
NL1	Fungi, D1/D2 domain of the 26S rRNA gene	Sequencing	GCATATCAATAAGCGGAGGAAAAG	22
NL4	Fungi, D1/D2 domain of the 26S rRNA gene	Sequencing	GGTCCGTGTTTCAAGACGG	22
Luc_1495-1514 (F) and Luc_1636-1618 (R)	Control RNA, luciferase gene	Real-time PCR	F: GTGGATTACGTCGCCAGTCA R: CGCCCTTCTTGGCCTTTAT	This study
fs15 (F) and fs17 (R)	*C. casei*, 16S rRNA gene	Real-time PCR	F: CCGCAAGGCTAAAACTCAAAGGAAT R: ACCGACCACAAGGGAAAGACT	23
mqo_Cc1075-1094 (F) and mqo_Cc1182-1163 (R)	*C. casei*, *mqo* gene (malate: quinone oxidoreductase)	Real-time PCR	F: TTCGGTCTGACCAAGTACCT R: GGTCTCCCAGTCATCACCAT	This study
16S_Aa766-786 (F) and 16S_Aa943-925 (R)	*A. arilaitensis*, 16S rRNA gene	Real-time PCR	F: CCGTAGCTAACGCATTAAGTG R: CCGAAGGGAAACTCCATCT	This study
mqo_Aa1035-1055 (F) and mqo_Aa1135-1119 (R)	*A. arilaitensis*, *mqo* gene (malate:quinone oxidoreductase)	Real-time PCR	F: GCACAACATCATACCGATGCT R: ACTCGTCCTTCTTGGCG	This study
16S_Ba838-856 (F) and 16S_Ba921-904 (R)	*B. aurantiacum*, 16S rRNA gene	Real-time PCR	F: GTACGGTCGCAAGGCTAAA R: TCCAGAACGGTCTGGTGT	This study
mqo_Ba105-121 (F) and mqo_Ba220-199 (R)	*B. aurantiacum*, *mqo* gene (malate:quinone oxidoreductase)	Real-time PCR	F: CCGGGTCTACGAGAAGC R: CGTTCTCGTCCTCAGGAGTGTA	This study
26S_Dh418-434 (F) and 26S_Dh517-494 (R)	*D. hansenii*, 26S rRNA gene	Real-time PCR	F: CTCGCAGCTTACTGGGC R: GGCAGTATCAACCAAGGCTATAAC	This study
tub_Dh833-856 (F) and tub_Dh964-943 (R)	*D. hansenii*, beta tubulin gene	Real-time PCR	F: CCAAATCATTCAGATCCTTAACCG R: AAACCTTACCTCTGAAGAACGC	This study
26S_Gc368-389 (F) and 26S_Gc474-450 (R)	*G. candidum*, 26S rRNA gene	Real-time PCR	F: TTGTTCAACTGTGTTTCGGCAT R: AGGCTATAACACTCCGAAGAGTTAC	This study
tub_Gc737-755 (F) and tub_Gc836-817 (R)	*G. candidum*, beta tubulin gene	Real-time PCR	F: AGCTCAATGGTGATCTGCG R: GAGTTGGCAGCAAAGAGAGG	This study

[a] F, forward; R, reverse.

since the amount of RNA being reverse transcribed is the same for all the samples. For the calibrator sample, the A_{RNA} value is equal to 1. For each gene of interest, the calibrator sample was chosen as the sample with the highest abundance value.

For normalization against the amount of cheese, the relative abundance value was designated A_{cheese} and calculated as follows: $A_{cheese} = A_{RNA} \times Y_{samp}/Y_{cal}$, where Y_{samp} and Y_{cal} are the RNA extraction yields (in μg of RNA per gram of cheese) for the considered sample and the calibrator sample, respectively (the amount of RNA that is reverse transcribed is the same for all the samples). For the calibrator sample, the A_{cheese} value is equal to 1.

For normalization against 26S rRNA (for yeast genes), the abundance value was designated $A_{26S\ rRNA}$ and calculated with the Pfaffl equation method (25):

$$A_{26S\ rRNA} = \{E_{GOI}^{[calCq(GOI)-sampCq(GOI)]}\}/\{E_{26S\ rRNA}^{[calCq(26S\ rRNA)-sampCq(26S\ rRNA)]}\}$$

where E_{GOI} and $E_{26S\ rRNA}$ are the PCR efficiencies for the gene of interest (GOI) and 26S rRNA targets, and Cq(GOI) and Cq(26S rRNA) are the corresponding Cq values for the considered sample (samp) or the calibrator sample (cal). For the calibrator sample, the $A_{26S\ rRNA}$ value is equal to 1.

For normalization against 16S rRNA (for bacterial genes), the abundance value was designated $A_{16S\ rRNA}$ and calculated as described for $A_{26S\ rRNA}$, except that the 16S rRNA gene was chosen as the reference gene.

Sequencing of real-time PCR amplicons. The mixture resulting from real-time PCR amplification was diluted 1,000 times in water and amplified by conventional PCR as described above, except that primer annealing conditions were set at 58°C for 60 s. The resulting amplicon was sequenced by GATC Biotech using the same primers as those used for PCR amplification.

RESULTS

Microbial analyses of retail cheeses. In the curd of four of the retail cheeses (C2, C3, C4, and C5), the lactic acid bacteria count was much lower ($P < 0.05$) than that of the other bacteria (Table 2). In cheese C1, the concentration of aerobic ripening bacteria was the lowest (5.0×10^8 CFU/g; $P < 0.05$ when comparing this concentration to that of each of the four other cheeses) and that of yeasts was the highest (6.8×10^8 CFU/g; $P < 0.05$). In this cheese, the most abundant colony morphotype on brain heart infusion

TABLE 2 Quantification of yeasts and bacteria in the rind of retail smear-ripened cheeses

Cheese sample	Lactic acid bacteria (CFU/g)[a]	Aerobic ripening bacteria (CFU/g)[a]	Yeasts (CFU/g)[a]	Bacterial count/yeast count
C1	3.0×10^8	5.0×10^8	6.8×10^8	1.2
C2	1.8×10^8	5.8×10^9	1.4×10^7	44.7
C3	$<10^8$	3.6×10^9	4.0×10^7	90.0
C4	3.2×10^8	2.2×10^9	1.3×10^8	19.4
C5	$<10^8$	5.9×10^9	2.8×10^8	21.1

[a] Values are means of three determinations. The standard errors were less than 30%.

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TABLE 3 Identification of the major aerobic ripening bacteria and yeast species in the rind of retail smear-ripened cheeses by sequencing the 16S rRNA gene[a] or the D1/D2 domain of the 26S rRNA gene[b]

Cheese and representative clone	Colony morphotype		% of aerobic ripening bacteria or yeast count	Closest phylogenic affiliation (GenBank accession no.)	% Identity
	Color	Diameter (mm)			
Aerobic ripening bacteria					
Cheese C1					
BA	Orange	1	4.0	*B. aurantiacum* NCDO 739^T (NR_044854)	99
BB	Cream	7	96.0	*P. celer* SW-238^T (NR_043225)	99
Cheese C2					
BC	Yellow	5	1.7	*A. arilaitensis* Re117^T (NR_025611)	99
BD	Cream	4	10.3	*Corynebacterium ammoniagenes* Cooke J.V 9.6^T (NR_037039)	98
BE	Cream, translucent edge	3	70.7	*Microbacterium gubbeenense* LMG S-19263^T (NR_025098)	99
BF	Cream	7	1.7	*A. arilaitensis* Re117^T (NR_025611)	100
BG	White	2	15.5	*B. aurantiacum* NCDO 739^T (NR_044854)	99
Cheese C3					
BH	Yellow	5	7.1	*A. arilaitensis* Re117^T (NR_025611)	99
BI	Cream	7	57.1	*C. casei* LMG S-9264^T (NR_025101)	99
BJ	Cream	3	14.3	*Staphylococcus warneri* AW 25^T (NR_025922)	99
BK	Orange	1	21.4	*B. aurantiacum* NCDO 739^T (NR_044854)	98
Cheese C4					
BL	Cream	6	95.5	*P. fragi* ATCC 4973^T (NR_024946)	99
BM	White	2	4.5	*S. warneri* AW 25^T (NR_025922)	98
Cheese C5					
BN	Orange	1	5.1	*B. aurantiacum* NCDO 739^T (NR_044854)	98
BO	Yellow	5	5.1	*A. arilaitensis* Re117^T (NR_025611)	100
BP	Slightly yellow	5	20.3	*A. arilaitensis* Re117^T (NR_025611)	98
BQ	Cream	7	59.3	*Psychrobacter cibarius* JG-219^T (NR_043057)	98
BR	Cream, semitranslucent	12	3.4	*Psychrobacter cibarius* JG-219^T (NR_043057)	99
BS	Cream	11	6.8	*P. cibarius* JG-219^T (NR_043057)	99
Yeasts					
Cheese C1					
LA	Pink	4	0.1	*D. hansenii* QD10.1 (EU543682)	99
LB	Slightly pink	10	0.1	*G. candidum* LMA-74 (JF262190)	99
LC	Slightly pink	15	99.8	*G. candidum* LMA-74 (JF262190)	99
Cheese C2					
LD	Pink, white edge	4	14.3	*Candida catenulata* NRRL Y-1508^T (U45714)	100
LE	Slightly pink	10	57.1	*G. candidum* LMA-74 (JF262190)	99
LF	White	15	28.6	*G. candidum* LMA-1025 (JF262196)	100
Cheese C3					
LG	Pink, white edge	8	30.0	*D. hansenii* NRRL Y-7426^T (U45808)	100
LH	Slightly pink	10	25.0	*G. candidum* LMA-1025 (JF262196)	100
LI	Pink	4	45.0	*D. hansenii* NRRL Y-7426^T (U45808)	100
Cheese C4					
LJ	Pink, white edge	8	15.4	*D. hansenii* NRRL Y-7426^T (U45808)	99
LK	Pink	4	23.1	*Kluyveromyces lactis* NRRL Y-8279^T (U94922)	100
LL	White	14	15.4	*G. candidum* LMA-74 (JF262190)	99
LM	Pink, white edge	16	46.2	*D. hansenii* NRRL Y-7426^T (U45808)	100
Cheese C5					
LN	Pink, white edge	8	49.6	*D. hansenii* NRRL Y-7426^T (U45808)	100
LO	Pink	4	35.5	*D. hansenii* NRRL Y-7426^T (U45808)	100
LP	Pink, white edge	12	0.7	*Yarrowia lipolytica* NRRL YB-423^T (U40080)	100
LQ	White	20	14.2	*G. candidum* LMA-74 (JF262190)	99

[a] For each cheese sample, the 16S rRNA gene of one representative clone of each morphotype (brain heart infusion agar supplemented with 50 mg/liter amphotericin) was sequenced.
[b] For each cheese sample, the D1/D2 domain of one representative clone of each morphotype (YGCA supplemented with 0.01 g/liter TTC) was sequenced.

agar plates was assigned to *Psychrobacter celer* (Table 3), a Gram-negative species. Colony morphotypes belonging to the *Psychrobacter* genus were also detected in cheese C5. In cheese C4, the most abundant morphotype was assigned to another Gram-negative species, *Pseudomonas fragi*. Colony morphotypes assigned to *Arthrobacter arilaitensis* and *Brevibacterium aurantiacum* were found in cheeses C2, C3, and C5 and in cheeses C1, C2, C3, and C5, respectively. *Corynebacterium casei* and the related species *Corynebacterium ammoniagenes* were detected in cheeses C3 and C2, respectively. Colony morphotypes assigned to *Geotrichum candidum* were present in the five cheeses, and morphotypes assigned to *Debaryomyces hansenii* were present in cheeses C1, C3, C4, and C5.

RNA extraction from retail cheeses. Three extractions of RNA were performed for each of the five types of cheese rinds. The RNA extraction yield varied up to a 5-fold factor, depending on the type

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TABLE 4 RTE values measured for the exogenous RNA (luciferase mRNA) added to the cheese RNA samples before reverse transcription[a]

Sample	RTE	
	Mean	SD
Control C0 (water)	1.00	0.08
Cheese C1	0.88	0.09
Cheese C2	0.80	0.01
Cheese C3	0.83	0.07
Cheese C4	0.59	0.05
Cheese C5	0.87	0.17

[a] Means and standard deviations are calculated from replicated RNA extractions. RTE values are expressed relative to a control in which no cheese RNA is present, C0, according to the following formula, RTE = $E_{LUC}^{(Cq\,C0\,-\,Cq)}$, where E_{LUC} is the PCR efficiency for the luciferase gene and *Cq* C0 is the mean *Cq* value obtained for the three control C0.

of cheese. Values of RNA per gram of cheese were 380 ± 57, 212 ± 51, 225 ± 38, 358 ± 41, and 1,002 ± 55 μg for the samples C1, C2, C3, C4, and C5, respectively. There was no obvious correlation between the values of the extraction yields and the viable cell counts of bacteria or yeasts (Table 2). Capillary electrophoresis analyses showed the presence of small peaks resulting from degradation of rRNA (see Fig. S1 in the supplemental material). However, peaks corresponding to intact 16S, 18S, 23S, and 26S rRNA were present. Most RNA from C1, C2, and C3 was of bacterial origin (16S and 23S), whereas it was mostly of fungal origin for C4 (18S and 26S). Peaks corresponding to bacterial and fungal rRNA were nearly equivalent for sample C5. There was no correlation between the proportion of rRNA of bacterial origin and the ratio of the viable bacterial count divided by the viable yeast count (Table 2).

Reverse transcription-quantitative PCR. In reverse transcription-quantitative PCR analyses, normalization is generally performed against internal reference genes to compensate for variations in reverse transcription efficiencies. We measured the reverse transcription efficiency of exogenous luciferase control RNA, which was added before reverse transcription. Interestingly, there were no major differences of RTE between the five types of cheese RNA samples under our conditions (Table 4). The highest standard deviation was obtained for sample C5, 0.17, which corresponds to a standard error of 20%. There was no major reverse transcription inhibition by compounds present in the cheese RNA samples, since the RTE of these samples represented from 59 to 88% (mean value, 79.4%) of the RTE obtained in water. We observed that higher concentrations of RNA during the reverse transcription step had a detrimental effect, on both the values of RTE and their standard deviations (results not shown). This is why all the reverse transcriptions were performed at an RNA concentration of 25 ng/μl. We did not observe lower RTE values for samples with lower levels of RNA integrity. For example, the RNA sample from C2 had a lower RNA integrity than that of C4 (see Fig. S1 in the supplemental material) but did not exhibit a lower RTE (Table 4).

Real-time PCR primers targeting genes present in five of the species detected in the retail cheeses (*Geotrichum candidum*, *Debaryomyces hansenii*, *Brevibacterium aurantiacum*, *Arthrobacter arilaitensis*, and *Corynebacterium casei*) were designed. The abundances of RNA transcripts corresponding to rRNA (26S rRNA for yeasts and 16S rRNA for bacteria) and to mRNA (beta tubulin for yeasts and malate:quinone oxidoreductase for bacteria) were quantified as described in Materials and Methods, and the results were normalized against RNA (A_{RNA}) or against the amount of cheese (A_{cheese}). For mRNA, normalization was also performed against 26S rRNA ($A_{26S\ rRNA}$) or against 16S rRNA ($A_{16S\ rRNA}$). *Geotrichum candidum* 26S rRNA transcripts were detected in the five cheeses (Table 5), which is in accordance with the fact that viable cells of *Geotrichum candidum* were found in all of these cheeses. Repeated analyses (separate RNA extractions from the same type of cheese) resulted in similar results (standard deviation values are given in Table 5). The abundance of 26S rRNA transcripts varied from one sample to another. The highest abundance was found for sample C4 when results were normalized against total RNA, whereas it was found for sample C5 when results were normalized against the amount of cheese. As for 26S rRNA, beta tubulin transcripts from *Geotrichum candidum* were detected in the five cheeses. When the results were normalized against 26S rRNA, there were smaller differences of beta tubulin transcript

TABLE 5 Abundances of *G. candidum* and *D. hansenii* 26S rRNA and beta tubulin gene transcripts in the rind of retail smear-ripened cheeses[a]

Species and sample	Detection of the species by analysis of colony morphotype[b]	Transcript abundance ± SD				
		A_{RNA} for 26S rRNA	A_{cheese} for 26S rRNA	A_{RNA} for beta tubulin	A_{cheese} for beta tubulin	$A_{26S\ rRNA}$ for beta tubulin
G. candidum						
Cheese C1	+	$2.2 \times 10^{-2} \pm 3.7 \times 10^{-3}$	$1.9 \times 10^{-2} \pm 4.6 \times 10^{-3}$	$1.6 \times 10^{-2} \pm 2.0 \times 10^{-3}$	$1.7 \times 10^{-2} \pm 3.5 \times 10^{-3}$	$7.1 \times 10^{-1} \pm 3.1 \times 10^{-2}$
Cheese C2	+	$4.1 \times 10^{-1} \pm 3.9 \times 10^{-2}$	$1.9 \times 10^{-1} \pm 3.8 \times 10^{-2}$	$8.8 \times 10^{-2} \pm 7.1 \times 10^{-3}$	$5.3 \times 10^{-2} \pm 1.6 \times 10^{-2}$	$2.1 \times 10^{-1} \pm 2.5 \times 10^{-2}$
Cheese C3	+	$1.5 \times 10^{-1} \pm 5.6 \times 10^{-2}$	$7.3 \times 10^{-2} \pm 1.9 \times 10^{-2}$	$2.9 \times 10^{-2} \pm 6.1 \times 10^{-3}$	$1.8 \times 10^{-3} \pm 2.1 \times 10^{-3}$	$2.0 \times 10^{-1} \pm 3.6 \times 10^{-2}$
Cheese C4	+	$\mathbf{1.0 \times 10^0} \pm 8.7 \times 10^{-2}$	$7.9 \times 10^{-1} \pm 7.9 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 5.9 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 6.1 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 8.9 \times 10^{-2}$
Cheese C5	+	$4.5 \times 10^{-1} \pm 3.2 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 1.6 \times 10^{-2}$	$2.2 \times 10^{-1} \pm 2.4 \times 10^{-2}$	$6.2 \times 10^{-1} \pm 5.0 \times 10^{-2}$	$4.9 \times 10^{-1} \pm 3.9 \times 10^{-2}$
D. hansenii						
Cheese C1	+	$2.7 \times 10^{-1} \pm 1.6 \times 10^{-2}$	$1.0 \times 10^{-1} \pm 1.7 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 1.2 \times 10^{-1}$	$4.5 \times 10^{-1} \pm 1.2 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 1.1 \times 10^{-1}$
Cheese C2	−	$8.9 \times 10^{-3} \pm 5.1 \times 10^{-4}$	$1.9 \times 10^{-3} \pm 3.8 \times 10^{-4}$	$1.2 \times 10^{-2} \pm 1.2 \times 10^{-3}$	$2.8 \times 10^{-3} \pm 7.0 \times 10^{-4}$	$3.5 \times 10^{-1} \pm 5.1 \times 10^{-2}$
Cheese C3	+	$4.2 \times 10^{-1} \pm 1.4 \times 10^{-1}$	$9.1 \times 10^{-2} \pm 2.1 \times 10^{-2}$	$5.1 \times 10^{-1} \pm 9.2 \times 10^{-2}$	$1.3 \times 10^{-1} \pm 2.1 \times 10^{-2}$	$3.5 \times 10^{-1} \pm 6.8 \times 10^{-2}$
Cheese C4	+	$2.4 \times 10^{-1} \pm 5.9 \times 10^{-3}$	$8.4 \times 10^{-2} \pm 1.1 \times 10^{-2}$	$4.7 \times 10^{-1} \pm 8.8 \times 10^{-2}$	$2.0 \times 10^{-1} \pm 4.5 \times 10^{-2}$	$5.4 \times 10^{-1} \pm 8.8 \times 10^{-2}$
Cheese C5	+	$\mathbf{1.0 \times 10^0} \pm 1.5 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 1.2 \times 10^{-1}$	$8.6 \times 10^{-1} \pm 1.2 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 1.1 \times 10^{-1}$	$2.3 \times 10^{-1} \pm 2.1 \times 10^{-3}$

[a] A_{RNA}, abundances normalized against the amount of RNA; A_{cheese}, abundances normalized against the weight of cheese; $A_{26S\ rRNA}$, beta tubulin gene transcript abundances normalized against 26S rRNA. For each type of normalization, the abundance values are expressed as the fold change relative to the sample with the highest abundance value (the calibrator, whose value is equal to 1, indicated in boldface). The A_{RNA} and A_{cheese} abundance values are corrected by a factor that takes the differences of reverse transcription efficiency between samples into account, as determined by analysis of the exogenous RNA (luciferase mRNA) added to the cheese RNA samples.
[b] See Table 3.

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TABLE 6 Abundances of *B. aurantiacum*, *A. arilaitensis*, and *C. casei* 16S rRNA and malate:quinone oxidoreductase gene transcripts in the rind of retail smear-ripened cheeses[a]

Species and sample	Detection of the species by analysis of colony morphotype[b]	Transcript abundance ± SD				
		A_{RNA} for 16S rRNA	A_{cheese} for 16S rRNA	A_{RNA} for malate:quinone oxidoreductase	A_{cheese} for malate:quinone oxidoreductase	$A_{16S\ rRNA}$ for malate:quinone oxidoreductase
B. aurantiacum						
Cheese C1	+	$7.3 \times 10^{-3} \pm 5.7 \times 10^{-4}$	$1.3 \times 10^{-2} \pm 2.9 \times 10^{-3}$	$2.2 \times 10^{-2} \pm 1.0 \times 10^{-2}$	$3.7 \times 10^{-2} \pm 1.3 \times 10^{-2}$	$8.9 \times 10^{-1} \pm 4.7 \times 10^{-1}$
Cheese C2	+	$\mathbf{1.0 \times 10^0} \pm 2.1 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 2.6 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 1.2 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 3.7 \times 10^{-1}$	$2.9 \times 10^{-1} \pm 3.0 \times 10^{-2}$
Cheese C3	+	$4.0 \times 10^{-1} \pm 9.3 \times 10^{-2}$	$4.1 \times 10^{-1} \pm 5.5 \times 10^{-2}$	$9.6 \times 10^{-1} \pm 3.5 \times 10^{-1}$	$9.6 \times 10^{-1} \pm 2.3 \times 10^{-1}$	$6.8 \times 10^{-1} \pm 1.2 \times 10^{-1}$
Cheese C4	−	NS	NS	NS	NS	
Cheese C5	+	$5.1 \times 10^{-3} \pm 2.7 \times 10^{-4}$	$2.4 \times 10^{-2} \pm 4.9 \times 10^{-4}$	$1.8 \times 10^{-2} \pm 9.8 \times 10^{-3}$	$8.3 \times 10^{-2} \pm 4.2 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 4.9 \times 10^{-1}$
A. arilaitensis						
Cheese C1	−	NS	NS	NS	NS	
Cheese C2	+	$2.7 \times 10^{-1} \pm 8.9 \times 10^{-3}$	$1.0 \times 10^{-1} \pm 2.5 \times 10^{-2}$	$4.3 \times 10^{-1} \pm 1.4 \times 10^{-1}$	$3.1 \times 10^{-1} \pm 1.7 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 3.2 \times 10^{-1}$
Cheese C3	+	$\mathbf{1.0 \times 10^0} \pm 2.3 \times 10^{-1}$	$4.0 \times 10^{-1} \pm 5.8 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 8.1 \times 10^{-2}$	$7.3 \times 10^{-1} \pm 1.8 \times 10^{-1}$	$6.5 \times 10^{-1} \pm 1.8 \times 10^{-1}$
Cheese C4	−	$3.7 \times 10^{-6} \pm 1.2 \times 10^{-6}$	$2.4 \times 10^{-6} \pm 6.2 \times 10^{-7}$	NS	NS	
Cheese C5	+	$5.5 \times 10^{-1} \pm 6.0 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 6.0 \times 10^{-2}$	$3.1 \times 10^{-1} \pm 7.2 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 2.2 \times 10^{-1}$	$3.5 \times 10^{-1} \pm 1.7 \times 10^{-2}$
C. casei						
Cheese C1	−	$1.7 \times 10^{-5} \pm 3.5 \times 10^{-6}$	$3.1 \times 10^{-5} \pm 1.1 \times 10^{-5}$	ND	ND	
Cheese C2	+	$\mathbf{1.0 \times 10^0} \pm 1.2 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 1.4 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 1.3 \times 10^{-1}$	$\mathbf{1.0 \times 10^0} \pm 3.5 \times 10^{-1}$	$8.9 \times 10^{-1} \pm 1.8 \times 10^{-1}$
Cheese C3	+	$5.4 \times 10^{-1} \pm 1.2 \times 10^{-1}$	$5.7 \times 10^{-1} \pm 5.1 \times 10^{-2}$	$6.1 \times 10^{-1} \pm 1.2 \times 10^{-1}$	$6.3 \times 10^{-1} \pm 6.9 \times 10^{-2}$	$\mathbf{1.0 \times 10^0} \pm 7.0 \times 10^{-2}$
Cheese C4	−	$1.6 \times 10^{-5} \pm 2.5 \times 10^{-6}$	$2.7 \times 10^{-5} \pm 5.2 \times 10^{-6}$	ND	ND	
Cheese C5	−	$2.8 \times 10^{-5} \pm 8.6 \times 10^{-6}$	$1.3 \times 10^{-4} \pm 4.5 \times 10^{-5}$	ND	ND	

[a] A_{RNA}, abundances normalized against the amount of RNA; A_{cheese}, abundances normalized against the weight of cheese; $A_{16S\ rRNA}$, malate:quinone oxidoreductase gene (*mqo*) transcript abundances normalized against 16S rRNA. For each type of normalization, the abundance values are expressed as the fold change relative to the sample with the highest abundance value (the calibrator, whose value is equal to 1, indicated in boldface). The A_{RNA} and A_{cheese} abundance values are corrected by a factor that takes the differences of reverse transcription efficiency between samples into account, as determined by analysis of the exogenous RNA (luciferase mRNA) added to the cheese RNA samples. NS, nonspecific PCR amplification (melting-curve analysis); ND, not detected (absence of amplification or *Cq* value of >35).
[b] See Table 3.

abundances between the samples (C3 represented 20% of C4) than when results were normalized against RNA (C1 represented 1.6% of C4) or against the amount of cheese (C3 represented 0.18% of C4). 26S rRNA and beta tubulin transcripts from *Debaryomyces hansenii* were also detected in the five cheese RNA samples (Table 5), despite the fact that no colony morphotypes from cheese C2 were assigned to that species. When the results were normalized against RNA or against the amount of cheese, the abundances of 26S rRNA and beta tubulin transcripts were the lowest in cheese C2. When the beta tubulin transcript abundance was normalized against 26S rRNA, there was less difference between the samples (C5 represented 23% of C1) than when results were normalized against RNA (C2 represented 1.2% of C1) or against the amount of cheese (C2 represented 0.28% of C5). 16S rRNA and malate:quinone oxidoreductase transcripts from *Brevibacterium aurantiacum* were detected in all of the samples except C4, which corresponded to the only cheese in which no colony morphotypes were assigned to *Brevibacterium aurantiacum* (Table 6). The three cheeses for which colony morphotypes were assigned to *Arthrobacter arilaitensis* (C2, C3, and C5) were also those in which *Arthrobacter arilaitensis* malate:quinone oxidoreductase transcripts were detected (Table 6). Likewise, the two cheeses for which colony morphotypes were assigned to *Corynebacterium casei* (C3) or *Corynebacterium ammoniagenes* (C2) were also those in which *Corynebacterium casei* malate:quinone oxidoreductase transcripts were detected (Table 6). No good correlation was found between the abundances of mRNA or rRNA transcripts and the concentration of the corresponding species determined by colony morphotype analysis (see Fig. S2 in the supplemental material). However, there was good agreement between the abundance of mRNA and that of rRNA (Fig. 1).

To assess the amplification specificities, we sequenced the *Arthrobacter arilaitensis* and *Debaryomyces hansenii* amplicons that had the expected melting-curve profile (see Table S1 in the supplemental material). These amplicons very closely matched the sequences used for the primer design (99 to 100% identity).

DISCUSSION

The physiology and behavior of microorganisms inside food products such as cheeses are poorly understood. We know a great deal about microorganisms growing in pure culture in laboratory media, but their study is more complicated in foods due to the

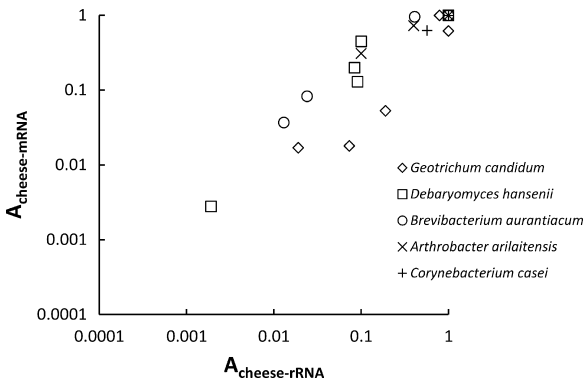

FIG 1 Abundances of mRNA transcripts ($A_{cheese\text{-}mRNA}$:beta tubulin for yeasts and malate:quinone oxidoreductase for bacteria) versus the abundances of rRNA transcripts ($A_{cheese\text{-}rRNA}$:26S rRNA for yeasts and 16S rRNA for bacteria). Abundances were normalized against the amount of cheese. Each point represents one type of cheese.

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complex food matrix and to the presence of a more or less well characterized microbiota. Quantification of specific microbial gene transcripts in food products is a very promising tool for the food microbiologist, and it will benefit from the rapidly growing availability of genome sequences from food microorganisms (26). The objective of the present study was to establish an approach for gene transcript quantification in retail cheeses.

For all the cheeses that were investigated, microbial RNA could be successfully extracted using a method that does not involve prior separation of microbial cells (13). The main advantage of this method is that the cellular processes, including RNA degradation by RNases, are stopped at the very beginning of the procedure. Unlike gene expression analyses performed on pure cultures in laboratory media, in which a good level of RNA integrity is achieved, the RNA samples from cheeses also contain degraded RNA. This is not due to the extraction procedure but to the lysis of part of the cells during the ripening or storage of the cheeses. The typical ripening time of smear cheeses is about 1 month, and during this period, several successions of microbial populations occur. Interestingly, under our conditions, we did not observe any detrimental effect of the RNA integrity level on the reverse transcription efficiency calculated from exogenous control luciferase mRNA. In reverse transcription-quantitative PCR experiments, it is recommended to use only intact RNA to obtain meaningful gene expression data (27–29). However, in our opinion, this recommendation is not appropriate for RNA extracted from ripened cheeses. Indeed, the presence of degraded RNA in these samples does not necessarily mean an inadequate sampling or extraction procedure, and the degraded RNA does not, *per se*, interfere with the quantification of transcripts that are intact. In addition, no major reverse transcription inhibition by compounds present in the cheese RNA samples occurred. Even if the reverse transcription efficiencies from one cheese sample to another were not very different, we suggest that these differences should be taken into account by including exogenous control RNA (such as luciferase control RNA) in all reverse transcription reactions.

Normalization strategy is a key issue in gene expression analysis. Unlike what occurs in classical gene expression analysis, the cheese RNA samples being compared do not correspond to different gene expression profiles of the same strain, and as a result, it is not possible to demonstrate that selected reference genes have a stable expression level by using tools such as geNorm or Bestkeeper (2, 3). Normalization against the quantity of RNA does not involve reference genes. When the abundances of a gene transcript in different cheeses are calculated by normalization against the amount of RNA, it is assumed that it is a reflection of the differences of proportion of this transcript among the whole RNA pool. The biological interpretation of such data is not easy, especially since the RNA pools represent mixtures of RNA from different species with unknown proportions. If we consider that the RNA pools represent a kind of global transcription activity of the cheese microbiota, the abundances normalized against the amount of RNA may be considered to reflect the transcription activity of the selected gene transcript among the global transcription activity. However, since most RNA represents rRNA, such analyses seem more suitable for rRNA measurements than for mRNA measurements.

Normalization of reverse transcription-quantitative PCR data may also be done against the weight of the cheese. In that case, the transcript abundances are assumed to reflect differences of proportion of this transcript among the whole cheese. For genes involved in the generation of functional properties such as proteolysis, lipolysis, or aroma compound production, the calculation of abundances normalized against the weight of the cheese may be considered as an indicator of that particular activity. The purpose of such measurements could be to better control or understand the generation of key functional properties during cheese ripening and storage. It may also be interesting to perform these types of analyses for transcripts of genes involved in the production of undesirable compounds such as biogenic amines or mycotoxins. One potential bias in normalization against the weight of cheese is that it does not compensate for differences of RNA extraction efficiencies from the cells of the target species from one cheese to another. While the occurrence of this bias cannot be excluded under our conditions, we assume that it is not very significant due to the extensive lysis of the cells induced by the combination of TRIzol addition and bead beating.

The third type of normalization that has been considered in the present study is against a reference gene that belongs to the same population as the target gene. It is assumed that the potential differences of RNA extraction efficiencies of the cells from one cheese to another do not interfere with this normalization method since the effect will be the same for the transcripts of the target and of the reference gene. The reference gene may be a protein-encoding gene or a ribosomal gene. If a ribosomal gene is used as a reference, the abundances are assumed to reflect differences of target gene transcripts among the amount of ribosomes of the target species. One advantage of using ribosomal genes as reference genes is that their transcript abundances are far higher than for protein-encoding genes and, in many cases, rRNA may be detected in samples in which transcripts of protein-encoding genes are below the threshold. For example, for *Corynebacterium casei*, malate:quinone oxidoreductase transcripts were not detected in three of the cheeses investigated, whereas 16S rRNA could still be quantified in these samples (Table 6). A noteworthy observation is that the differences in malate:quinone oxidoreductase or tubulin transcript abundances in the five types of cheeses did not represent more than a 5-fold difference when these abundances were normalized against rRNA (see the difference between cheese C3 and C4 for *Geotrichum candidum* in Table 5). Data resulting from normalization against reference genes cannot be easily used to establish repression or induction of genes in cheeses because, as previously mentioned, it is not possible to demonstrate that the reference genes have a stable expression level. However, an adequate choice of the target and reference genes may help to better understand the physiology of microorganisms in the cheese matrix.

We did not find a correlation between the abundances of mRNA or rRNA transcripts and the concentration of the corresponding species determined by colony morphotype analysis. However, it is noteworthy that when the target species was not detected by colony morphotype analysis (which reveals only the dominant species), there was also no detection of the corresponding malate:quinone oxidoreductase or tubulin transcript, except for *Debaryomyces hansenii* in cheese C2. This good correspondence corroborates the specificity of the real-time PCR amplifications. A simple way to assess the amplification specificities is to analyze the melting curves of the amplicons. A better but more labor-intensive method is to sequence the amplicons. In the present study, the sequences obtained for

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the *Arthrobacter arilaitensis* and *Debaryomyces hansenii* amplicons very closely matched the sequences used for the primer design (99 to 100% identity). Even if this sequencing does not cover the sequence corresponding to the primer pairs (only the inner part of the amplicons is sequenced), this is an indication that there was probably a perfect match between the primers and the target sequences. The design of the real-time PCR primers is a critical step in gene expression analysis of cheeses with an undefined microbiota. Indeed, for the same gene, there may be some sequence differences between the strains that belong to the target population. To limit this problem, it is better to know the gene sequence of several strains of the target population. It is also advisable to know the genome sequences of strains that are phylogenetically close to the target population in order to identify *in silico* possible problems of cross-hybridization of the primers. In the present study, since such sequences are not available, we cannot exclude the possibility that the amplicons correspond instead to distinct but closely related species in some cases. This emphasizes the importance of obtaining the genome sequences of large collections of food microorganisms.

Reverse transcription-quantitative PCR requires the development of specific primers for each of the target genes, which explains why it can be used for the analysis of only a limited set of genes. This technique will therefore not provide a global picture of the activity of food microbiota. In the future, the analysis of all the transcripts of this microbiota through the use of next-generation sequencing technologies will probably expand and help us to better understand how functions are carried out by the food microbiota.

In conclusion, the present study shows examples of quantification of gene transcripts from selected species in cheeses containing an undefined microbiota. Such analyses are useful for a better understanding of the physiology of the microorganisms in the cheese matrix and their contribution to ripening. However, several issues have to be considered, especially the type of normalization of the transcript abundances and the assessment of the amplification specificity.

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