Phenotypic, genetic and technological characterisation of *Lactococcus garvieae* strains isolated from a raw milk cheese

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Abstract

A series of Lactococcus garvieae strains isolated as the majority population of a Spanish traditional, starter-free cheese made from raw milk were phenotypically and genotypically characterised in order to address their biochemical potential, safety requirements, and technological properties. As expected, all L. garvieae cheese strains fermented lactose but grew slowly in UHT-treated milk. Enzymatic activities of L. garvieae were similar to those of Lactococcus lactis, although higher esterase and lipase activities were recorded for L. garvieae strains. Profiles of the volatile compounds produced from milk by L. garvieae and L. lactis strains were also comparable. L. garvieae strains did not produce haemolysin, gelatinase and the biogenic amines tyramine and histamine. Five L. garvieae strains showed tetracycline resistance encoded by a tet(M) gene. The use of L. garvieae strains as starter or adjunct cultures might be recommended for certain cheese types, provided that the safety of the strains has been demonstrated.

1. Introduction

The sensorial properties of cheeses made from raw milk depend on a number of key factors, such as type of milk, animal breed and nutrition and technology of manufacture. Traditional cheeses made from pasteurised milk used to lack the intense flavour of cheeses made from raw milk (Albenzio et al., 2001). Besides inactivation of native milk enzymes, pasteurisation reduces to a great extent the complex cheese microbiota which develops in raw milk cheeses (Corroler et al., 1998; Wouters et al., 2002). Microbial types which are not restored by the addition of commercial starter cultures, may play a pivotal role in the development of typical sensory profiles (Smit et al., 2005).
Although *Lactococcus garvieae* has been identified as the etiological agent of the lactococcosis in fish, it has been reported recently as a majority component of the autochthonous microbial populations of certain artisanal cheeses (Fortina et al., 2003; Foschino et al., 2006) and fermented milk products (El-Baradei et al., 2008) manufactured from raw milk. *L. garvieae* strains of dairy origin have been shown to be free of virulence determinants, such as haemolysins and gelatinase (Fortina et al., 2007), suggesting that *L. garvieae* dairy strains are unrelated to the pathogenic ones (Foschino et al., 2008). This agrees well with the lack of association between consumption of raw milk cheese and human diseases. In addition, it is believed that the activity of *L. garvieae* strains in dairy products may contribute to their final sensory characteristics. This has prompted some authors to propose the use of characterised strains as starter or adjunct cultures, provided that the safety of the strains has been determined unequivocally (Fortina et al., 2007).

However, scarce data are available on the biotechnological properties of *L. garvieae* strains from dairy origin, strengthening the need for a complete phenotypic and genetic characterization of more strains.

During a recent microbial typing of Casín, a Spanish traditional, starter-free cheese made from raw milk, high populations of *L. garvieae* strains were observed at day three by both culturing and culture-independent analyses (Alegría et al., 2009). As this species might be associated with the authenticity and originality of the cheese and could be valuable for the designing of new starter and/or adjunct cultures for other cheese types, this work was aimed to characterise a series of representative *L. garvieae* strains isolated throughout Casín manufacturing and ripening for their relevant physiological, genetic, and technological properties. In addition, a primary evaluation of their safety was also assessed.
2. Material and methods

2.1. Bacterial strains, media and culture conditions

Forty-seven *L. garvieae* isolates from Casín cheese were used in this study. The strains *L. garvieae* CECT 4531\textsuperscript{T}, *L. lactis* subsp. *lactis* LMG 6890\textsuperscript{T} and *L. lactis* subsp. *cremoris* NCIMB 700608 from the Spanish Type Culture Collection (CECT, Valencia, Spain), Belgian Co-ordinated Collections of Micro-organisms (BCCM\textsuperscript{TM}, University of Ghent, Ghent, Belgium), and National Collection of Industrial and Marine Bacteria (NCIMB Ltd., Aberdeen, UK), respectively, were used as controls in the different assays. *Listeria monocytogenes* Scott A (laboratory collection), *Staphylococcus aureus* CECT 4520\textsuperscript{T} and *Enterococcus faecalis* LMG 8222 were used as controls in the safety assays. Unless otherwise stated, isolates and control strains were cultured in liquid and agarised M17 medium (Scharlau, Barcelona, Spain) at 30\textdegree C for 48 h.

2.2. Genotypic typing

2.2.1. Isolation of total DNA.

Total genomic DNA from isolates was purified from overnight cultures using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich Inc., St. Louis, MO, USA), following the supplier’s recommendations.

2.2.2. Amplified ribosomal DNA restriction analysis (ARDRA)

Total DNA from isolates was used as a template to amplify a segment of the 16S rRNA gene by the polymerase chain reaction (PCR) technique using the universal prokaryotic primers S-D-Bact-0008-a-S-20 (27F) (5’-AGAGTTTGATCCTGGCTCAG-3’) and S-*Univ-1492R-b-A-21 (1492R) (5’-GGTACCTTGTTACGACTT-3’). Amplicons were purified through GenElute\textsuperscript{TM} PCR Clean-Up columns (Sigma-Aldrich), digested with
HaeIII and HhaI restriction enzymes (Invitrogen Ltd., Pasley, UK) and electrophoresed in agarose gels. Gels were stained with ethidium bromide (0.5 μg mL⁻¹) and photographed under UV light.

2.2.3. Typing analysis.

Isolates were grouped by combined analysis of repetitive element (REP) typing using primer BoxA2R (5’-ACGTGGTTTGAAGAGATTTTCG-3’) as reported by Koeuth et al. (1995), and random amplification of polymorphic DNA-PCR (RAPD) typing with primers OPA-18 (5’-AGGTGACCCT-3’) and M13 (5’-GAGGTTGCGGTCTT-3’), as reported by Mättö et al. (2004) and Rossetti and Giraffa (2005), respectively.

2.3. Phenotypic characterization

Carbohydrate fermentation profiles of isolates and control strains were determined using the commercial PhenePlate™ system (Bactus, Stockholm, Sweden) as recommended by the supplier. Enzyme activities were measured by the commercial, semi-quantitative API-ZYM system (bioMérieux, Montalieu-Vercieu, France) following the manufacturer’s recommendations. Sixty-five μL of a cellular suspension corresponding to McFarland standard 5 (spectrophotometric equivalent of 3 × 10⁹ cfu mL⁻¹) were inoculated into each well of the API-ZYM strips, incubated for 4 h at 30°C and developed as recommended.
2.4. Safety assessment

2.4.1. Production of gelatinase and haemolysins

Production of gelatinase was tested on Todd-Hewitt agar containing 30 g of gelatin/litre, as described by Coque et al. (1995). The production of haemolysins was analysed by a plate assay in Columbia agar plates containing 5% defibrinated human blood (Merck, Darmstadt, Germany). The presence of haemolysis is indicated by the formation of clear zones surrounding the colonies.

2.4.2. Production of biogenic amines

Production of the biogenic amines tyramine and histamine was analysed by an agar plate assay, as reported by Bover-Cid and Holzapfel (1999). Amino acid precursors, tyroxine and histidine, respectively, were added at 1% to the growth medium.

2.4.3. Antibiotic resistance

The minimum inhibitory concentration (MIC) was determined by microdilution in VetMIC<TM> plates for lactic acid bacteria (National Veterinary Institute of Sweden, Uppsala, Sweden), containing two-fold serial dilutions of 14 antibiotics. Colonies grown in LSM (Klare et al., 2005) agar plates were suspended in 2 mL of sterile saline (Oxoid, Basingstoke, Hampshire, UK) to obtain a density corresponding to McFarland standard 1 (spectrophotometric equivalent $3 \times 10^8$ cfu mL$^{-1}$). The suspension was further diluted 1:1000 with LSM (final cell concentration $3 \times 10^5$ cfu mL$^{-1}$). One hundred microlitres of this inoculum were added to each well of the VetMIC<TM> plate, and the plate was incubated at 28°C for 48 h. The MICs were defined as the lowest antibiotic concentration at which no visual growth was observed.

The presence of tetracycline resistance genes was checked by PCR using the universal primers for genes encoding ribosomal protection proteins DI (5’-
GYACICCGICAYRTIGAYTT-3’) and DII (5’-G
CCCARWAIGGRTTIGGIGGIACYTC-3’) (Clermont et al., 1997), and following the
reported PCR conditions. Amplicons were purified and sequenced by cycle extension in an
ABI 370 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were
finally compared with others in databases.

2.5. Technological characterisation

2.5.1. Growth and acidification of milk

Acid production was determined in UHT-treated milk (CAPSA, Siero, Spain). A 1%
inoculum from an overnight culture was used to inoculate UHT milk, which was then
incubated at 22ºC; samples were scored for clotting at 15, 18 and 24 h. The pH was
measured at 24 h with a Crison pH-meter (Crison Instruments S.A., Barcelona, Spain).
Appearance of the coagulum by visual inspection was also recorded.

2.5.2. Production of volatile compounds

Analysis of volatile compounds was performed after growth of the strains at 30ºC in
UHT-treated milk (CAPSA) for 48 h. Cultures were supplied with 100 μL of internal
standard (cyclohexanone, 0.36 mg mL⁻¹) and stored at −80ºC until analysis. The separation
and quantification of volatile compounds was carried out by head space/gas
chromatography/mass spectrometry analysis in an Agilent equipment composed of a G
1888 HS, a 6890 GC and a 5975B MSD (Agilent Technologies, Wilmington, DE, USA),
and equipped with a capillary column HP-Innovax 60 m × 0.25 μm (Agilent). Sample
preparation and gas chromatographic separation were performed as recently described by
Salazar et al. (2008). Peaks were quantified as the relative total ionic count abundance with
respect to the internal standard. Concentration (mM) of some volatile compounds (acetaldehyde, diacetyl, 2-propanone, acetic acid, 2-butanoate and ethanol) was calculated using linear regression equations \(R^2 \geq 0.99\) from the corresponding curves of standards obtained using five representative concentrations.

3. **Results and discussion**

3.1. **Identification and typing of *L. garvieae* strains**

*L. garvieae* isolates had been identified previously by amplifying a 16S rDNA region, sequencing and comparing the sequences against those in databases (Alegría et al., 2009). As ribosomal DNA had been amplified from cell extracts obtained directly from the counting plates, isolates were subcultured twice in M17 plates and isolated colonies were utilised to inoculate M17 broth. Total DNA was purified from overnight cultures in this medium. The identity of the isolates was verified by partial ARDRA, for which ribosomal DNA was amplified with primers 27F and 1492R and digested with the restriction enzymes *Hae* III and *Hha*I. As judged by a previous in silico analysis, the latter enzyme generated recognisably different restriction patterns for *L. garvieae* species and its relative *L. lactis*. All isolates utilized in this study displayed ARDRA patterns for these two enzymes identical to those of *L. garvieae* CECT 4531\(^T\), as shown in Fig. 1 for three isolates.

Once the identity of the isolates was assured, they were all subjected to REP typing with primer BoxA2R, and to RAPD analysis with primers OPA-18 and M13. *L. garvieae* isolates had been obtained from a single cheese batch, which anticipated the isolation of replicates. Surprisingly, the REP profiles with primer BoxA2R were found to be identical (as shown for 15 isolates in Fig. 2A), suggesting isolates could also be the same strain. However, four RAPD patterns were obtained with primer M13; although the profiles were
highly similar and shared over 80% identity, they differed in a few faint bands (data not shown). Production of strain-specific bands in bacterial species with primer BoxA2R has been repeatedly reported (Ko euth et al., 1995; Malatham et al., 1998; Esteves et al., 2006). Furthermore, homogeneous RAPD groups of \textit{L. garvieae} isolates from different cheeses obtained with primer M13 has been reported recently (Foschino et al., 2008).

In the search for a more discriminating RAPD analysis, the OPA-18 primer was identified, which yielded three clear-cut distinct profiles (Fig. 2B). Thirty six isolates where shown to belong to genotype (a), nine to genotype (b) and the remaining two to genotype (c) (Fig. 2B). These results demonstrated the unambiguous presence of at least three different genetic strains. Parallel genetic variability and relatedness has recently been reported for \textit{L. garvieae} strains isolated from traditional Italian cheeses (Foschino et al., 2008). Furthermore, the \textit{L. lactis} population of Casín (Alegría et al., 2009) and other traditional cheeses made from raw milk has been reported repeatedly to consist of a similar number of strains (Corroler et al., 1998; Mannu et al., 2000; Delgado and Mayo, 2004).

3.2. Phenotypic analysis

Based on the genotypic similarity, a set of eight representative strains of the different RAPD profiles [5 of genotype (a), 2 of genotype (b), and 1 of genotype (c) in Fig. 2B] was selected for further analysis. For comparison, three control strains were also subjected to all assays, namely \textit{L. garvieae} CECT 4531\textsuperscript{T}, \textit{L. lactis} subps. \textit{lactis} LMG 6890\textsuperscript{T} and \textit{L. lactis} subsp. \textit{cremoris} NCIMB 700608. The carbohydrate fermentation profiles were assayed by the PhenePlate\textsuperscript{TM} system, by which the fermentation of 23 different substrates was analysed (Table 1). The profiles of the different \textit{L. garvieae} strains showed small, if any, differences. All strains assimilated galactose, maltose, cellobiose, trehalose, lactose, mannose, inosine,
mannitol, amygdalin, tagatose and salicin, but showed variability in the utilisation of
palatinose and sorbitol (weak reaction by one strain each), arbutin (two positive strains and
four weak utilisers) and gluconate (utilised weakly for all but one strains). Profiles of *L.
garieae* cheese strains were more similar to that of *L. garvieae* type strain compared with
the fermentation profiles of *L. lactis* strains. However, *L. garvieae* CECT 4531T was
recorded as lactose negative. The number of carbohydrates utilised by the *L. lactis*
strains analysed was shorter; in particular, only four substrates from the PhenePlates were utilised
by *L. lactis* subsp. *cremoris* NCIMB 700608.

As measured by the semi-quantitative API-ZYM method, the enzymatic profiles
displayed by the *L. garvieae* strains were rather weak (Table 2). All strains presented
moderate esterase (C4), esterase-lipase (C8) and leucine arylamidase activities, and low
levels of acid phosphatase and naphtol-AS-BI-phosphohydrolase. Though positive strains
showed low activity, variability among strains was found for alkaline phosphatase, valine
arylamidase, trypsin, α-chymotrypsin, β-galactosidase, β-glucuronidase, and α-
glucosidase. Similar reduced enzymatic profiles were observed for control strains. In spite
of this similarity, *L. lactis* subsp. *lactis* LMG 6890T showed high α- and β-glucosidase
activities. The latter activity was absent in the assayed *L. garvieae* strains.

Scarce data are available on the phenotypic and biochemical properties of *L. garvieae*
strains from dairy sources. In spite of a high genetic similarity among strains, a certain
degree of variability was observed for carbohydrate utilisation and some enzymatic
activities. In contrast with fish pathogens, *L. garvieae* strains from dairy origin are all
lactose fermenters (Teixeira et al., 1996; Fortina et al., 2007). This agrees well with the
results obtained in this work, in which the only lactose negative strain was *L. garvieae*
CECT 4531\textsuperscript{T}, which is a fish isolate. However, Fortina et al. (2007) further described a large variation in the utilisation of many other carbohydrates, such as melibiose, L-arabinose, tagatose and N-acetyl-glucosamine. These authors assayed a large number of strains from milk, curd and cheeses of the Toma Piemontese (cows’ milk) and Caprino Lombardo (goats’ milk) varieties, which may explain the larger variations. Moreover, the use of a different microdilution system (API 50 CHL) may further account for some of the observed differences. Minor discrepancies were also observed among the enzymatic activities displayed by strains from Casín and those isolated from Italian cheeses (Fortina et al., 2007). Interestingly, esterase and esterase-lipase activities of \textit{L. garvieae} strains seem to be stronger than those displayed by the two \textit{L. lactis} control strains analysed in this work and those of wild isolates from traditional cheeses (unpublished results). Casín cheese is characterised by an intense lipolysis, which may partially be due to a weekly manual kneading step during ripening (Alegría et al., 2009), but activity of bacterial esterases and lipases could also contribute to the degradation of fats.

3.3. Safety assays

All 47 wild isolates were assayed for the presence of haemolysin and gelatinase activity, as well as for the production of biogenic amines. Haemolysis (of either $\beta$- or $\alpha$-type) and gelatinase activities were never detected. In addition, none of the strains was shown to produce either tyramine or histamine on the plate assays. These results agree well with those reported by Fortina et al. (2007), except for the presence in their isolates of 11 strains from Caprino Lombardo cheese producing tyramine. Thought \textit{L. garvieae} is a well recognized fish pathogen (Vendrell et al., 2006), and has been implicated in subclinical
cases of mastitis in cows (Teixeira et al., 1996), true factors of pathogenesis in this species
have never been reported, except for the presence of capsule, which is associated with
pathogenicity in fish (Vendrell et al., 2006). Initial microscopic analysis of cheese strains
indicates that they all are non-capsulated (data not shown). In addition, genome sequences
of L. garvieae strains are not yet available, hampering the search for virulence determinants
that should be absent in strains intended to be used in food systems.

The antibiotic resistance/susceptibility profiles of the strains were analysed by
microdilution. The MIC values obtained for the 14 antibiotics assayed with the VetMIC™
system are summarised in Table 3. On one hand, the MICs were low and similar for
erthyromycin, chloramphenicol, ampicillin, penicillin G, vancomycin, linezolid and
ciprofloxacin. On the other hand, the MICs of aminoglycosides were rather high (8–16 µg
mL⁻¹ for gentamicin and 32–256 µg mL⁻¹ for kanamycin, streptomycin and neomycin) in all
but the L. lactis subsp. cremoris control strain (which proved to be very susceptible to all
antibiotics). High MIC values in the L. garvieae strains were also scored for clindamycin (≥
16 µg mL⁻¹), trimethoprim (64–≥64 µg mL⁻¹) and rifampicin (34–≥64 µg mL⁻¹). However,
equal distribution of these MICs between strains suggested the presence of inherent
resistance mechanisms to all these antibiotics. In fact, the differential resistance of L.
garvieae strains to clindamycin as compared with L. lactis has been proposed as a
phenotypic test for distinguishing between the two species (Elliot and Facklam, 1996;
Zlotkin et al., 1998). However, the recent spread of the macrolides, lincosamides, and
streptogramin B resistance (MLS_B phenotype) encoded by *erm*(B) among L. lactis makes
this test inappropriate (Walther et al., 2008).
The MICs to tetracycline were shown to behave differently, as values varied from 0.5–2 μg mL\(^{-1}\) in all control strains and three wild *L. garvieae* strains, to ≥ 64 μg mL\(^{-1}\) in five wild strains [all of which belonged to OPA-18 genotype (a); Fig. 2B]. Positive amplification was obtained in all resistant strains with universal primers for tetracycline resistance genes encoding ribosomal protection proteins (Fig. 3). Sequencing of the amplicons and analysis of the sequences showed that a *tet*(M) gene, identical to each other and to many others in databases, was responsible for the high tetracycline resistance. Genes *tet*(M) and *tet*(S) encoding ribosomal protection proteins already have been identified in both dairy and fish isolates (Kim et al., 2004; Fortina et al., 2007; Maki et al., 2008; Walther et al., 2008). Tetracycline resistance in *L. garvieae* has been associated to transferable plasmids (Maki et al., 2009) or transposons of the Tn1545-Tn916 family (Fortina et al., 2007). Association of the *tet*(M) gene identified in this work with transferable elements has yet to be determined. However, resistant strains should not be used as cheese cultures in order to avoid spread of antibiotic resistance genes via the food chain.

### 3.4. Technological characterization

In contrast to *L. lactis* LMG 6890\(^T\) and NCIMB 700608, none of the *L. garvieae* strains coagulated UHT-treated milk after 24 h of incubation at 22°C. Indeed, *L. garvieae* strains lowered the pH of the milk from 6.6–6.7 to only 5.50–5.35 compared with a final pH of 4.52 and 4.22 for LMG 6890\(^T\) and NCIMB 700608, respectively. Despite this, at least seven volatile compounds could be detected by GC/MS after growth of the strains in milk at 30°C for 48 h (Table 4). Furthermore, volatile profiles of *L. lactis* and *L. garvieae* strains were shown to be similar in both qualitative and quantitative terms. Though differences
among strains were found, ethanol was the major volatile compound of the profiles and was produced by all strains. Small amounts of acetaldehyde and 2-propanone were also produced by all strains. Marginal levels of acetoin and diacetyl were detected in samples fermented by all eight and three *L. garvieae* strains, respectively. The repeatability of this analysis was shown to be high, as the coefficient of variation for the different volatile compounds and strains varied from 1 to 8%.

*L. garvieae* cheese isolates from this work and others (Fortina et al., 2007) have shown to present a slow rate of acidification, but still comparable to wild lactococcal isolates (Delgado et al., 2002; Wouters et al., 2002; Topisirovic et al., 2006). *L. garvieae* dairy strains have been found to present other desirable technological traits, such as assimilation of lactose and low proteolytic activity (Fortina et al., 2007), which makes their use as starter or adjunct cultures feasible.

4. Conclusions

Phenotypic and genetic studies of *L. garvieae* strains are still necessary in order to understand the functional and ecological significance of this species in dairy products. In this work, a series of 47 *L. garvieae* isolates from the manufacturing and ripening stages of a traditional cheese made from raw milk and a set of *L. garvieae* and *L. lactis* control strains were subjected to several assays to assess their phenotypic and genotypic diversity, as well as address their safety and technological properties. These analyses allowed an initial characterization of the *L. garvieae* cheese strains and their comparison with *L. lactis*. Similar functional properties were observed for strains of the two species, which included qualitative and some quantitative volatile profiles from milk. In addition, no particular undesirable traits were encountered in the *L. garvieae* strains analysed in this work, except
for the presence of tet(M) in five the isolates. The suitability of the wild L. garvieae strains free of tetracycline resistance, as starters for cheese making is currently being addressed either alone or in combination with L. lactis. The small biochemical differences shown by L. garvieae strains as compared to those of L. lactis could be amplified along cheese ripening giving rise to distinctive or unique sensory properties.

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Table 1.- Carbohydrate fermentation profiles of L. garvieae strains assayed by the PhenePlate system (Bactus).

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<th>Q1-21</th>
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<th>LMG 6890&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>CECT 4531<sup>T</sup> is the L. garvieae type strain, while LMG 6890<sup>T</sup> and NCIMB 700608<sup>a</sup> are L. lactis subsp. lactis (type strain) and L. lactis subsp. cremoris, respectively.

<sup>b</sup>W, weak reaction.
Table 2.- Enzymatic activities of *L. garvieae* strains measured with the API ZYM system (bioMérieux).

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<tr>
<th>Enzymatic activity&lt;sup&gt;a&lt;/sup&gt;</th>
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<th></th>
<th></th>
<th></th>
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<th>LMG 6890&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>T2-17</td>
<td>T3-18</td>
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<sup>a</sup>Activity was recorded as the approximate nanomoles of hydrolysed substrate. Strains were all negative for α-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase.

<sup>b</sup>CECT 4531<sup>T</sup> is the *L. garvieae* type strain, while LMG 6890<sup>T</sup> and NCIMB 700608 are *L. lactis* subsp. *lactis* (type strain) and *L. lactis* subsp. *cremoris*, respectively.
Table 3.- Minimum Inhibitory Concentration (MIC) in μg mL⁻¹ of fourteen antibiotics to *L. garvieae* strains by broth microdilution.

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<th>Strain</th>
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<th>T1-41</th>
<th>T2-4</th>
<th>T2-17</th>
<th>T3-18</th>
<th>T3-22</th>
<th>Q1-21</th>
<th>Q2-41</th>
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<th>LMG 6890Ta</th>
<th>NCIMB 700608a</th>
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<td>8</td>
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<td>32 2</td>
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</table>

*a*CECT 4531<sup>T</sup> is the *L. garvieae* type strain, while LMG 6890<sup>T</sup> and NCIMB 700608 are *L. lactis* subsp. *lactis* (type strain) and *L. lactis* subsp. *cremoris*, respectively.
Table 4.- Relative abundance of volatile compounds in three independent cultures as detected by Head Space, Gas Chromatography, Mass Spectrometry (HS/GC/MS) after growth of the strains at 30°C for 48 h in UHT-treated milk.

<table>
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<tr>
<th>Volatile compound</th>
<th>Strain</th>
<th>CECT 4531&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LMG 6890&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NCIMB 700608&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1-1</td>
<td>T1-41</td>
<td>T2-4</td>
<td>T2-17</td>
</tr>
<tr>
<td>Methanethiol</td>
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<sup>a</sup>CECT 4531<sup>T</sup> is the *L. garvieae* type strain, while LMG 6890<sup>T</sup> and NCIMB 700608 are *L. lactis* subsp. *lactis* (type strain) and *L. lactis* subsp. *cremoris*, respectively.

<sup>b</sup>In parenthesis, concentration of the compounds in the fermented milk in µg mL<sup>-1</sup>.
Figure 3
Figure Legends

**Figure 1.** Partial ARDRA profiles of *Lactococcus garvieae* strains. Amplicons were obtained with primers S-D-Bact-0008-a-S-20 (27F) and S-^a^-Univ-1492R-b-A-21 (1492R) and digested with restriction enzymes *Hae*III and *Hha*I. Order: Line 1-3, amplicons from *L. garvieae* CECT 4531^T^, *L. lactis* subsp. *lactis* LMG 6890^T^, and *L. lactis* subsp. *cremoris* NCIMB 700608, respectively, digested with *Hha*I. Lines 4-9, amplicons from three wild *L. garvieae* strains digested with *Hha*I (4-6) and *Hae*III (7-9). M, molecular weight marker: Lambda DNA digested with *Hind*III.

**Figure 2.** Typing of fifteen *L. garvieae* isolates from Casín cheese with primers BoxA2R (A) and OPA-18 (B). The different profiles identified with the two primers are indicated by a letter code. On the left, the molecular weight (in kbp) of some amplicons is indicated.

**Figure 3.** Amplification of tetracycline resistance genes with the universal primers DI and DII (Clermont et al., 1997) for ribosomal protection genes from *L. garvieae* strains. Order: Lines 1-11, order of the strains as in the tables (T1-1, T1-41, T2-4, T2-17, T3-18, T3-22, Q1-21, Q2-41, CECT 4531^T^, LMG 6890^T^, and NCIMB 700608); C+, positive amplification control (DNA from *Lactococcus lactis* AA29, which carries a functional tet(M) gene; Flórez et al., 2008); B, blank to which DNA was not added; M, Gene Ruler Express™ DNA ladder (Fermentas GmbH., Germany); the molecular weight (kbp) of some bands is indicated on the left.