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Rough and smooth morphotypes isolated from *Lactobacillus farciminis* CNCM I-3699 are two closely-related variants

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ABSTRACT

This study focused on a pleomorphic strain *Lactobacillus farciminis* CNCM I-3699 known as probiotic for animal applications. On plating, this strain was characterized by the presence of rough and smooth morphotypes depending on experimental conditions. Dominant smooth (S) form, bright white, having smooth edges with moist, ropy, and creamy along with rough (R) form, pale white, having irregular edges and a dry and granular aspect were always obtained from the parent strain under aerobic culture conditions. In anaerobic conditions, only S form growth was observed. Biochemical dosage of capsular exopolysaccharides showed a significant difference between S and R forms ($p < 0.01$), in agreement with a ropy or non ropy phenotype for the S or R form, respectively. These differences were confirmed by transmission electronic microscopy. The auto-aggregation profile revealed major differences in cultural behaviors. The R morphotype presented a highly auto-aggregative ability contrary to the S morphotype. However, biochemical and molecular analyses revealed that R and S morphotypes: 1) shared the same sugar fermentation pattern; 2) belonged to *L. farciminis* species using 16S rDNA sequencing; 3) had identical PFGE patterns using NotI and ApaI endonucleases; and 4) had identical CRISPR loci but different from those of other *L. farciminis* strains. Furthermore, the novelty and uniqueness of CRISPR spacer sequences in CNCM I-3699 provides a genetic support for the development of a molecular tracking tool for CNCM I-3699 and its variants. In conclusion, *L. farciminis* CNCM I-3699 is a pleomorphic strain giving reproducibly rise to two phenotypically distinct morphotypes R and S. This phenomenon may explain survival and growth abilities in *in vitro* fluctuating aerobic–anaerobic conditions along with modulation of exopolysaccharide synthesis and autoaggregation profile.

1. Introduction

Phenotypic variations within single-strain populations are known as pleomorphism (Wainwright et al., 1999), which is common in the biological world, especially in bacteria. Observed on solid media, it is expressed as heterogeneous colony morphotypes ranging from rough (R) to smooth (S) (Klaenhammer and Kleeman, 1981). The generation of such variants maximizes bacteria fitness for optimizing population level in response to fluctuating environments and is considered to be an important adaptive strategy (Davidson and Surette, 2008; Wolf et al., 2005). These variations may arise as a response to limiting or changing growth conditions. Temperature, pH, oxygen level, and some components of the growth medium have been shown to induce changes in cell and colony morphology. In these cases, the whole bacterial population suffers from these variations, the original morphological type reappearing when the original growth conditions are restored (Reinheimer et al., 1995).

These morphological changes can also occur in some strains in prolonged optimal culture conditions, leading new morphotypes to appear spontaneously with a low frequency (Monk et al., 2004). Colonial heterogeneity actually reflects differential expression of components on bacteria cell surfaces within the colony (Horn et al., 2013). Some of these surface components are often involved in the colonization of the host, virulence of pathogenic microorganisms, interactions with the immune system, biofilm formation, and stress resistance (Monk et al., 2004). For a long time these properties have been targeted to study and understand the mechanisms of virulence and host colonization in pathogens such as *Brucella* spp. (Zygmunt et al., 2009), *Candida* spp. (França et al., 2011), *Escherichia coli* (Dong et al., 2009), *Pseudomonas aeruginosa* (Putignani et al., 2008), and *Shigella* spp. (Coimbra et al., 1999).

Similarly, phenotypic variations within lactic acid bacteria (LAB) populations have been observed early on (Rogosa and Mitchell, 1950) and noted in the systematic investigation of LAB (Kandler and Weiss,

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1986). Their natural habitats vary widely, encompassing plant materials, soil, and the gastro-intestinal tract (GIT) of human and farm animals (Claesson et al., 2007). Within the LAB group, *Lactobacillus* species are Gram-positive bacteria known mostly for their technological importance in the feed and food industry, providing interesting properties such as preservative action, enhancement of flavor, texture and nutritional effects or probiotic effects on the host (Bernardeau et al., 2006, 2008; Kumari et al., 2011). Species forming the *Lactobacillus* genus are typically subjected to variable environments during their technological production. Many stresses are encountered in industrial processes and include oxidative, temperature, osmotic, phage, and/or solvent contact stresses during industrial fermentation, as well as industrial processing stresses such as freeze-drying or freezing (Bron and Kleerebezem, 2011). Some pleomorphic traits giving to these strains technological advantages were described in this genus (Klaenhammer and Kleeman, 1981). Morphological and phenotypic variants of *Lactobacillus delbrueckii* subsp. *bulgaricus* LB6 were reported and associated with variable phage resistance phenotype (Vescovo et al., 1990). *L. helveticus* ATCC 15807 was shown to have a heterogeneous cell constitution, which could be a source of spontaneous, stable variants with particular technological characteristics such as mannose and fructose fermentation ability, phage resistance, and proteolytic activity (Reinheimer et al., 1995). For probiotics strains that are defined as "living microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (WHO/FAO, 2002), the impressive variety of technological stresses encountered during production, was enhanced during shelf-life of the product to be able to reach gut in living conditions, and after that to survive in the gut. In the consumer's gastro-intestinal (GI) tract, stress conditions include low-gastric pH, bile and digestive enzyme exposure in the duodenum, and elevated microbial competition and osmolarity in the colon (Kleerebezem et al., 2010). All these variable stress conditions induce an adaptative strategy for survival including pleomorphism. For example, variable antagonistic properties against pathogens according to the rough or smooth form of *Lactobacillus crispatus* 104 were reported (Conway and Kjelleberg, 1998). Other studies have demonstrated variable morphotype-dependent abilities such as aggregation and adhesion properties, host colonization, and immunomodulation (Cesena et al., 2001; Voltan et al., 2007), exopolysaccharide synthesis (Bouzar et al., 1996; Nikolic et al., 2012), and sugar fermentation (Reinheimer et al., 1995; Whitley and Marshall, 1999).

Lactobacillus farciminis CNCM I-3699 is a probiotic strain with a scientific and commercial interest. Mixed with *Lactobacillus rhamnosus* CNCM I-3698, these two strains have been approved by EFSA as zootechnical feed additive for post-weaning piglets (Commission regulation, 2008). Antimicrobial and livestock properties have been demonstrated *in vivo* in pigs (Owusu-Asiedu et al., 2012; Sánchez et al., 2008). Antagonistic activities against zoonotic pathogens of swine *Brachyspira* spp. (Bernardeau et al., 2009) and *Campylobacter* spp. (Tareb et al., 2013) were demonstrated *in vitro* and suggested to be linked to aggregation properties, thus highlighting the role of surface components in probiotic properties. Furthermore *L. farciminis* CNCM I-3699 is a pleiomorphic strain, able to produce concomitantly distinct colonial morphologies on solid media *in vitro*. In this study, from the parental strain CNCM I-3699, we isolated two variants that were identified and compared for main phenotypic and genotypic traits including aggregation abilities.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus strains were stored in de Man Rogosa Sharpe broth (MRS, AES, Combourg, France) with 30% glycerol at −80 °C and subcultured twice in appropriate broth at 37 °C in aerobic conditions for subculturing. Strains investigated in this study are detailed in Table 1.

Experimental culture conditions consisted in incubations in MRS broth during 18 h at 37 °C without agitation leading to a final concentration of 10^9 cfu·ml^{-1}. Atmospheric conditions for broth cultures were either aerobic, microaerobic or anaerobic to check their ability to produce different morphotypes. Variation of oxygen content of the atmospheric culture conditions was done using GENbox generators (Biomerieux). Plating was done on MRS agar (1.5% agar, Merck) incubated during 48 h at 37 °C either in aerobic, microaerobic, or anaerobic conditions, and was used for observations and enumeration.

2.2. Stability and storage of morphotypes

The reversion potential of each morphotype under different atmospheric environments was examined to define conditions under which the shift to the other morphotype was triggered and to test their stability. For all conditions, plates containing 30 to 300 colonies were examined, and the entire content of the plates was counted with respect to colony morphology. Serial passages were performed in 10 ml MRS broth for 24 h at 37 °C in aerobic conditions. The stability of the obtained rough and smooth variants was checked before and after storage at −80 °C.

2.3. Macroscopic description of colonies

The different features of colony morphology were recorded from MRS agar plates incubated for 48 h at 37 °C in aerobic or anaerobic conditions. Ropy phenotype was determined by touching the surface of the colony with a plastic tip and stretching.

2.4. Electron microscopy

L. farciminis CNCM I-3699 cells from the S and R colonies were grown under aerobic conditions in MRS broth as described above. For scanning electron microscopy the liquid cultures were centrifuged for 10 min at 10,000 ×*g*. The resulting cell pellets were fixed, dehydrated, and dried as previously described (Bernardeau et al., 2009). All preparations were observed under a JEOL 6400 F scanning electron microscope. For transmission electron microscopy (TEM), the protocol was adapted from (Schär-Zammaretti and Ubbink, 2003). Briefly, bacteria were suspended in a mixture of 2.5% glutaraldehyde in RR cacodylate buffer (0.1 M sodium cacodylate buffer at pH = 7.0 containing 0.04% Ruthenium Red) and incubated at 4 °C. After 1 h, the sedimented part of the suspensions was microencapsulated in agar gel tubes. The samples were fixed by incubation in 2.5% glutaraldehyde in RR cacodylate buffer and incubated for 16 h at 4 °C. The samples were washed 3 times with RR cacodylate buffer and incubated in 2% osmium tetroxide in the same buffer for 2 h at room temperature. The samples were next washed as described above before dehydration in a series of solutions with an

Table 1
References and culture conditions of microbial strains used in this study.

Strains	Culture collection	Origin of the isolate when known
L. acidophilus DSM 9126	DSMZ[a]	Unknown
L. farciminis CNCM-I-3699	DGCC[c]	Goat rumen isolate
L. farciminis CIP 103136[T]	CIP[b]	Sausage
L. farciminis DSM 20180	DSMZ[a]	Sausage
L. rhamnosus CNCM-I-3698	DGCC[c]	Goat rumen isolate
L. rhamnosus GG	Valio Finland	Intestinal tract of a healthy human

[a] DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.
[b] CIP Collection Institut Pasteur, France.
[c] Danisco Culture Collection.
[T] Type strain CIP 103136.

ethanol concentration increasing from 50% to 100%. The samples were then embedded by three successive incubations for 16 h at 4 °C in 50% Spurr resin in ethanol, 75% Spurr resin in ethanol, and finally in 100% Spurr resin. After polymerization of the resin (70 °C, 48 h), ultra-thin sections were cut with ultra-microtome (Leïca Ultra Cut R). Ultra-thin sections (70 nm thickness) stained with aqueous uranyl acetate and lead citrate were examined under a transmission electron microscope (TEM-JEOL 1011/micrograph MegaviewIII SIS).

2.5. Aggregation assays

Aggregation abilities of the two morphotypes were screened by spectrophotometric assay according to the method of Collado et al. (2007) using bacteria cells washed with phosphate buffer saline (PBS) and resuspended in PBS to obtain 10^8 cfu·ml^{-1} as described previously (Tareb et al., 2013). The presence of soluble clumping factors in the cell culture supernatant which accelerate aggregation rate was evaluated by visual observation according to the method of Reniero et al. (1992) by adding 10% (v/v) of freshly prepared, filter-sterilized (0.2 μm diameter membrane pore size, Millipore) MRS supernatant (from a 18 h cell culture at 37 °C) to the corresponding PBS-washed *Lactobacillus* suspension (10^8 cfu·ml^{-1}). Cultures were then immobilized at room temperature and visualized after 2 and 24 h of incubation.

2.6. Assessment of carbohydrate fermentation patterns

Carbohydrate fermentation patterns were determined using API 50CHL galleries (BioMérieux, Marcy-l'Etoile, France) following the manufacturer's instructions. The API test system did not include *L. farciminis* as a reference species.

2.7. Isolation and quantification of capsular polysaccharides (CPS)

L. farciminis S and R variants were grown at 37 °C for 18 h in 10 ml of MRS broth to obtain a final population of 10^9 cfu·ml^{-1}. The culture was centrifuged at 10,000 ×*g* for 15 min at 4 °C. To isolate CPS, the cells were washed twice with 0.9% NaCl, and further resuspended in 360 μl of 0.5 M EDTA by pipetting up and down. CPS was dissociated from cells by two times sonication for 5 s at 4 °C at 30 W with 2 s/1 s pulse (Vibracell TM 72408, Bioblock Scientific, France). The suspension was then transferred to a microtube and boiled at 100 °C for 5 min. Samples were then centrifuged at 18,000 ×*g* for 10 min at 4 °C, cell debris were removed, and 300 μl of the supernatant containing the CPS was transferred to a cold microtube. Proteinase K (20 mg·ml^{-1} final concentration) was added and samples were incubated at 60 °C for 1 h. The proteinase K was heat-inactivated at 80 °C for 30 min. CPS were recovered by precipitation upon the addition of three volumes of chilled absolute ethanol and incubation at 4 °C for 18 h. The precipitated product (crude CPS) was collected by centrifugation (16,000 ×*g*, 20 min, 4 °C) and was resuspended in 2 ml of distilled water. The total amount of carbohydrates in the CPS was determined using the phenol/sulfuric acid method (Dubois et al., 1956) with glucose as a standard. The total amount of CPS from the bacterial cell pellet was expressed as μg CPS per 10^9 cells.

2.8. 16S rRNA gene analysis

The rough and smooth variants of *L. farciminis* CNCM I-3699 were identified by sequencing of the 16S rDNA (Genoscreen, Lille, France). The obtained sequences were compared to existing sequences in the NCBI GenBank database using BLASTn (Altschul et al., 1997) and BIBI (http://pbil.univ-lyon1.fr/bibi/) (Devulder et al., 2003).

2.9. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis was adapted from the method previously described (Coeuret et al., 2004). Overnight cultures (10 ml) of *L. farciminis* in MRS broth at 37 °C were adjusted to an OD_{600} of 0.5 with Tris–EDTA. Cells were centrifuged at 7000 ×*g* at 4–8 °C for 10 min, washed once in 5 ml TES buffer (10 mM Tris, 1 mM EDTA, 0.5 M saccharose), and suspended in 400 μl of suspension buffer (10 mM Tris–HCl, 50 mM EDTA, 20 mM NaCl). The resulting suspensions were mixed with 500 μl low-melting point agarose (2% in suspension buffer at 50 °C) and poured into plug molds. The molds were then incubated at 4 °C for 20 min. Plug lysis was done in flasks containing 5 ml lysis buffer (30 mg·ml^{-1} lysozyme, 50 U·ml^{-1} mutanolysin, 0.5% N-lauryl sarcosine, 0.2% sodium deoxycholate, 10 mM Tris, 50 mM NaCl) incubated for at least 18 h at 37 °C. After washing twice with 25 ml TE (10 mM Tris, 1 mM EDTA) for 20 min, incubation with proteinase K was done at 50 °C for 18 h in 4 ml proteinase K buffer (2 mg/ml proteinase K, 1% N-lauryl sarcosine, 0.2% sodium deoxycholate, 100 Mm EDTA, pH 8). The plugs were washed twice with 20 to 30 ml TE for 20 min followed by two successive additions of 50 μl PMSF (phenylmethylsulfonyl fluoride, 17.5 mg in 1 ml propanol-2), each for a 30-min incubation at 55 °C. The TE and PMSF were then discarded and the inserts washed twice with 20 to 30 ml TE for 20 min. Finally, the TE was discarded and the plugs placed in 50 mM EDTA and stored at 4 °C. Each plug was equilibrated during 1 h at 4 °C in a reaction tube (1.5 ml) containing 1 ml of TE and then during 1 h at 4 °C in 200 μl of 1× enzyme buffer. Digestion was done in a distinct tube with 200 ml 1× enzyme buffer and 20 units of restriction enzyme for 4 h at the appropriate temperature in a water bath. The following restriction enzymes were used: NotI and ApaI (Sigma-Aldrich). PFGE was performed with a CHEF DR III apparatus (Biorad) with 1% pulsed-field certified agarose in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.3). The voltage was adjusted to 6 V·cm^{-1} and the gels were maintained at 14 °C for 18 h; the pulse time was 2–25 s.

2.10. CRISPR analyses

The draft genome sequence of *L. farciminis* KCTC 3681^T is available under accession number AEOT00000000 (Nam et al., 2011). CRISPRFinder (Grissa et al., 2007a,b) was used for *in silico* analysis to identify CRISPR loci and then to retrieve CRISPR repeat and spacer sequences loci. For experimental CRISPR typing, the reference strain CIP 103136^T, and CNCM I-3699 and its morphotypes R and S were used. Genomic DNAs were extracted and purified using FTA paper (Whatman), and submitted to two distinct PCR amplifications using primers flanking each CRISPR locus. The CRISPR1 locus was amplified with primers CR1Lfar-F1 (5′-ttttaggatatgagacgtcg-3′) and CR1Lfar-R2 (5′-ataccaacgtgatcatgacc-3′), whereas the CRISPR2 locus was amplified with primers CR2Lfar-F3 (5′-cattggtcgcctattgaacg-3′) and CR2Lfar-R4 (5′-gatttgaaacgtgtccggac-3′). Each PCR product was then subjected to DNA sequencing from each end with the same primers on a CEQ8000 system (Beckman–Coulter). The spacer sequences of CRISPR loci were analyzed and graphically represented using color schemes, as previously described (Horvath et al., 2008).

2.11. Statistics

The results are expressed as the mean ± standard deviation of three independent experiments. Statistical analysis was done using StatGraphicPlus software. Data were subjected to a one-way analysis of variance (ANOVA).

3. Results

3.1. Isolation of the rough and smooth morphotypes of L. farciminis CNCM I-3699 and description of their phenotypic traits

Different atmospheric conditions (aerobic, anaerobic, microaerobic) of successive broth and agar plate cultivations were tested in order to select and isolate “pure” rough and smooth morphotypes (named

R and S, respectively) from the *L. farciminis* strain CNCM I-3699 (Fig S1A). In anaerobic conditions, only S form growth was observed. However, R and S morphotypes along with intermediate morphotypes were regularly observed when growing the parental strain under aerobic culture conditions, as a mix of dominant S forms along with rough R morphotypes (R/S ratio range from 1 to 2%). In aerobic culture conditions, bright white, convex colonies with smooth edges corresponded to the S morphotype, while large, flat colonies with irregular edges and pale white color corresponded to the R morphotype. Those two morphotypes were isolated and sub-cultured in standard aerobic conditions and both were stable over 30 successive subcultures, and thus were thought to be pure strains. At that point, frozen vials of each S or R morphotype were produced and named CNCM I-3699S and CNCM I-3699R, respectively, and stored at −80 °C in order to be used as a primary stock culture for all subsequent experiments. The isolated R form was stably propagated over 123 serial subcultures in aerobic conditions before and after freezing. In the same conditions the S form was stable, although at a low frequency (less than 10%) it could produce, a mix of dominant S form along with intermediates and or R forms, and thus it is reminiscent of the parent strain plating pattern. Morpho-macroscopic characteristics of CNCM I-3699S and CNCM I-3699R are given in Table 2. Touching the R or S colonies on agar plates with plastic tips revealed that the moist and creamy S colonies were ropy (Fig S1B), whereas R colonies were dry, granular, and not ropy.

3.2. Microscopy observations

Scanning microscopy observations of *L. farciminis* CNCM I-3699 revealed microscopic differences and confirmed macroscopic heterogeneity (Fig 1). Culture of the parental *L. farciminis* strain CNCM I-3699 presented two types of cells (Fig 1A): one type, present in majority, exhibited heterogeneity on the cell surface whereas the other type had a regular uniform surface aspect. Microscopic observations of the two stable S and R morphotypes cultivated separately revealed that cell surfaces of the R morphotype were uniform, as shown in Fig 1B, whereas the cell surfaces of the S morphotype were heterogeneous and covered by a high amount of globular and filamentous exopolymers (Fig 1C). Those observations suggest that the parental population of *L. farciminis* CNCM I-3699 contains both morphotypes S and R. The ropy phenotype observed for the S form could be explained by abundant surface exopolymer secretion. Transmission electron microscopy observations of CNCM I-3699S and CNCM I-3699R also revealed major differences in cell surface structure (Fig 2). The cells of the S morphotype exhibited surfaces with high amount of granular and filamentous exopolymers (Fig 2A) whereas the cell surface of the R morphotype appeared uniform (Fig 2B). This is reminiscent of the aspect of capsular polysaccharide surfaces described in a previous study (Dabour et al., 2005) in which ruthenium red coloration was used also to enhance capsule resolution.

3.3. Biochemical analysis

The amounts of CPS (expressed in $mg \cdot ml^{-1}$) extracted from each R or S morphotype of *L. farciminis* CNCM I-3699 were 9.22 ± 0.04 and 14.33 ± 0.37 $\mu g/10^9$ cells, respectively. The significant difference in values ($p < 0.01$) obtained with the R and S morphotypes might be correlated with the above visual differences observed between the two capsules, especially for the external heterogeneous part observed for the S morphotype. These results also corroborate the smooth appearance and sticky, viscous ropes observed with S colonies (Fig S1B).

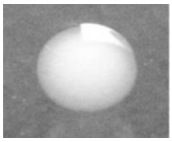
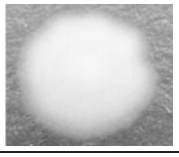
3.4. Fermentation profiles

Carbohydrates fermentation profiles were identical for the parent strain CNCMI-3699 and its variants (Table S1). Both variants were able to ferment D-glucose, galactose, fructose, mannose, salicine, N-acetylglucosamine, amygdaline, arbutin, esculin, cellobiose, maltose, lactose, gentiobiose, D-turanose, D-tagatose, saccharose, and trehalose.

3.5. Auto-aggregation profiles

A significant difference between R and S morphotypes was easily visible after growth in MRS broth incubated at 37 °C for 18 h in aerobic conditions. Broth cultures of the S morphotype appeared turbid whereas the R morphotype spontaneously aggregated at the bottom of the tube, leaving a clear supernatant (Fig S1C). The auto-aggregation profiles were also checked visually according to the method of Reniero et al. (1992) where supernatant of an 18-hour culture at 37 °C in MRS of a bacterium was added at 10% to the corresponding bacteria in suspension in PBS. The two morphotypes were compared to other strains of *L. farciminis* and to other *Lactobacillus* species recognized for their aggregating potential, such as *L. rhamnosus* GG (Tripathi et al., 2013), and *Lactobacillus acidophilus* DSM 9126 (unpublished data) (Fig S2). It was possible to differentiate the strains suspensions by visual observation of gradual turbidity and transparency after only 2 h. After this time, positive controls such as GG and DSM 9126 did not even initiate their auto-aggregation process whereas the R morphotype expressed the fastest auto-aggregating pattern of all the strains tested. After 24 h (data not shown) the turbid suspension of the S morphotype was maintained. In additional experiments and according to Reniero et al.'s (1992) method, we exchanged the supernatants from R and S cultures, but this leads to similar results as those described when no exchange was done. This means that soluble factors are not the main cause of the differential aggregation behavior observed with R and S cultures, suggesting that the aggregative behavior is directed by bacterial cells in PBS rather than by specific soluble factors which could have been present when added at 10%. These qualitative observations were completed by a quantitative determination of auto-aggregation using the method of Collado et al. (2007) involving only rinsed bacterial cells, without any culture

Table 2
Morpho-macroscopic characteristics of smooth and rough morphotypes of *L. farciminis* CNCM I-3699 grown on MRS agar at 37 °C under aerobic conditions.

Morpho-macroscopic characteristics	Morphotype smooth (S)	Morphotype rough (R)
Colony size	Diameter 0.5 mm–1.5 mm;	Diameter 1.5 mm–4 mm
Colony shape	Convex	Flat
Colony circumference	Regular	Irregular
Overall surface shape degree of color texture	Smooth, shiny, ropy	Flat, matte, rough, pale, dry
Color	White	White to gray
Translucency	Opaque center and translucent edges, bright	Opaque, cloudy
Photography		

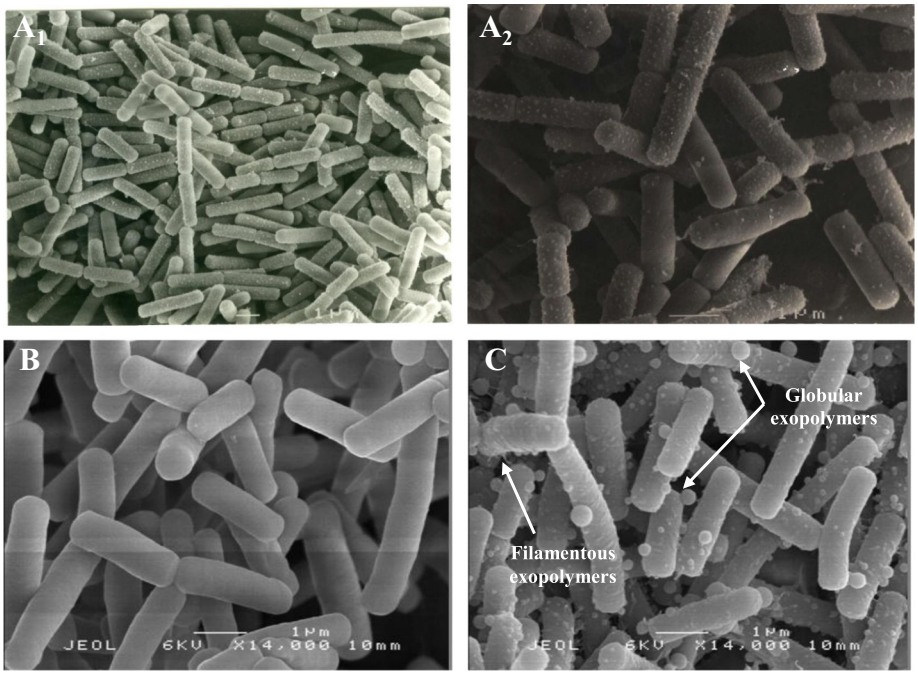


Fig. 1. Cells of parent culture of *L. farciminis* CNCM I-3699 (A_1 & A_2) and its rough (B) and smooth (C) morphotypes grown in MRS broth at 37 °C, 18 h, under aerobic atmosphere and observed by scanning electron microscopy.

supernatant added. The two morphotypes as well as *L. acidophilus* DSM 9126, *L. rhamnosus* CNCM I-3698 and GG, and *L. farciminis* CIP 103136 and DSM 20180 were included in this study. The S and R morphotypes of CNCM I-3699 exhibited significantly different auto-aggregative profiles ($p < 0.01$) after 24 h. R was two-fold more auto-aggregative after 24 h compared to S (% of auto-aggregation after 24 h: 81.35% ± 2.69 and 43.42% ±6.10, respectively) (Fig 3). This quantitative characterization demonstrated that the well-known auto-aggregative *L. rhamnosus* GG and the R morphotype exhibited similar auto-aggregation abilities, that were significantly higher compared to all the other strains tested (Fig 3). Furthermore, strain ranking according to their auto-aggregative profiles after 24 h was congruent with that obtained with the qualitative method after visual observation at 2 h (Fig S2). It suggests that auto-aggregative parameters are mainly associated

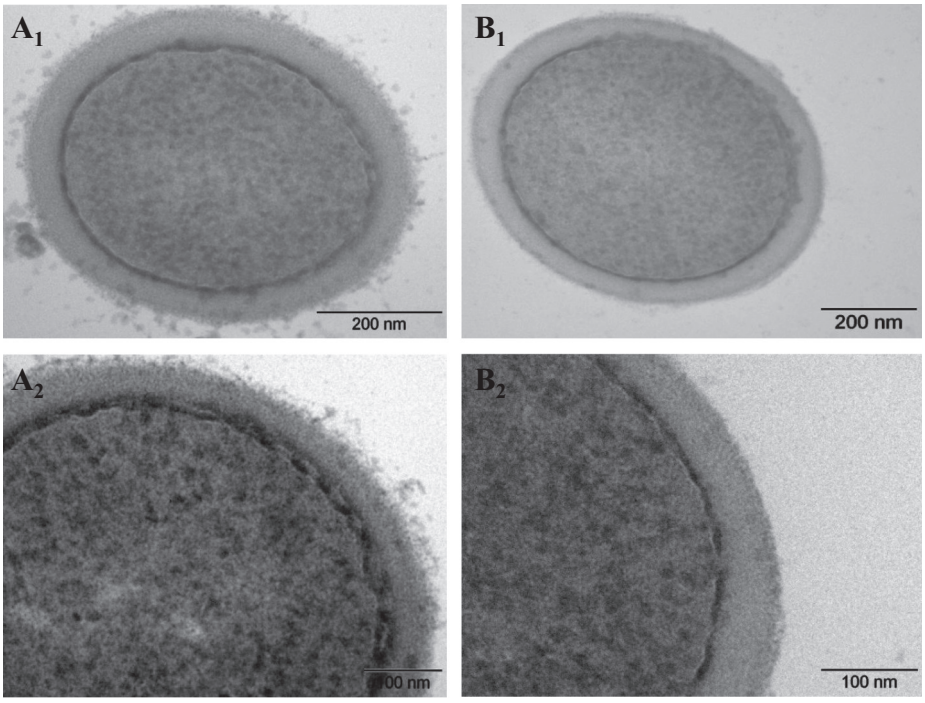


Fig. 2. Transmission electron micrographs of the *Lactobacillus farciminis* CNCM I-3699 type smooth (A_1 & A_2) and *L. farciminis* CNCM I-3699 type rough (B_1 & B_2) grown in MRS broth at 37 °C, 18 h, under aerobic condition. In micrograph A, fibrillose materials attached to the bacterial wall can be observed.

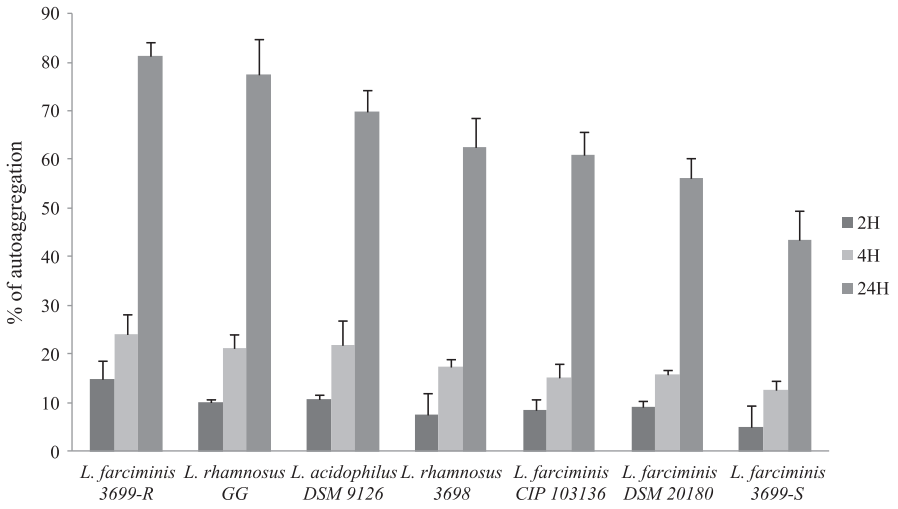


Fig. 3. Autoaggregation percentages over time (2, 4, 24 h) of *Lactobacillus* strains determined by the spectrophotometry assay method.

with strain-specific cell membrane factors, while soluble factors present in the supernatant only accelerate the auto-aggregative cell process, as observed by Reniero et al. (1992).

3.6. Molecular analyses and comparison of the rough and smooth morphotypes

The 16S rDNA sequences obtained for the S and R morphotypes (1051 and 1069 bp, respectively) confirmed their identification to the *L. farciminis* species, since they both showed 99.7% identity to the *L. farciminis* CIP 103136^T 16S rDNA sequence.

The PFGE patterns obtained either with Not*I* or Apa*I* endonuclease on different *L. farciminis* strains, including DSM 20180, CIP 103136, and the R and S morphotypes of CNCM I-3699 are shown in Fig 4. Regardless of the enzyme used, PFGE typing provided undistinguishable pulsotypes for the R and S morphotypes, while these were different for the other *L. farciminis* strains (Fig 4: lanes 1, 2 and 3–4).

Due to the high evolutionary turnover of their spacer content, CRISPR loci constitute "first-class" chromosomal regions for strain identification and differentiation (Barrangou and Horvath, 2012; Horvath and Barrangou, 2010). Based on the draft genome sequence of *L. farciminis* KCTC 3681^T (Nam et al., 2011) we identified two distinct Type II CRISPR-Cas systems belonging to the previously described families Sthe1 or Lsal1 (Horvath et al., 2009) (Fig 5; see also S3). Downstream of a set of four *cas* genes including the Type II signature *cas9* and the universal *cas1* and *cas2* genes, the CRISPR1 locus (Sthe1 family) comprises 6 repeats that define 5 unique spacers. The CRISPR2 locus is located downstream of another set of four analogous *cas* genes (Lsal1 family) and is composed of 10 repeats defining 9 unique spacers. None of these 14 spacers show significant sequence similarity to any sequence in GenBank, which might be explained by the limited information currently available on *L. farciminis* mobile genetic elements such as phages and plasmids. From this preliminary analysis, we designed flanking primers for PCR amplification and DNA sequencing of each CRISPR locus (Fig S3). In addition to the above *in silico* analysis of *L. farciminis* KTCC 3681, the type-strain CIP 103136^T, CNCM I-3699, and its two morphotypes R and S were subjected to CRISPR typing. All strains were positive for both amplifications, producing CRISPR1 amplicons with a size between 700 bp (for *L. farciminis* CIP 103136) and 2 kb (for CNCM I-3699 and its R and S isolates), while PCR amplification of CRISPR2 generated a product of 1 kb for strain CIP 103136 and 1.4 kb for strain CNCM I-3699 and its variants (Fig S4). All these PCR products were sequenced from both ends using the same primers. Sequence analysis showed that strains CIP 103136, and KCTC 3681 contain almost identical CRISPR1 and CRISPR2 loci (with the exception of a single nucleotide polymorphism in the fifth spacer of CRISPR2), indicating that these strains are closely related, if not identical (Fig 6). In contrast, CNCM I-3699 contains totally different spacer content at both CRISPR1 and CRISPR2 loci, suggesting that CNCM I-3699 is not closely related to the type strain. Its CRISPR1 locus contains only two spacers, and appears to be truncated by an insertion sequence (IS) belonging to the IS*L3* family. Downstream of the second spacer, the presence of a truncated repeat followed by a complete repeat may be a trace of the recombination event that occurred during integration of the IS element. CRISPR2 contains 13 unique spacers. Only one of the total 15 CRISPR spacers of CNCM I-3699 shows partial but significant sequence identity

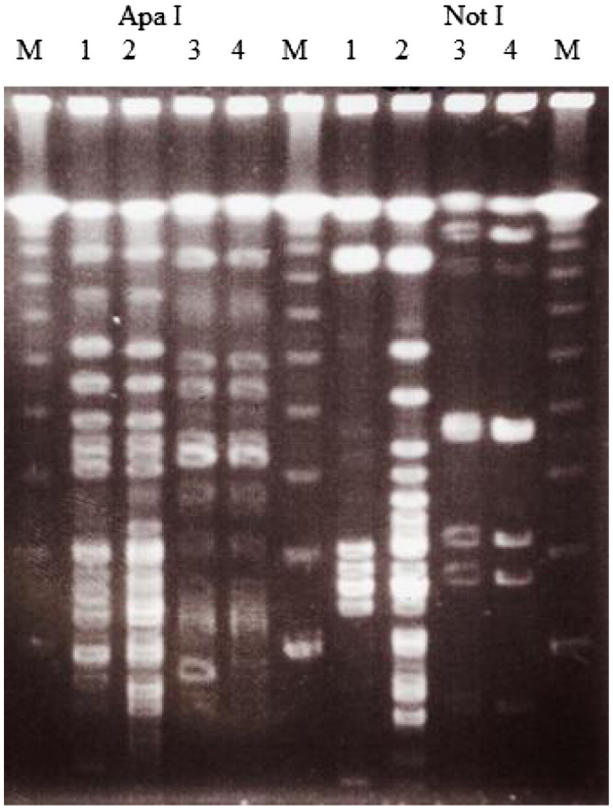


Fig. 4. Pulsed field gel electrophoresis patterns of *Apa*I and *Not*I digested genomic DNA of *L. farciminis* DSM 20180 (lane 1), *L. farciminis* CIP 103136 (lane 2), *L. farciminis* CNCM I-3699-S (lane 3) and *L. farciminis* CNCM I-3699-R (lane 4), molecular weight marker (phage *lambda* λ) (M).

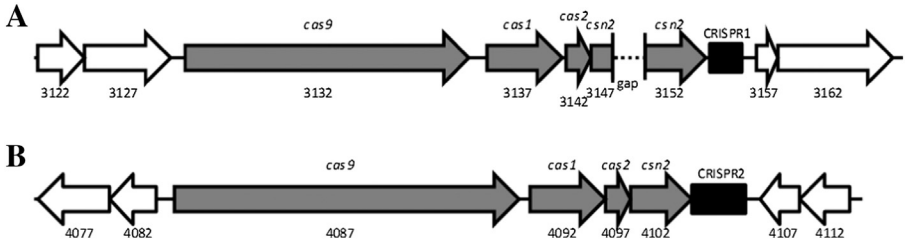


Fig. 5. Overview of the two *L. farciminis* CRISPR loci in the KCTC 3681^T genome: A) CRISPR1/Cas loci (Sthe1 family) and B) CRISPR2/Cas loci (Lsal1 family). The repeat-spacer region were represented as black boxes and cas genes represented as gray arrows.

with current entries in GenBank. Specifically, the fourth CRISPR2 spacer contains a stretch of 21 consecutive bases that are 100% identical to a sequence found in *E. coli* T5-like bacteriophages (*e.g.* accession number AY543070, or AJ604530). Both R and S morphotypes and their pleomorphic parental strain CNCM I-3699 share identical CRISPR1 and CRISPR2 loci, with exactly the same spacer content and sequence as shown in Fig 6, which confirms that the R and S isolates are derivatives of the parental strain CNCM I-3699, and not just different strains of the same species. This analysis also demonstrates that *L. farciminis* CNCM I-3699 is genetically different from the type strain. CRISPR sequences obtained in this study constitute a valuable strain-specific molecular signature which can be used to detect and track CNCM-I-3699 and its derivatives, notably by using PCR or qPCR.

4. Discussion

L. farciminis CNCM I-3699 presents heterogeneous colonies on MRS agar and is described here as a pleomorphic strain constituted of well-defined rough and smooth variants (along with intermediate forms) whose presence depends on environmental atmospheric conditions. Although this phenomenon was initially described as being associated to pathogen adaptation and virulence, spontaneous R and S morphotypes were observed in some lactobacilli having technological or beneficial interests such as *L. acidophilus* RL8K (Klaenhammer and Kleeman, 1981), *L. acidophilus* ATCC 4356 (Khaleghi et al., 2011), *Lactobacillus amylovorus* NCFB 2745 (Whitley and Marshall, 1999), *L. brevis* ATCC 14869 (Jakava-Viljanen et al., 2002), *Lactobacillus johnsonii* FI9785 (Horn et al., 2013), *L. rhamnosus* ATCC 9595 (Péant et al., 2005), *Lactobacillus plantarum* WCFS1 (Bron et al., 2004), or *Lactobacillus sakei* (Chiaramonte et al., 2010). Furthermore, similar to the present work, R and S morphotypes of *Lactobacillus brevis* ATCC 14869 were obtained in the presence or absence of O_2 during cultivation (Jakava-Viljanen et al., 2002). Anaerobic or aerated cultures of *L. brevis* ATCC 14869 in rich medium resulted in morphotype-specific surface-layer (S-layer) protein (Slp) patterns: under aerobic conditions, R cells produced SlpB and SlpD proteins, whereas under anaerobic conditions S cells synthesized essentially only SlpB (Jakava-Viljanen et al., 2002). However other cultivation parameters have been shown to allow morphological changes in *Lactobacillus* strains. In other cases, R and S morphotypes of *Lactobacillus* strains were isolated by the use of varying energy source carbohydrates (Whitley and Marshall, 1999), or in the presence of penicillin (Khaleghi et al., 2011) or bile salts in broth culture (Bron et al., 2004), or more recently by deleting a small heat shock gene of *L. plantarum* WCFS1 (Capozzi et al., 2011).

In this study we compared the growth in MRS broth of CNCM I-3699 R and S morphotypes and their aggregation potential. The R morphotype was shown to aggregate and sediment at the bottom of the tube within only 2 h, whereas the S morphotype still exhibited a planktonic growth after 24 h. Those results are reminiscent of previous studies which showed that a change from R to S morphotype of *L. acidophilus* isolated from an adult fowl coincided with a change from aggregative to planktonic growth in liquid medium, and a marked loss of ability to adhere (Schneitz et al., 1993). Cell surface structures of lactobacilli are expected to contribute in several ways to their interaction with the host gastrointestinal tract and the gut microbiota, affecting their survival, adherence to the host tissue (Cesena et al., 2001; Voltan

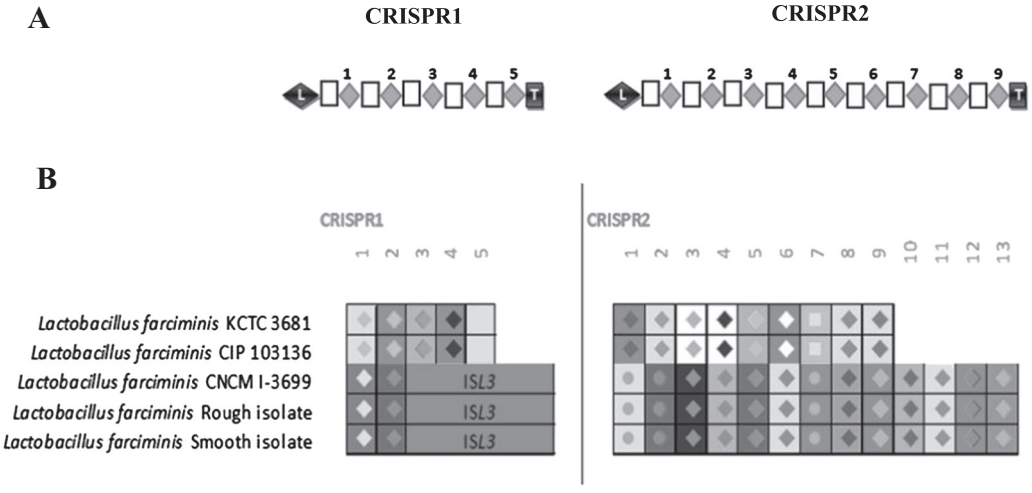


Fig. 6. Graphic representation of CRISPR1 and CRISPR2 in different *Lactobacillus farciminis* strains. A: The repeat-spacer region found by *in silico* analysis is shown at the top of this figure for KTCC 3681 CRISPR1 and CRISPR2, with repeats shown as white boxes and spacers shown as numbered gray diamonds; the leader (L) was shown as a black diamond, and the trailer end (T) shown as a black box. B: The spacer content of CRISPR1 and CRISPR2 found by molecular analysis of different strains KTCC 3681, CIP 103136, CNCM I-3699, CNCM I-3699-R and CNCM I-3699-S is shown. Repeats are not included. Each spacer is represented by a combination of one selected character in a particular font color, on a particular background color. The color combination allows unique representation of a particular spacer: squares with similar color schemes (combination of character color and background color) represent identical spacers, whereas different color combinations represent distinguishable spacers. Downstream of CRISPR1 in CNCM I-3699, an insertion sequence of the IS*L3* family is present between the last repeat and the trailer.

et al., 2007; Weiser et al., 2001) and interactions with themselves and with other bacteria (Kleerebezem et al., 2010), resistance to phage (Suárez et al., 2008) and technological properties linked to the formation of texture in food products (De Vuyst and Degeest, 1999). In this study, the *L. farciminis* CNCM I-3699S variant showed a ropy phenotype, denoted by the formation of a long filament each time a colony was picked. SEM and TEM observations performed on the two CNCM I-3699 variants revealed marked cell surface differences with ornamental exopolymers present only around S cells. This specificity has been associated with the production of exopolysaccharides (EPS) (Nikolic et al., 2012) which often presents a technological interest for food LABs (Badel et al., 2011; Ruas-Madiedo et al., 2006). As recently reviewed by Lee et al. (2013) polysaccharides are ubiquitously found in *Lactobacillus* cell walls. They can be covalently linked to the peptidoglycan (wall polysaccharides), loosely attached to the cell envelope (capsular polysaccharides) or released into the environment (exopolysaccharides). Depending on their structure and their location, they are involved in different biological functions. Interestingly, a recent study correlated a mutation in the *epsC* gene of a smooth variant of *L. johnsonii* FI10386 to a less dimpled surface and a high frequency of small lumps on the surface (Horn et al., 2013).

Here, the *L. farciminis* CNCM I-3699S variant also presents significantly higher dosage of CPS compared to the R variant. As the R variant showed a high auto-aggregation potential while the S form did not, it suggests that the higher CPS production observed for the smooth variant is not required for the formation of aggregates. On the contrary, CPS seem to have a negative impact on auto-aggregation, possibly by shielding off receptor-like adhesins, as already suggested for streptococci (Morona et al., 2004). Similarly, an EPS-negative mutant of *L. johnsonii* NCC533 was reported to have a slightly increased residence time in the murine gut, possibly due to an enhanced exposure of adhesins (Denou et al., 2008), and an EPS negative mutant of *L. rhamnosus* GG showed an increased biofilm formation capacity (Lebeer et al., 2008) by less shielding of adhesins such as fimbria-like structures (Lebeer et al., 2009). It is known that the presence of capsular polysaccharide can mask clumping factors that mediate adherence in other bacterial genera (Risley et al., 2007). Thus the close correlation between morphotype variation, aggregation ability, and CPS production in *L. farciminis* CNCM I-3699 and its variants agreed with results published in the literature.

In order to determine the reasons of this colony polymorphism, and particularly to differentiate the pleomorphism hypothesis from a mixed population case, it has been decided to perform strain differentiation using molecular typing method(s), with the idea that pleomorphs should have (almost) identical genetic backgrounds, while a mixed population of unrelated strains would more likely to display distinct patterns. Strain typing was achieved using PFGE with two distinct restriction enzymes as a broad, global approach, which produced undistinguishable patterns for these three strains, whereas other *L. farciminis* strains could be easily differentiated. In the absence of any other published, robust typing method for the *L. farciminis* species, and because CRISPR typing was previously shown to be highly discriminant for strain differentiation, notably in several *Lactobacillus* species, a dual CRISPR-based typing scheme was developed which relies on CRISPR spacer sequence comparison. Based on the identification of two CRISPR loci within the draft genome sequence of *L. farciminis* KCTC 3681, a sequence-based CRISPR analysis was performed. Indeed, over the last years, CRISPR typing has settled as one of the most powerful approaches for bacterial strain differentiation (Barrangou and Horvath, 2012) and typing (Fabre et al., 2012). Obtaining identical sequences for both CRISPR1 and CRISPR2 loci from CNCM I-3699 and their R and S morphotypes, while other *L. farciminis* strains displayed different CRISPR spacer content, unquestionably shows that CNCM I-3699 and its R and S morphotypes are related. In addition, as all 15 CRISPR spacers identified in CNCM I-3699 are unique and novel, CRISPR sequences obtained in this study provide genetic support for the development of a molecular tracking tool for CNCM I-3699 and its variants since the pleiomorphic CNCM I-3699 strain (including its variants) has been recognized and authorized as a microbial feed additive.

In conclusion, *L. farciminis* CNCM I-3699 is a pleomorphic strain giving reproducibly rise to two phenotypically distinct morphotypes R and S. This phenomenon may explain survival and growth abilities in *in vitro* fluctuating aerobic–anaerobic conditions along with modulation of exopolysaccharides production and autoaggregation profile. These significant differences in capsular polysaccharide production shown for these two variants, should logically be supported by some genetic differences, as it is the case for *L. johnsonii* FI9785 and its mutant FI10386 (Horn et al., 2013). Comparative genomics analyses are currently underway in order to identify the putative mutational events that explain the phenotypic instability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.08.036.

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