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Development of antifungal ingredients for dairy products: From *in vitro* screening to pilot scale application

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ABSTRACT

Biopreservation represents a complementary approach to traditional hurdle technologies for reducing microbial contaminants (pathogens and spoilers) in food. In the dairy industry that is concerned by fungal spoilage, biopreservation can also be an alternative to preservatives currently used (e.g. natamycin, potassium sorbate). The aim of this study was to develop antifungal fermentates derived from two dairy substrates using a sequential approach including an *in vitro* screening followed by an *in situ* validation. The *in vitro* screening of the antifungal activity of fermentates derivating from 430 lactic acid bacteria (LAB) (23 species), 70 propionibacteria (4 species) and 198 fungi (87 species) was performed against four major spoilage fungi (*Penicillium commune, Mucor racemosus, Galactomyces geotrichum* and *Yarrowia lipolytica*) using a cheese-mimicking model. The most active fermentates were obtained from *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus casei/paracasei* and *Lactobacillus plantarum* among the tested LAB, *Propionibacterium jensenii* among propionibacteria, and *Mucor lanceolatus* among the tested fungi. Then, for the 11 most active fermentates, culture conditions were optimized by varying incubation time and temperature in order to enhance their antifungal activity. Finally, the antifungal activity of 3 fermentates of interest obtained from *Lactobacillus rhamnosus* CIRM-BIA1952, *P. jensenii* CIRM-BIA1774 and *M. lanceolatus* UBOCC-A-109193 were evaluated in real dairy products (sour cream and semi-hard cheese) at a pilot-scale using challenge and durability tests. In parallel, the impact of these ingredients on organoleptic properties of the obtained products was also assessed. In semi-hard cheese, application of the selected fermentates on the cheese surface delayed the growth of spoilage molds for up to 21 days, without any effect on organoleptic properties, *P. jensenii* CIRM-BIA1774 fermentate being the most active. In sour cream, incorporation of the latter fermentate at 2 or 5% yielded a high antifungal activity but was detrimental to the product organoleptic properties. Determination of the concentration limit, compatible with product acceptability, showed that incorporation of this fermentate at 0.4% prevented growth of fungal contaminants in durability tests but had a more limited effect against *M. racemosus* and *P. commune* in challenge tests. To our knowledge, this is the first time that the workflow followed in this study, from *in vitro* screening using dairy matrix to scale-up in cheese and sour cream, is applied for production of natural ingredients relying on a large microbial diversity in terms of species and strains. This approach allowed obtaining several antifungal fermentates which are promising candidates for dairy products biopreservation.

1. Introduction

Fungal spoilage of dairy products is responsible for significant food waste and economic losses (Pitt and Hocking, 2009). Dairy products, despite their acidic pH and high content in organic acids, may provide a favorable environment for the development of undesirable micro-organisms including fungi. Fungal spoilage is usually easy to detect due to the presence of colonies or thalli at the surface of the product.

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Nonetheless, fungal spoilage may also be non-visible resulting in flavor or texture alteration via fungal metabolism (Ledenbach and Marshall, 2010), thus preventing product consumption. According to the literature, species belonging to the *Penicillium*, *Mucor, Cladosporium, Candida*, *Galactomyces* and *Yarrowia* genera have been identified as the most common contaminating molds and yeasts, respectively (Pitt and Hocking, 2009; Garnier et al., 2017a).

In order to avoid or delay fungal spoilage, and hence extend product shelf life, prevention and control methods are currently used by industrials. Prevention methods applied in the dairy industry include good manufacturing and hygiene practices, implementation of Hazard Analysis Critical Control Point (HACCP) system, and use of air filtration or packaging in aseptic conditions (Garnier et al., 2017a). Control methods can involve the use of heat treatment, modified atmosphere packaging or fermentation with beneficial microorganisms (Garnier et al., 2017a; Leyva Salas et al., 2017; Nguyen Van Long et al., 2016; Sakkas et al., 2014; Bourdichon et al., 2012). In specific dairy products such as sour cream or semi-hard cheese, the addition of defined chemical preservatives with antifungal properties (e.g. natamycin or organic acids and their salts) is permitted depending on the regulation in place (Leyva Salas et al., 2017; Regulation (EC) No 852/2004). However, there is a strong societal demand for “preservative-free” food products since consumers are looking for more “natural” (i.e. without chemical preservatives) and less heavily processed foods. Therefore, industrial groups are looking for efficient solutions to replace chemical preservatives while maintaining the current shelf-life of food products (Varsha et al., 2016).

In this context, biopreservation, defined as the use of natural or added microbiota and/or their antimicrobial compounds to extend food shelf-life and increase food safety (Stiles, 1996), could represent a complementary approach to reduce fungal spoilage and when applicable, an alternative to chemical preservatives. The use of lactic acid bacteria (LAB) and propionibacteria and/or their metabolites is particularly interesting for reducing fungal spoilage in dairy products (Leyva Salas et al., 2017). Indeed, LAB and propionibacteria are naturally present in many foods and have a long history of safe use in fermented foods (Bourdichon et al., 2012). They produce a wide range of antifungal compounds including organic acids such as acetic, lactic, phenyllactic, propionic and fatty acids as well as cyclic dipeptides or proteinaceous compounds (Crowley et al., 2013; Schnürer and Magnusson, 2005; Lavermicocca et al., 2000). Fungal inhibition generally results from the additive and/or synergistic activities of several of these compounds (Varsha et al., 2016). In addition to LAB and propionibacteria, some fungi can also produce antifungal compounds such as alcohols, acids, esters, and proteinaceous compounds (Delgado et al., 2016; Leyva-Salas et al., 2017), and their utilization could also be compatible with some dairy technologies.

Commercial antifungal solutions for dairy products are currently available on the market. All of them involve LAB and/or propionibacteria in single or culture combinations. However, their efficiency varies according to the dairy technologies in which they are used (Leyva-Salas et al., 2017; Garnier et al., 2017b). These microorganisms can be used as adjunct cultures (i.e. co-inoculated with the starter culture during product manufacturing) or to produce fermentates (e.g. resulting from dairy substrate fermentation) added to food as an ingredient (Varsha et al., 2016; Garnier et al., 2017b).

While there is an increasing number of studies showing the *in vitro* antifungal activity of selected microorganisms and/or their metabolites for application in dairy products or other foods (Leyva-Salas et al., 2017), very few have dealt with the efficiency of such antifungal ingredients in real products and their potential impact on product organoleptic properties (Lynch et al., 2014; Delavenne et al., 2015). Moreover, to our best knowledge, no work on the development of a biopreservation ingredient to replace natamycin (E235 in the European Union) for cheese surface-treatment has been reported yet in the literature.

The aim of this study was to develop dairy antifungal biopreservation ingredients obtained by fermentation, by selected microorganisms, of 2 dairy substrates (low-heat milk -LH- and milk permeate -—UF—). In this framework, a sequential approach based on *in vitro* screening and *in situ* validation at a pilot scale on actual dairy products, namely sour cream and semi-hard-cheese, was used.

2. Materials and methods

2.1. Fermentate production

Two different dairy substrates, UF and LH, were used to prepare fermentates. The UF substrate consisted of an ultrafiltration milk permeate (T.I.A., Bollene, France) supplemented with 10 g/L yeast extract (Biokar Diagnosis) and 0.5% litmus, and filtrated at 0.22 µm (VWR, Radnor, USA). The LH substrate was obtained as described by Garnier et al. (2018). Both substrates were then individually inoculated by 430 LAB, 70 propionibacteria or 198 fungal strains obtained from the Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (LUBEM, Plouzané, France), the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France, www.univ-brest.fr/ubocc) and the CIRM-BIA (Centre International de Ressources Microbienne-Bactéries d’Intérêt Alimentaire, Rennes, www.collection-cirmbia.fr) culture collection (Supplementary Table S1). For LAB and propionibacteria strains, respectively, LH and UF substrates were inoculated with bacterial MRS and YEL broth pre-cultures (Difco, Le Pont de Chaix, France) according to Garnier et al. (2018), except for *Lactobacillus amylovorus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* strains which were pre-cultured and incubated at 37 °C. *Lactobacillus sanfranciscensis* and *Lactococcus lactis* strains were pre-cultured in MRS and M17 supplemented with 20 g/L maltose, respectively (Difco, Le Pont de Claix, France). For yeasts, LH and UF media were inoculated (1% v/v) after 2 pre-cultures (24 h at 25 °C, 120 rpm agitation speed) in Yeast Glucose Chloramphenicol (YGC) broth (Difco, Le Pont de Claix, France), and incubated for 48 h in the same conditions. Concerning molds, they were first cultivated on Potato Dextrose Agar (PDA) slant for 7 d at 25 °C. After spore recovery with 0.01% Tween 80 and enumeration using a Malassez cell, LH and UF media were inoculated at 10^4 spores/ml and incubated for 96 h at 25 °C on a rotary shaker at 120 rpm. Yeast and mold fermentates were centrifuged three times (8000 g at 15 min), the cell pellets were discarded and the final supernatant was filter-sterilized at 0.45 µm (VWR, Radnor, USA). Fermentates were then stored at −80 °C until use. For pilot scale applications, fermentates were produced in 2–5-L flasks. Bacterial fermentates were lyophilized (Virtis, Fisher, France, final residual water < 2% after freeze drying under vacuum) while fungal fermentates were prepared as previously described.

2.2. In vitro screening of antifungal activity

The antifungal activity of the obtained fermentates against *Mucor racemosus* UBOCC-A-116002, *Penicillium commune* UBOCC-A-116003, *Galactomyces geotrichum* UBOCC-A-216001 and *Yarrowia lipolytica* UBOCC-A-216006, isolated from spoiled dairy products and representative of the most common dairy product spoilers (Garnier et al., 2017b), was evaluated *in vitro* using a high-throughput screening method in a cheese-mimicking model as described by Garnier et al. (2018).

2.3. Optimization of culture conditions for antifungal activity enhancement

Bacterial (n = 12) and fungal (n = 1) fermentates exhibiting the highest antifungal activity were selected for an optimization step of culture conditions. Depending on the considered microorganism, 5 time/temperature combinations were tested: 20, 48 and 72 h at 30 °C as

Table 2
Antifungal activity of selected fermentates against *M. racemosus* UBOCC-A-116002 and *P. commune* UBOCC-A-116003 in semi-hard cheese and sour cream.

Product	Fermentate (fermentation substrate)	Time before visible fungal growth (in days)		
		Concentration	*M. racemosus*	*P. commune*
Semi-hard cheese	None (negative control)	–	5	7
	P. jensenii CIRM-BIA1774 (UF) and *M. lanceolatus* UBOCC-A-109193 (LH)	0.63 g/dm^2 and 5 ml/dm^2	20	14
	P. jensenii CIRM-BIA1774 (UF)	0.63 g/dm^2	26	21
	L. rhamnosus CIRM-BIA1952 (LH) and *M. lanceolatus* UBOCC-A-109193 (LH)	1.26 g/dm^2 and 5 ml/dm^2	8	9
	Natamycin (positive control)	1 mg/dm^2	> 33	> 33
Sour cream	None (negative control)		7	8
	L. rhamnosus CIRM-BIA1952 (LH)	5%	7	8
	L. rhamnosus CIRM-BIA1952 (LH)	2%	7	8
	P. jensenii CIRM-BIA1774 (UF)	5%	30	30
	P. jensenii CIRM-BIA1774 (UF)	2%	30	19
	P. jensenii CIRM-BIA1774 (UF)	0.4%	10	12
	P. jensenii CIRM-BIA1774 (UF)	0.1%	8	11
	L. rhamnosus CIRM-BIA1952 (LH) and *P. jensenii* CIRM-BIA1774 (UF)	2.5% each	> 30	> 30
	Potassium sorbate (positive control)	0.08%	> 30	> 30

well as 48 h at 30 °C followed by 24 h at either 12 °C or 43 °C for LAB; 48, 72 and 96 h at 30 °C as well as 96 h at 30 °C followed by 24 h at either 12 °C or 43 °C for propionibacteria and 48, 96 and 168 h at 25 °C as well as 96 h at 25 °C followed by 48 h at either 12 °C or 43 °C for filamentous fungi. Cell concentration at the end of incubation was determined on MRS and YEL agar for LAB and propionibacteria, respectively. Antifungal activities of the resulting fermentates were tested as described above against the four selected target fungal spoilers.

2.4. In situ antifungal activity

2.4.1. Antifungal activity by fermentate surface-spraying on semi-hard cheese

Fermentates with promising antifungal activities were tested *in situ* by surface-spraying on semi-hard cheeses (Table 2). Two replicate cheese trials were carried out at a pilot scale on two separate weeks at the Dairy Platform of INRA (Rennes, France). Cow's milk was pasteurized at 72 °C for 20 s, standardized to 30 g fat/kg milk and pumped into a cylindrical jacketed, stainless steel cheese vat, with variable speed cutting and stirring (Frominox, Assat, France). Milk was supplemented with 36 mg Ca per kg (20 ml per 100 kg milk of a 500 g/L $CaCl_2$ solution). A commercial starter culture consisting of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (MA016, Elimeca, Thoissey, France) was resuspended in UHT semi-skimmed milk, left to rehydrate for 1 h at 20 °C and added to the cheese milk at 0.5 U/100 kg, i.e. ~10^6 CFU/mL. After 2 h at 12 °C followed by 30 min pre-ripening at 33 °C (pH 6.57), 0.25 mL/kg of animal rennet (520 mg/L chymosin, Carlina 145/80, Dupont Danisco, Dangé, Saint Romain, France), diluted in deionized water, was added. After coagulation for 29 min, curd was cut at the size of corn grains, washed with hot water, cooked for 20 min at 35 °C and drained (pH 6.54). Curd was pre-pressed at 120 kPa for 30 min, moulded (700 g curd per mold) and pressed at 300 kPa for 30 min then 600 kPa for 90 min. The cheeses remained overnight in the mold, the temperature decreased from ~25 °C to ~20 °C and the pH reached 5.07 at demoulding. Cheese composition at this step was 50.8% dry matter and 49% fat in dry matter. Cheeses were salted by immersion in a sterile brine containing 33% (w/w) NaCl, 36 mg Ca per kg, pH 5 at 20 °C for 2 h. Cheeses were left to dry for one night at room temperature (20–25 °C). Then, in a laminar air flow cabinet, the external surface (3 mm in thickness) was removed to limit surface contamination. Cheeses were then smeared once with a mixture of the following commercial cultures: *Debaryomyces hansenii* CHOOZIT DH, *Kluyveromyces lactis* CHOOZIT KL71, and *Brevibacterium linens* CHOOZIT SR3 (Danisco, Dangé Saint-Romain, France). After 2 h drying at room temperature in a laminar air flow cabinet, 3 ml of fermentates (bacterial lyophilized fermentates being resuspended either in sterile distilled water or in filter-sterilized *M. lanceolatus* fermentate), alone or in combination, were sprayed on the cheese surface. On day 2, after a 12 h drying period at room temperature following the first spraying, cheese surface was inoculated with 50 spores of either *P. commune* UBOCC-A-116003 or *M. racemosus* UBOCC-A 116002. Fermentate spraying was then repeated on day 3 and 5. Cheeses were ripened for 6 weeks in ripening chambers at 12 °C and 96% relative humidity (one ripening chamber per fungal target). The ripened semi-hard cheeses had a final dry matter of 60% and pH 5.5. Fungal growth was visually evaluated every day during the 6 ripening weeks. A negative control (cheese without surface fermentate spraying) and a positive control (cheese surface treated with 1 mg/dm^2 natamycin on day 2) were also included.

2.4.2. Antifungal activity by fermentate incorporation in sour cream

Various fermentates, alone or in combination, were also tested by incorporation in sour cream (Table 2). To do so, cream (final fat 33%, dry matter 41%) was produced at a pilot scale from pasteurized milk (16 s at 75 °C) and fermented at 23 °C for 22 h using 2.5 U/100 kg (10^6 CFU/L) of commercial starter cultures MM100 (Elimeca, Thoissey, France). Sour cream (pH 4.5) was then packaged in individual 100 g containers. Various fermentate concentrations ranging from 0.1 to 5% were tested according to the selected fermentate (Table 2). The fermentates were added directly in each container, gently mixed and stored at 10 °C overnight before fungal target inoculation. A negative control (sour cream without fermentate) and a positive control (sour cream containing 0.08% potassium sorbate) were also included. For challenge tests with filamentous fungi, 50 fungal spores of *P. commune* UBOCC-A-116003 or *M. racemosus* UBOCC-A 116002 were inoculated on the sour cream surface followed by incubation at 10 °C for 4 weeks. Fungal growth was visually evaluated every day. For challenge test with *Rhodotorula mucilaginosa* UBOCC-A-216004, 2 CFU/g were inoculated in the sour cream, followed by gentle mixing and incubation at 10 °C. Yeast growth was evaluated by surface-plating after 1, 2 and 3 weeks on YGC agar incubated at 25 °C for 2 days.

2.4.3. Durability tests

Durability tests were performed to evaluate fermentate antifungal activity in naturally contaminated environments. For semi-hard cheese, growth of spoilage fungi was evaluated on the surface of 3 different cheeses sprayed or not with selected fermentates and after ripening for 6 weeks at 12 °C in a ripening chamber. For sour cream, 100 g of sour cream with or without fermentate, and a control containing 0.08% potassium sorbate were spread out in 10 Petri dishes, exposed for 20 min to indoor air of the lunch room at INRA STLO and then sealed using parafilm. Fungal spoilage at the surface was visually evaluated after 2-weeks incubation at 10 °C.

2.4.4. Sensory analyses

To evaluate the potential impact of antifungal fermentates on the sensorial properties of tested products, a sorting task was performed by a panel of 39 and 29 untrained judges for cheese and sour cream, respectively. Sour cream, packed in 100 g containers, was kept at 4 °C for 2 weeks before sensory analysis and semi-hard cheese were tested after a 6 week ripening period. Products were left for 30 min at room temperature before being served and a random 3-digit code was assigned to each sample. Panelists were asked to group together the samples perceived as the most similar, taking into account the characteristics they considered as important to differentiate the products (free sorting task). Once groups were made, panelists were asked to associate specific descriptors to each group (verbalization task). To ensure the panelist ability to group similar samples, a random sample was tested in duplicate. Sorting task data were analyzed by the Factorial Approach for Sorting Task data (FAST) using multiple correspondence analysis (MCA) via the FactoMineR and SensoMineR packages implemented in the R environment, according to Cadoret et al. (2009). In particular, confidence ellipses were drawn to also provide validation elements.

To define the concentration of the selected fermentate at which no sensory impact could be perceived in sour cream, an acceptability test was performed with a panel of 26 untrained judges. Eight fermentate concentrations ranging from 0 to 0.7% were tested. As for sensory tests, a random 3-digit code was assigned to each sample and products were left for 30 min at room temperature before being served. Panelists were asked to indicate whether each sour cream sample was acceptable or not for consumption.

3. Results

3.1. Antifungal activity of fermentates after in vitro screening

In the first part of this work, the antifungal activity of fermentates obtained by individual culture of 698 LAB, propionibacteria or fungi in both LH and UF substrates (thus for a total of 1396 fermentates) was evaluated *in vitro* against 4 fungal targets in a cheese-mimicking model. The number of fermentates exhibiting antifungal activity largely varied according to the substrate used for fermentation, the tested taxon, and the fungal target (Supplementary table S2).

LH fermentates were overall notably more inhibitory than UF fermentates with LAB being the most represented microbial group among active strains. Regarding LH fermentates, 81 (18.9%), 13 (3%), and 1 (0.2%) LAB fermentates out of the 430 tested ones, were active against *P. commune, M. racemosus*, and *Y. lipolytica,* respectively, while no propionibacteria out of the 70 tested showed any activity. For fungal fermentates, 2 (1%) and 6 (3%) out of the 198 tested ones were active against *M. racemosus* and *G. geotrichum,* respectively. In contrast, for UF fermentates, only fermentates from 8 LAB (1.8% of tested LAB), 1 propionibacteria (1.4% of tested propionibacteria) and 4 fungi (2% of tested fungi) showed an antifungal activity, and this against only one target.

Among the 27 tested bacterial species, *Lactobacillus buchneri* (n = 34), *Lactobacillus plantarum* (n = 115) and *Lactobacillus brevis* (n = 8) represented the most active species with 44.1, 40.8 and 37.5% of active LH fermentates against at least one target. Noteworthy, 9 LAB LH fermentates showed an antifungal activity against both *M. racemosus* and *P. commune*, while a *L. buchneri* L162 fermentate inhibited the latter 2 fungi and *Y. lipolytica* as well. In contrast, in the tested conditions, only a few strains of *Lactobacillus reuteri* (n = 42) and *Propionibacterium jensenii* (n = 20) showed antifungal activity, with 1 (2.4%) and 1 (5%) active strains for LH and UF fermentates, respectively. In addition, *Leuconostoc mesenteroides* (n = 71), *Propionibacterium acidipropionici* (n = 17) and *Propionibacterium thoenii* (n = 14) strains did not show any antifungal activity whatever the substrate used.

As mentioned above, only a few fungal fermentates showed antifungal activities and there was no particular over-represented species whatever the substrate used. Indeed, for LH fermentates, one isolate of *Aureobasidium pullulans* and *M. lanceolatus,* and *Exophiala* sp., *Rhodotorula minuta*, *Rhodotorula mucilaginosa*, *Torulaspora delbrueckii* and *Trichoderma viride*, led to fermentates active against *M. racemosus* and *G. geotrichum,* respectively. For UF fermentates, one isolate of *Actinomucor elegans*, *Verticillium dahliae* and *Candida tenuis*, and *Trichoderma harzianum* produced fermentates active against *P. commune* and *Y. lipolytica,* respectively.

Concerning the susceptibility of the fungal targets, *P. commune*, followed by *M. racemosus* were the most frequently inhibited fungi, while *G. geotrichum* and *Y. lipolytica* were only inhibited by a very small number of LAB or propionibacteria fermentates. Interestingly, for fungal fermentates, *M. racemosus* and *G. geotrichum* were the most sensitive targets with LH as a growth substrate whereas it was *P. commune* and *Y. lipolytica* with UF substrate.

After *in vitro* screening, 11 strains exhibiting the most promising antifungal activity, namely 9 *Lactobacillus* strains (*L. buchneri* L151, L162, L164 and L233, *L. harbinensis* L172, *L. paracasei* L117 and L194, *L. plantarum* L244 and *L. rhamnosus* CIRM-BIA1952), *P. jensenii* CIRM-BIA1774 and *M. lanceolatus* UBOCC-A-109193 were selected for further investigation.

3.2. Optimization of culture conditions for antifungal activity enhancement

The 11 strains previously selected were inoculated and cultivated in LH and/or UF substrate with 5 different time/temperature combinations, and the antifungal activity of the obtained fermentates were tested against the 4 selected fungal targets in the cheese model. Increases in antifungal activity against at least one fungal target were observed for all strains when culture conditions were modified (Table 1). Activity improvement was more pronounced against *P. commune*, followed by *R. mucilaginosa* and *M. racemosus,* whereas there was little improvement against *Y. lipolytica*. The increase in time before visible growth, which varied between 1 and 4 days as compared to the control depending on the tested strain, was generally higher in conditions with prolonged incubation time with the exception of *L. paracasei* L117 fermentate for which incubation for 20 h at 30 °C yielded the best antifungal activity. Increases in antifungal activity were also generally related to the final cell concentration at the end of the incubation even though this was not systematically observed. Finally, a final incubation step at suboptimal temperatures did not improve the antifungal activity except for *L buchneri* L233, *M. lanceolatus* UBOCC-A-109193 and *P. jensenii* CIRM-BIA1774 fermentates for which a significant increase in antifungal activity against *P. commune* and *M. racemosus,* and *M. racemosus* and *R. mucilaginosa*, respectively, was observed (Table 1).

After this step, 3 fermentates were selected for *in situ* applications at a pilot scale, namely those of *L. rhamnosus* CIRM-BIA1952 (cultivated in LH for 48 h at 30 °C) which was able to delay *P. commune* and *R. mucilaginosa* growth, *P. jensenii* CIRM-BIA1774 (cultivated in UF for 96 h at 30 °C followed by 24 h at 12 °C), which was able to delay the growth of the 4 tested fungal targets, and *M. lanceolatus* UBOCC-A-109193 (cultivated in LH for 96 h at 30 °C followed by 48 h at 12 °C) which was able to delay *M. racemosus* and *P. commune* growth.

3.3. Antifungal activity and organoleptic impact of fermentates after surface-spraying on semi-hard cheeses

The 3 fermentates selected after the optimization step were used, alone or in combination (either *L. rhamnosus* or *P. jensenii* fermentate associated with *M. lanceolatus* fermentate), for surface treatment of semi-hard cheeses manufactured at a pilot-scale. The observed times to visible growth of *M. racemosus* and *P. commune* after the challenge test are presented in Table 2. Surface-spraying of each of the tested fermentates led to an increase in time to visible growth of both fungal targets as compared to the negative control (untreated cheese), but their efficiency varied. For example, the *L. rhamnosus/M. lanceolatus*

Table 1
Effect of culture conditions on the antifungal activity of fermentates from selected lactic acid bacteria, propionibacteria and fungi against *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003, *R. mucilaginosa* UBOCC-A-216004 and *Y. lipolytica* UBOCC-A-216006. The antifungal activity is expressed as increase in time to visible growth of the fungal target (days) as compared to a control without fermentate.

Fermentation substrate	Increase in time to visible fungal growth (in days)						
	Strain	Culture conditions[a]	Cell concentration (log $_{10}$ CFU/ml)	*M. racemosus*	*P. commune*	*R. mucilaginosa*	*Y. lipolytica*
Low heat milk	*L. buchneri* L151	1-a	5.10^7	0	0	0	0
		2-a	6.10^7	0	0	+1	0
		3-a	2.10^8	0	+1	+2	0
		4-a	6.10^7	0	0	+1	0
		5-a	7.10^7	0	0	+2	0
	L. buchneri L162	1-a	1.10^7	0	0	+1	0
		2-a	4.10^8	0	+1	+1	0
		3-a	1.10^8	0	+3	+1	0
		4-a	8.10^8	0	+3	+1	0
		5-a	1.10^9	0	+4	+2	0
	L. buchneri L164	1-a	6.10^8	0	+1	+1	0
		2-a	3.10^8	0	+1	+1	0
		3-a	2.10^8	0	0	+1	0
		4-a	4.10^8	0	+1	+1	0
		5-a	2.10^8	0	0	+1	0
	L. buchneri L233	1-a	6.10^7	0	+1	0	0
		2-a	7.10^6	0	+1	0	0
		3-a	5.10^8	+1	+3	+1	0
		4-a	2.10^8	0	+2	+1	0
		5-a	4.10^8	+1	+4	+2	0
	L. harbinensis L172	1-a	2.10^8	+1	0	+1	0
		2-a	7.10^7	0	0	0	0
		3-a	8.10^7	0	0	+2	0
		4-a	2.10^8	0	0	+1	0
		5-a	1.10^8	+1	0	+1	0
	L. paracasei L117	1-a	2.10^9	+1	+4	+2	0
		2-a	2.10^9	0	+1	+2	0
		3-a	1.10^8	0	+1	+1	0
		4-a	1.10^9	0	+1	+2	0
		5-a	2.10^9	0	0	+1	0
	L. paracasei L194	1-a	3.10^9	0	+2	+2	0
		2-a	2.10^9	0	+1	+1	0
		3-a	3.10^8	0	+1	+1	0
		4-a	2.10^9	0	+1	+1	0
		5-a	2.10^9	0	+1	+1	0
	L. plantarum L244	1-a	7.10^8	0	+1	+1	0
		2-a	5.10^8	0	+1	+1	0
		3-a	2.10^8	0	0	+1	0
		4-a	6.10^8	0	+1	+1	0
		5-a	1.10^8	0	+1	+1	0
	L. rhamnosus CIRM-BIA1952	1-a	2.10^9	0	+2	+2	0
		2-a	1.10^9	0	+3	+2	0
		3-a	1.10^9	0	+3	+1	0
		4-a	1.10^9	0	+1	+1	0
		5-a	2.10^9	0	+1	+2	0
	M. lanceolatus UBOCC-A-109193	1-b	nd†	+1	0	nd	0
		2-b	nd	+2	+1	nd	0
		3-b	nd	+1	+1	nd	0
		4-b	nd	+4	+1	nd	0
		5-b	nd	0	+1	nd	0
UF milk permeate	*L. buchneri* L162	1-a	1.10^7	0	0	+1	0
		2-a	4.10^8	0	0	+1	0
		3-a	1.10^8	0	0	0	0
		4-a	8.10^8	0	0	0	0
		5-a	1.10^9	0	0	0	0
	P. jensenii CIRM-BIA1774	1-c	1.10^7	+1	+1	+1	0
		2-c	2.10^8	+2	+1	+1	0
		3-c	6.10^8	+2	+1	+2	0
		4-c	8.10^8	+2	+1	+2	+1
		5-c	8.10^8	+2	+1	+1	0

[a] Culture conditions for lactobacilli: 1-a, 24 h/30 °C; 2-a, 48 h/30 °C; 3-a, 72 h/30 °C; 4-a, 48 h/30 °C followed by 24 h/12 °C; 5-a, 48 h/30 °C followed by 24 h/43 °C. Culture conditions for *M. lanceolatus* UBOCC-A-109193: 1-b, 48 h/25 °C; 2-b, 96 h/25 °C; 3-b, 168 h/25 °C; 4-b, 96 h/25 °C followed by 48 h/12 °C; 5-b, 96 h/25 °C followed by 48 h/43 °C. Culture conditions for *P. jensenii* CIRM-BIA1774: 1-c, 48 h/30 °C; 2-c, 72 h/30 °C; 3-c, 96 h/30 °C; 4-c, 96 h/30 °C followed by 24 h/12 °C; 5-c, 96 h/30 °C followed by 24 h/43 °C.†nd, not determined.

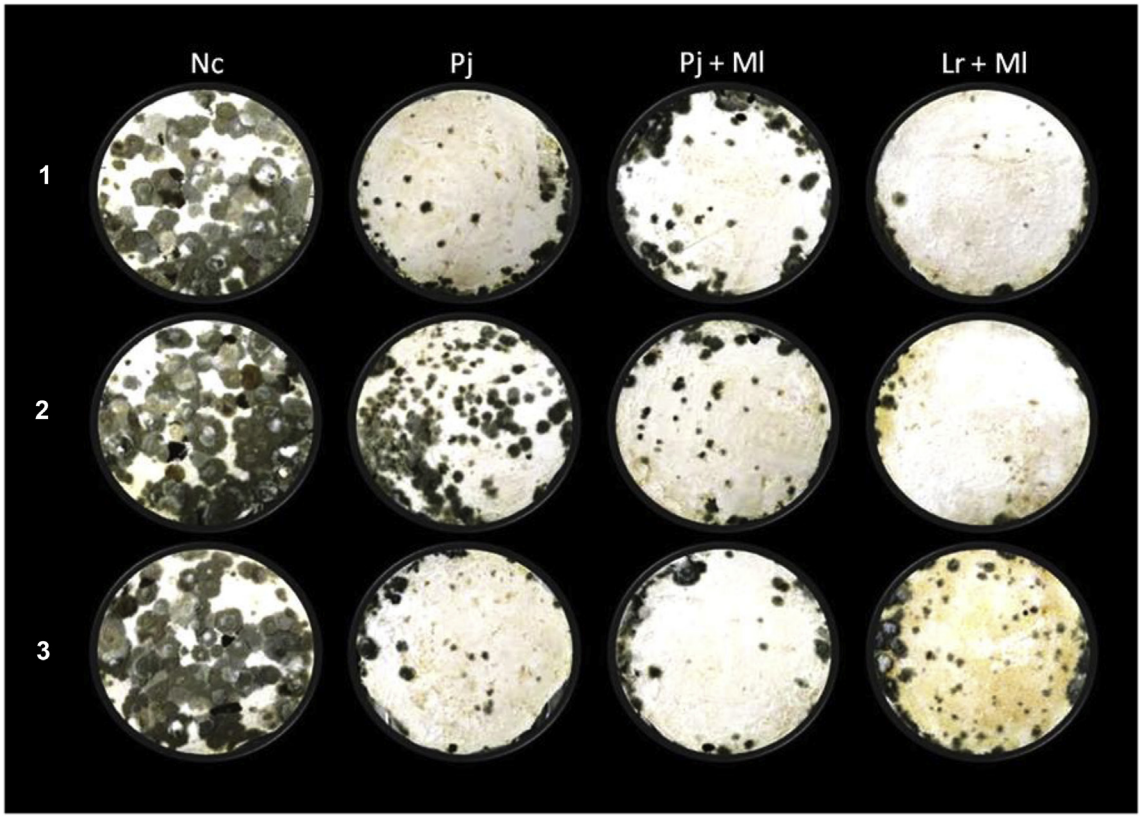

Fig. 1. Photographs showing semi-hard cheese surfaces treated or not (negative control, Nc) by surface-spraying using *P. jensenii* CIRM-BIA1774 (Pj), *P. jensenii* CIRM-BIA1774 associated with *M. lanceolatus* UBOCC-A-109193 (Pj + Ml) or *L. rhamnosus* CIRM-BIA1952 associated with *M. lanceolatus* UBOCC-A-109193 (Lr + Ml), after 6 weeks of ripening at 10 °C in a naturally contaminated environment. Numbers 1 to 3 are replicates.

and *P. jensenii*/*M. lanceolatus* combinations increased the time to visible growth of *M. racemosus* by 3 and 15 days, respectively. The same combinations increased the time to visible growth of *P. commune* by 2 and 7 days, respectively. The most efficient fermentate was that of *P. jensenii* CIRM-BIA1774, which inhibited *M. racemosus* and *P. commune* for up to 21 and 14 days, respectively. No visible growth of the fungal targets was observed after natamycin application during the time of the experiment (33 days). Interestingly, we did not observe any effect of the fermentates on the smear development, while that of the cheese treated with natamycin was considerably reduced (data not shown).

The strong antifungal activity of the fermentates after surface-treatment of semi-hard cheeses was further confirmed by the durability test results. As shown in Fig. 1, control cheeses (untreated) were almost completely covered with mold after 6 week incubation at 10 °C in a naturally contaminated ripening chamber, whereas the fermentate treated cheeses appeared with a much smaller surface covered by mold, with the exception of 1 out of 3 replicate treated with *P. jensenii* fermentate.

The results of the multiple correspondence analysis made from cheese sensory data are presented in Fig. 2. The total variance explained by the first two dimensions was 62%, with Dim 1 and Dim 2 representing 34 and 28% of variance, respectively. The panelists could differentiate all cheeses except the cheese treated with *P. jensenii* fermentate which could not be differentiated from other samples including the control cheese.

3.4. Antifungal activity and organoleptic impact of fermentates after incorporation in sour cream

Tested concentrations were initially 2 and 5% (w/w) of each fermentate and a mixture of both fermentates at 2.5% (w/w) each. Once again, the *P. jensenii* CIRM-BIA1774 fermentate, alone or in combination with *L. rhamnosus* CIRM-BIA1952 fermentate, showed a high antifungal activity (similar to that of potassium sorbate) against *M. racemosus* and *P. commune*. It delayed their growth up to 30 days (total duration of the experiment) as compared to the negative control (Table 2). All fermentates containing *P. jensenii* totally inhibited *R. mucilaginosa* growth (counts < 1 CFU/g) during storage, while that of *L. rhamnosus* did not show any antifungal activity (Fig. 3).

Durability tests confirmed the trends observed with the challenge tests as none of the samples containing *P. jensenii* CIRM-BIA1774 fermentate showed any visible fungal contamination (Fig. 4). In contrast, samples containing *L. rhamnosus* CIRM-BIA1952 fermentates at 2 and 5% were only slightly less contaminated than control samples with 90 and 80% of samples showing visible fungal contamination, respectively.

In the multiple correspondence analysis, the total variance explained by the two first dimensions was 61%, with Dim 1 and Dim 2 representing 37 and 24% of variance, respectively (Fig. 5). The overlapping of the confidence ellipses of the control sample and sour cream with 2% *L. rhamnosus* fermentate showed that these samples were perceived as similar by the panelists. Samples containing *P. jensenii* fermentates were grouped together and clearly separated from the control on Dim 1, while sour creams containing 5% *L. rhamnosus* CIRM-BIA1952 fermentate were separated from the control on Dim 2, indicating that panelists could perceive significant sensory differences between these samples. Sour creams with *P. jensenii* CIRM-BIA1774 fermentate were described as very unpleasant, strong, and acid, showing a high negative impact of this fermentate on the product flavor while sour creams with 5% *L. rhamnosus* CIRM-BIA1952 fermentate were described as dry, pasty or burnt showing an impact on texture and flavour.

Based on these results, using an acceptability test, we investigated the concentration of *P. jensenii* fermentate at which no organoleptic impact could be perceived in sour cream (Supplementary Figure 1).

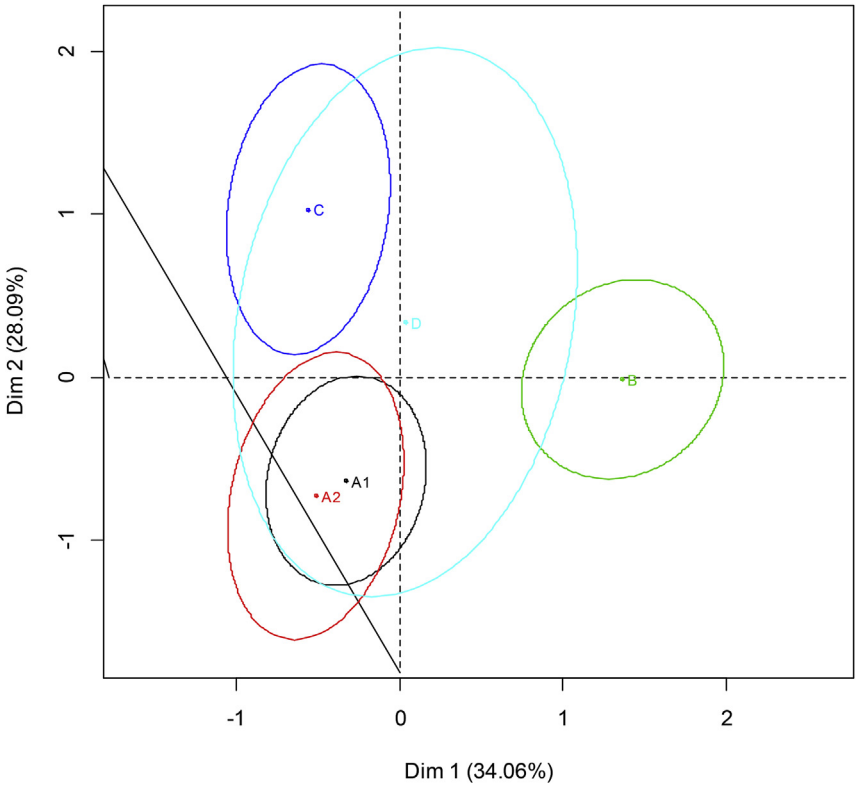

Fig. 2. Impact of cheese surface treatment with antifungal fermentates on sensorial properties of semi-hard cheese determined using a sorting approach: representation of the cheeses and their respective 95% confidence ellipses in the plane defined by Dim 1 and Dim 2 of a multiple correspondence analysis. A1 and A2, control cheeses (without fermentate); B, cheese surface-treated with a combination of 4.8 mg/dm^2 L. *rhamnosus* CIRM-BIA1952 and 10 ml/dm^2 M. *lanceolatus* UBOCC-A-109193 fermentates; C, cheese surface-treated with a combination of 2.4 mg/dm^2 *P. jensenii* CIRM-BIA1774 and 10 ml/dm^2 M. *lanceolatus* UBOCC-A-109193 fermentates and D, cheese surface-treated with 2.4 mg/dm^2 *P. jensenii* CIRM-BIA1774 fermentate.

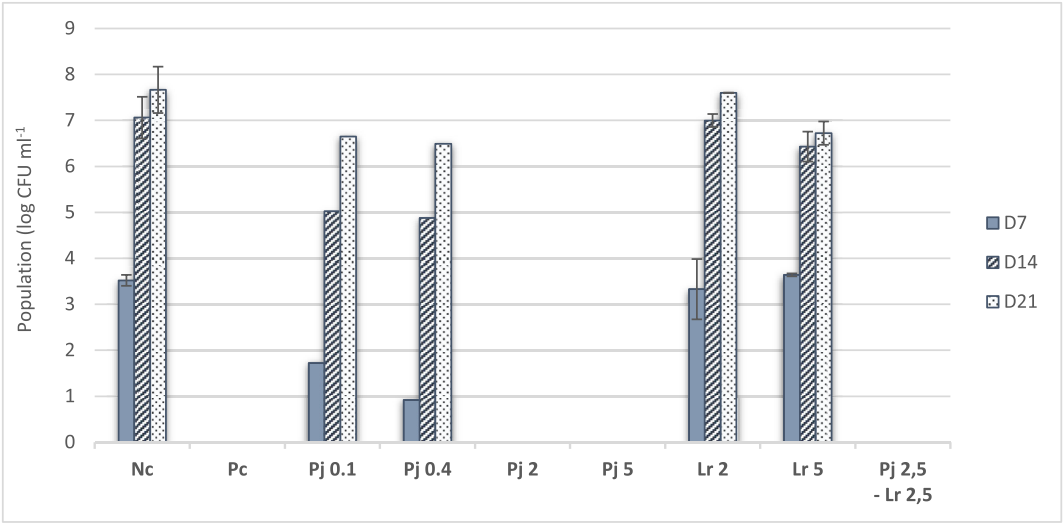

Fig. 3. Growth of *R. mucilaginosa* UBOCC-A-216004 in sour cream after incorporation or not of *P. jensenii* CIRM-BIA1774 and/or *L. rhamnosus* CIRM-BIA1952 fermentates. Nc, negative control (sour cream without fermentate); Pc, positive control (sour cream with 0.08% potassium sorbate); Pj-2, *P. jensenii* fermentate at 2% w/w; Pj-5, *P. jensenii* fermentate at 5% w/w, Lr-2, *L. rhamnosus* fermen-tate at 2% w/w; L-5, *L. rhamnosus* fermentate at 5% w/w; Pj-2.5 + Lr-2.5, mixture of *P. jensenii* and *L. rhamnosus* fermentates at 2.5% w/w each.

Acceptability decreased with increasing fermentate concentrations. Among the 26 panelists, 21 (80.8%) accepted the control sour cream while only 6 (23.1%) accepted the product with 0.7% *P. jensenii* fermentate. The 21 panelists who did not reject the sour cream also accepted the sour cream containing 0.1% *P. jensenii* fermentate, while 10 panelists (47.6%) accepted that containing 0.4% *P. jensenii* fermentate. These 2 concentrations were selected for further challenge and durability tests.

For challenge tests in sour creams containing 0.1 and 0.4% *P. jensenii* fermentate, fungal growth delays were much shorter than those observed at 2 and 5%. They corresponded to 1 and 3 days, and 3 and 4 days for *M. racemosus* and *P. commune,* respectively (Table 2). On the contrary, no antifungal activity was observed against *R. mucilaginosa* (Fig. 3). Concerning durability tests, all control sour creams were contaminated, as well as sour creams with 0.1% fermentate (Fig. 6). Interestingly, none of the samples with 0.4% fermentate showed any visible fungal contamination (Fig. 6).

4. Discussion

In order to develop dairy antifungal biopreservation ingredients, fermentation of LH and UF was performed by large array of

Fig. 4. Photographs showing sour cream surfaces in which fermentates of *P. jensenii* CIRM-BIA1774 (2% and 5%), *L. rhamnosus* CIRM-BIA1952 (2 % and 5%) and mixture of *P. jensenii* and *L. rhamnosus* at 2.5% each were incorporated. Negative controls corresponded to sour cream samples without fermentate. Samples were stored for 2 weeks at 4 °C, naturally contaminated in an indoor environment (20 min) and stored again for 2 weeks at 10 °C. Nc, Negative control; Pj-2, *P. jensenii* fermentate at 2% w/w; Pj-5, *P. jensenii* fermentate at 5% w/w, Lr-2, *L. rhamnosus* fermentate at 2% w/w; Lr-5, *L. rhamnosus* fermentate at 5% w/w; Pj-2.5 + Lr-2.5, mixture of *P. jensenii* and *L. rhamnosus* fermentates at 2.5% w/w each. Numbers 1 to 10 are replicates. Score at the bottom of the figure correspond to the number of contaminated cream/total number of cream.

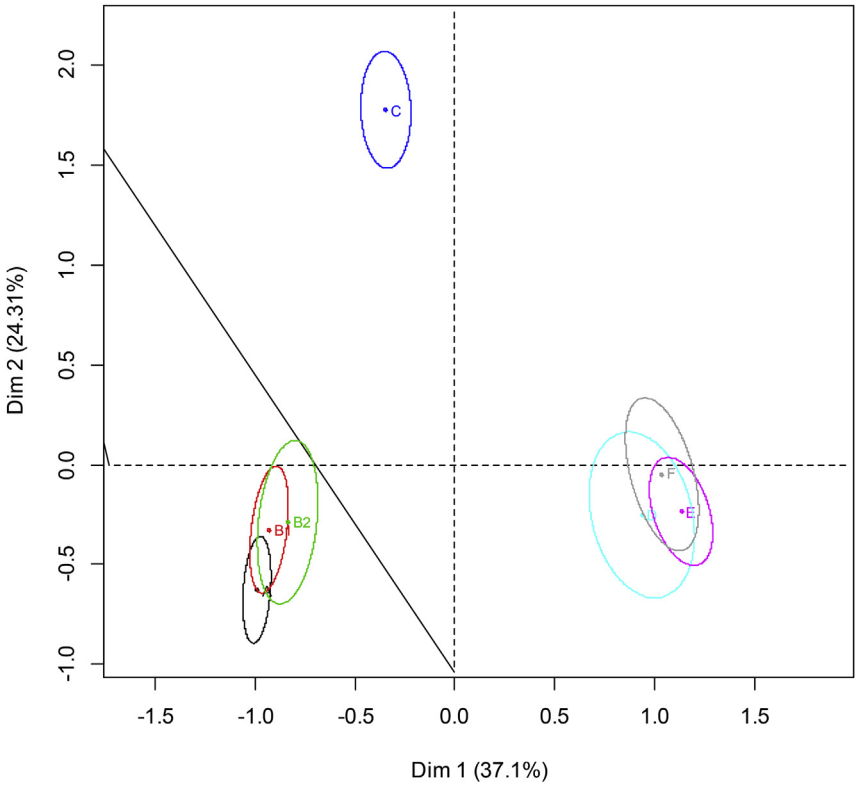

Fig. 5. Impact of the incorporation of antifungal fermentates on sensorial traits of sour cream determined using a sorting approach: representation of the sour creams and their respective 95% confidence ellipses in the plane defined by Dim 1 and Dim 2 of a multiple correspondence analysis. A, control sour cream (without fermentate); B1 and B2, sour cream with 2% *L. rhamnosus* CIRM-BIA1952 fermentate, C, sour cream with 5% *L. rhamnosus* CIRM-BIA1952 fermentate; D, sour cream with 2% *P. jensenii* CIRM-BIA1774 fermentate, E, sour cream with 5% of *P. jensenii* CIRM-BIA1774 fermentate; F, sour cream with 2.5% *L. rhamnosus* CIRM-BIA1952 and 2.5% of *P. jensenii* CIRM-BIA1774 fermentates.

microorganisms. LH and FH were chosen for their availability since they are current products of the dairy industry, food-grade status and compatibility with dairy food applications. The *in vitro* screening step of corresponding fermentates revealed that several fermentates exhibited a significant antifungal activity. Interestingly, the proportion of active fermentates and their antifungal activity varied according to the dairy substrate. Better expression of antifungal activity was observed for LH substrate (qualitatively and/or quantitatively) as compared to UF substrate. The antifungal activity was not correlated to the ability of the selected microorganisms to grow in each of these sustrates. Indeed, an assessment of the final biomass on the tested 27 bacterial and 18 fungal strains showed that similar populations were generally reached in both substrates after fermentation (10^7–10^9 CFU/ml for bacteria and 10^7 CFU/ml for yeasts), except for filamentous fungi which generally showed ~5 times more final biomass in LH than in UF (data not shown). Therefore, for most bacteria and yeasts, it is likely that the differential expression in the production of antifungal activity was related to the substrate composition, while, for filamentous fungi, both biomass level and/or substrate composition could be involved. The individual components which are responsible remain unclear. It can be underlined however that LH but not UF substrate contains milk fat and is richer in proteins, both qualitatively (presence of caseins) and quantitatively (374 g/kg total nitrogen and 190 g/kg nonprotein nitrogen in LH versus 5.7 g/kg total nitrogen and 5.5 g/kg non-protein nitrogen in UF). Fat and proteins are precursors of antifungal compounds such as fatty acids, peptides, organic acids, and volatile compounds (Hidalgo et al., 2015; Théolier et al., 2013). Similar observations were made in a recent study showing that production of lactic, acetic, succinic, propionic, formic, and butyric acids by LAB significantly varied according to the growth medium used (Özcelik et al., 2016). Concerning the proportion of antifungal strains observed in the present study, 17% of LH and 1% UF fermentates showed a significant antifungal activity against at least one fungal target. These proportions are much lower than those found in previous studies. Inglin et al. (2015) found that ~30% of 504 tested lactobacilli isolates, cultivated in MRS agar, inhibited at least one of the 4 tested fungal targets. More recently, Le Lay et al. (2016) reported that ~32% of the 270 tested LAB and 50 tested propionibacteria inhibited 5 fungal targets after growth in wheat flour hydrolysate agar. The lower number of active strains reported in the present study can be explained by the fact that

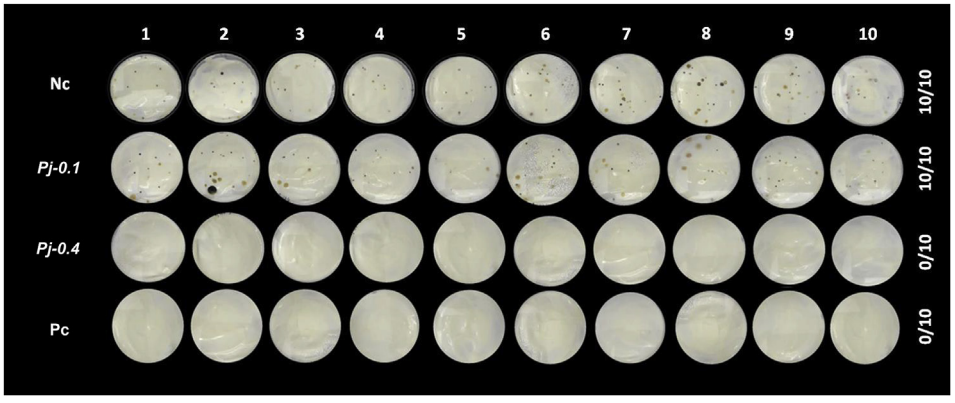

Fig. 6. Photographs showing sour cream surfaces in which *P. jensenii* CIRM-BIA1774 fermentates were incorporated at 0.1 (Pj-0.1) and 0.4 (Pj-0.4) %. Negative control (Nc) correspond to sour cream samples without fermentate and positive control (Pc) to sour cream samples containing 0.08% potassium sorbate. Samples were stored for 2 weeks at 4 °C, naturally contaminated in an indoor environment and stored again for 2 weeks at 10 °C. Numbers 1 to 10 are replicates. Scores correspond to number of contaminated sour cream/total number of sour cream.

fermentates instead of bacterial colonies (Inglin et al., 2015) or bacteria embedded in agar medium (Le Lay et al., 2016) were used, probably resulting in a more stringent selection of active isolates. In addition, the substrates used for screening were also different, for example, Inglin et al. (2015) utilized MRS medium containing acetate, which is known to potentialize antifungal activity (Stiles, 1996) while in Le Lay et al. (2016) work, pH of wheat flour hydrolysate medium after LAB growth was ~3.5 (1.5 unit lower to miniaturized cheeses's pH), which enhances the antifungal activity of organic acids. In this study the testing matrix (cheese-mimicking model) was more closely related to an actual food product.

Our results confirmed the strain-dependent character of antifungal activities in terms of percent of active strains as well as inhibited targets. Indeed, within each screened species, strains significantly differed in their antifungal activity as reported elsewhere (Le Lay et al., 2016; Cortés-Zavaletta et al., 2014; Thierry et al., 2015; Russo et al., 2017; Leyva-Salas et al., 2017). For example, for *L. buchneri* (n = 34), one of the most antifungal species, around 40% of strains were active, while in *L. reuteri* (n = 42), only 2% of the strains were active.

Antifungal fermentate-producing isolates mostly belonged to *Lactobacillus* species such as *L. brevis* (Axel et al., 2016), *L. buchneri* (Kharazian et al., 2017), *L. plantarum* (Cheong et al., 2014), *L. harbinensis* (Belguesmia et al., 2014), and *L. rhamnosus* (Fernandez et al., 2017). This can be explained by the fact that *Lactobacillus* spp. produce a large range of antifungal molecules such as organic acids (Brosnan et al., 2012), hydrogen peroxide, ethanol, cyclic dipeptides, proteinaceous compounds and fatty acids (Luz et al., 2017; Crowley et al., 2013; Lavermicocca et al., 2000). The additive or even synergistic action of these molecules, despite being produced at weak concentrations, could increase the antifungal activity. Interestingly, one *Propionibacterium* and 7 fungal strains also produced antifungal fermentates. Propionibacteria produce propionic acid, as well as hydrogen peroxide, azelaic, phenyllactic and hydroxyphenyllactic acids (Schwenninger et al., 2004; Lind et al., 2007; Fernandez et al., 2017; Le Lay et al., 2016) and fungi are known to produce antifungal compounds acting in synergy such as volatile compounds, killer proteins, antifungal peptides and lytic enzymes (Leyva-Salas et al., 2017; Nùñez et al., 2015; Izgu et al., 2011; Coelho et al., 2009; Weiler and Schmitt, 2003; Wheatley, 2002; Schmitt et al., 1996) (see also the review by Delgado et al., 2016). To our best knowledge, this is the first time that *A. pullulans*, *C. tenuis* and *M. lanceolatus* are reported for their antifungal activities. In contrast, *Trichoderma* spp. such as *T. harzianum* and *T. viride* are commonly used as biocontrol agents and are known to produce both non-volatile and volatile compounds like pyrones, butenolides, azaphylones, anthraquinones, trichothecenes, terpenoids and steroids, as well as non-ribosomal peptides, such as siderophores and peptaibols (Mukherjee et al., 2012). In future studies, it would be interesting to understand which antifungal molecules are involved in the the most active fermentates and to investigate their mode of action against different fungal targets.

In order to enhance antifungal activities an optimization step of culture conditions was included. In general, the longer the incubation time and the higher the cell concentration was, the more the antifungal activity was significant. In addition to incubation time, cold or hot thermal shock increased the antifungal activity of several strains. The reasons for this are unclear but suboptimal temperatures could trigger or increase the production of antifungal compounds as seen for example for bacteriocin production in LAB (Leal-Sánchez et al., 2002).

When selecting strains for food applications, microbial safety and regulatory considerations such as Generally Recognized as Safe (GRAS) or Qualified Presumption of Safety (QPS) must be considered (Bourdichon et al., 2012). Thus, the safety of the LAB and propionibacteria strains selected for *in situ* tests were verified, i.e. biogenic amines production and antibiotic resistance pattern. *L. brevis* L128 and *L. buchneri* L162 and L233, although showing interesting antifungal properties were not kept further for *in situ* step applications since they were shown to possess biogenic amines associated genes (*hdc* and *agdi* for L162 and L233, *agdi* and *tyrdc* for L128) and to produce histamine, putrescine and tyramine *in vitro* (Coton et al., 2018). On the contrary, *P. jensenii* CIRM-BIA1774 and *L. rhamnosus* CIRM-BIA1952, selected for the *in situ* tests were both considered as safe for dairy food applications. Concerning *Mucor*, while some species are mycosis agents, others are technological agents in food products and known not to produce mycotoxins (Morin-Sardin et al., 2017). More specifically, *M. lanceolatus* has a long history of safe use in food as it is utilized as a ripening culture in Tomme and Saint-Nectaire cheeses (Desmasures, 2014; Hermet et al., 2014). *In situ* tests including challenge and durability tests are a crucial step in the process of developing an antifungal ingredient since *in vitro* screening can lead to an overestimation of the antifungal activity as discussed above (see also Leyva-Salas et al., 2017)

For challenge tests, surface-spraying of *P. jensenii* CIRM-BIA1774 fermentate on the cheese surface was efficient for delaying fungal growth without any impact on cheese sensory characterisitics. Noteworthy, to our best knowledge, it is the first time that the use of a fermentate is shown to efficiently replace natamycin. Further work would be necessary in an industrial context to verify its antifungal activity in other cheese types as well as to assess whether it can be produced on a large scale with an acceptable cost. In sour cream, *P. jensenii* fermentate at 2 and 5% (w/w) harbored a very high antifungal activity against the 3 tested targets and prevented fungal contaminations of natural origin for up to one month. However, at these high concentrations, its negative impact on sensory attributes, yielding very acid sour cream with propionic odor and flavor mainly linked to high concentration of propionic acid, is a major limit for its application in this product category. Nevertheless, it is interesting to underline that it prevented natural contaminations at 0.4% (w/w) with a limited impact on consumer acceptance. Further work is still necessary to evaluate whether it could be used or not in this product type. These results also indicate that, before performing time-consuming sensorial analysis, a preliminary study to determine the maximum concentration to be used without any impact on sensorial properites could have been useful. Volatile profiles could also be determined on antifungal fermentates prior to sensory analyses and on products subjected to sensorial analysis to develop rapid screening and quality control approaches, using for example head space mass spectrometry (Makhoul et al., 2016). The use of *L. rhamnosus* CIRM-BIA1952 fermentate, incorporated in sour cream or sprayed on semi-hard cheeses, had no impact on product sensorial properties but yielded a limited antifungal activity. However, when considering durability tests results the *L. rhamnosus* fermentate were much more promising. It is worth mentioning that, for *in situ* challenge tests, 50 fungal spores were inoculated, which is a worst-case scenario in comparison to natural contamination and that tested products were incubated at 10 °C, which is also favorable to fungal growth.

Concerning *M. lanceolatus,* to our knowledge, this is the first time that the antifungal activity of a *M. lanceolatus* fermentate is demonstrated.

5. Conclusion

To our best knowledge, this study is the first one which describes the development of dairy-based antifungal ingredients for a large number of strains and species, from *in vitro* screening to pilot scale applications, including the evaluation of their impact on product organoleptic properties. The obtained results provided 3 promising antifungal dairy ingredients which may be used in dairy foods as part of the hurdle technology and to replace or at least limit the use of chemical preservatives such as natamycin or potasium sorbate. From a fundamental point of view, the identification of the molecules harboring antifungal activities and their mechanism of action should be explored.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.11.003.

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