Characterization of the antifungal activity of *Lactobacillus harbinensis* K.V9.3.1Np and *Lactobacillus rhamnosus* K.C8.3.1I in yogurt

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Abstract:

Few antifungal protective cultures adapted to fermented dairy products are commercially available because of the numerous constraints linked to their market implementation. Consumer's demand for naturally preserved food products is growing and the utilization of lactic acid bacteria is a promising way to achieve this goal. In this study, using a 2^{5-1} factorial fractional design, we first evaluated the effects of fermentation time, of initial sucrose concentration and of the initial contamination amount of a spoilage yeast, on antifungal activities of single and mixed cultures of *Lactobacillus rhamnosus* K.C8.3.1I and *Lactobacillus harbinensis* K.V9.3.1Np in yogurt. *L. harbinensis* K.V9.3.1Np, the most relevant strain with regard to antifungal activity was then studied to determine its minimal inhibitory inoculation rate, its antifungal stability during storage and its impact on yogurt organoleptic properties. We showed that *L. harbinensis* K.V9.3.1Np maintained a stable antifungal activity over time, which was not affected by initial sucrose, nor by a reduction of the fermentation time. This inhibitory activity was an all-or-nothing phenomenon. Once *L. harbinensis* K.V9.3.1Np reached a population of $\sim 2.5 \times 10^6$ cfu/g of yogurt at the time of contamination, total inhibition of the yeast was achieved. We also showed that an inoculation rate of 5×10^6 cfu/ml in milk had no detrimental effect on yogurt organoleptic properties. In conclusion, *L. harbinensis* K.V9.3.1Np is a promising antifungal bioprotective strain for yogurt preservation.

Highlights

► Two lactobacilli were selected for their strong antifungal activity in yogurt. ► The influence of different yogurt parameters on their activity was assessed. ► None of the tested parameters impacted the activity of *L. harbinensis* K.V9.3.1Np. ► We showed that its activity was an all-or-nothing phenomenon and was stable over time. ► The presence of this strain did not impact the organoleptic properties of yogurt.

Keywords : *Lactobacillus harbinensis* ; bioprotective culture ; antifungal activity

1. Introduction

Lactic acid bacteria (LAB) have a long history of use in the manufacture of a large variety of fermented foods where they contribute to their preservation and organoleptic properties. During the last decade, there has been increasing interest in the development of LAB bioprotective cultures as alternative to chemical additives in food. This growing interest is mainly driven by consumers' demand for food products without chemical preservatives and/or for preservatives from natural origin. This is why many industrials are moving towards the use of protective microbial cultures, mainly LAB, able to produce antagonistic metabolites such as bacteriocins, peptides and/or low-weight non-proteinaceous compounds (organic acids, fatty acids, H₂O₂, etc.). Many scientific evidences or proof-of-concept in literature underline the great potential of such an approach to combat pathogenic or spoilage microorganisms in various food products such as meat (Budde et al., 2003; Vermeiren et al., 2004; Castellano et al., 2008), fish (Brillet et al., 2005; Tomé et al., 2008; Chahad et al., 2012), bakery products (Dal Bello et al., 2007; Gerez et al., 2009; Ryan et al., 2011) and vegetables (Trias et al., 2008; Randazzo et al., 2009). However, in contrast to probiotics (Gregoret et al., 2013), only a limited number of commercial protective cultures are marketed today, and this statement is especially true for antifungal bioprotective cultures in dairy products. Yet, dairy products are particularly susceptible to fungal contaminations leading to food spoilage (off-flavour, deterioration of visual appearance) and important economic losses (Nelson, 1993).

The limited number of marketed bioprotective cultures in fermented dairy products can be first explained by the numerous constraints linked to their market implementation. Apart from being safe for human consumption, a selected strain must fulfill several criteria (Wessels et al., 2004) and it is often difficult to gather them all. Among them, the antimicrobial strain

must be active in the desired food without producing any detrimental effects on the growth and functionality of starter bacteria (Holzapfel et al., 1995). The antimicrobial activity should also be exhibited and/or maintained under conditions of production, during storage and once the packaging is opened at consumers' home. It is well established that a bacterial antimicrobial activity in food can vary positively or negatively according to environmental conditions including biotic (competing microbiota) and abiotic factors such as temperature, pH, oxygen and available nutrients (Gálvez et al., 2007; Pfeiler and Klaenhammer, 2007 ; Rouse et al., 2008 ; Schillinger and Villareal, 2010 ; Zhang et al., 2010). Indeed, Guerra et al. (2001) showed that nisin production by *Lactococcus lactis* in diluted whey was inhibited by an increase in nitrogen, and by an increase in sugar but in a lesser extent. On the other hand, Dallagnol et al. (2011) highlighted that higher concentrations of phenylalanine and citrate in *Lactobacillus plantarum* CRL 778 fermentation medium, significantly increased the production of phenyllactic and hydroxyphenyllactic acids, two well-known antifungal compounds. Furthermore, bioprotective cultures should be active at the lowest possible concentration in order to reduce the cost associated with their use. In *in situ* studies, Suomalainen and Mäyrä-Mäkinen (1999) and Schwenninger and Meile (2004) used initial cell numbers of 10^7 to 10^8 cfu/g to totally inhibit the fungal targets. Such high concentrations of bioprotective cultures would certainly induce an additional cost for their production. Finally, the protective culture should not impair the sensory attributes of the food product.

In a previous study, *Lactobacillus rhamnosus* K.C8.3.1I and *Lactobacillus harbinensis* K.V9.3.1Np were shown to respectively possess moderate and strong antifungal activities against 6 fungal species (*Debaryomyces hansenii*, *Rodothorula mucilaginosa*, *Yarrowia lipolytica*, *Penicillium brevicompactum*, *Kluyveromyces lactis* and *Kluyveromyces marxianus*) commonly encountered in yogurt spoilage (Delavenne et al., 2013). In the present study, these two strains were included in an experimental design to evaluate the effects of two yogurt

process parameters (fermentation time and initial sucrose) and of the initial contamination rate of *Yarrowia lipolytica*, on their antifungal activities in single and mixed cultures. Finally, the antifungal activity of *L. harbinensis* K.V9.3.1Np was studied in more details, by determining its minimal inhibitory inoculation rate in yogurt, the stability of its antifungal activity during storage and its impact on yogurt organoleptic properties using sensory analyses.

2. Materials and methods

2.1 Microorganisms and culture conditions

L. harbinensis K.V9.3.1Np and *L. rhamnosus* K.C8.3.1I were previously isolated from cow and goat milks, respectively (Delavenne et al., 2012). These bacterial strains were stored in MRS (AES Chemunex, Bruz, France) supplemented with glycerol (30%, v/v) at -80°C , and routinely cultivated at 30°C in MRS broth. They were used as antifungal protective cultures. Commercial freeze-dried yogurt starter cultures (Nat-Ali, Nantes, France), comprising *Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) were used for yogurts manufacture. *Yarrowia lipolytica* UBOCC 2.11.004=LMSA 2.11.004, a yeast particularly resistant to organic acids (Delavenne et al., 2013) and commonly encountered in yogurt spoilage, was obtained from the Culture Collection of Université de Bretagne Occidentale (UBOCC, Plouzané, France). This strain was stored in yeast extract (0.3%) and malt extract (2%) medium (YEMA) supplemented with glycerol (30%, v/v) at -80°C , and cultivated aerobically at 25°C for 2 days on YEMA agar. This strain was used for the artificial contamination of yogurts.

2.2 Cultivation of bacterial and fungal strains for yogurts inoculation

L. harbinensis K.V9.3.1Np and *L. rhamnosus* K.C8.3.1I were separately cultivated for 17 h in MRS broth at 30°C to reach populations of $\sim 2 \times 10^9$ cfu/ml. These cell suspensions were then

centrifuged for 10 min at $10000 \times g$ and washed with sterile 0.1% peptone water, before being suspended and diluted in half-fat pasteurized milk (Lait frais demi-écrémé pasteurisé GrandLait, Candia, France) to reach concentrations ranging from $\sim 10^3$ to 10^9 cfu/ml. Yeasts suspensions were prepared in sterile 0.1% peptone water by scraping colonies from the surface of YEMA agar after 2 days incubation at 25°C. Cells were then enumerated with a Malassez cell and adjusted to 10^4 , 5×10^4 or 10^5 cells/ml.

2.3 Production of yogurt samples

Yogurts were produced with sweetened (100 g/l sucrose) or unsweetened half-fat pasteurized milk supplemented with low-fat milk powder (4 %) (Lait en poudre écrémé, Casino, France) and litmus dye (0.6 %) (RAL, Martillac, France). After heat treatment for 30 min at 85°C, milk was rapidly cooled to 45°C before inoculation with the freeze-dried starter cultures according to the manufacturer's instructions, mixed and sampled into glass containers. Suspensions of antifungal strains (1.5 ml at the desired concentration) were added or not (controls) to milk. Fermentations were carried out at 42°C for 6, 4.5 or 3 hours. The final volume of each yogurt was of 30 ml. Yogurts were then artificially contaminated with the desired concentration of *Y. lipolytica*, either at the end of fermentation (experiments 1 and 2), or after 1 or 5 weeks of storage at 10°C (experiment 3).

2.4 Enumeration of bacteria in yogurts

Bacteria were enumerated before and after fermentation, during storage (once a week) and at the end of the storage periods. Lactobacilli were enumerated on acidified MRS agar (pH 5.5) (Randazzo et al., 2002). *L. bulgaricus* was differentiated from *L. harbinensis* K.V9.3.1Np or *L. rhamnosus* K.C8.3.1I based on colony morphotypes on MRS agar. However, in certain experiments, *L. bulgaricus* was not enumerated because *L. harbinensis* K.V9.3.1Np and/or *L.*

L. rhamnosus K.C8.3.1I were at higher concentrations. *L. harbinensis* K.V9.3.1Np or *L. rhamnosus* K.C8.3.1I were enumerated on LAMVAB agar (Hartemink et al., 1997) and *S. thermophilus* on M17 agar (AES Chemunex, Bruz, France) supplemented with 0.5 % lactose (Ashraf and Shah, 2011). For the assessment of process factors on the antifungal activity of yogurts (experiment 1), LAB were enumerated at the end of the storage period on adequate medium agar supplemented with 5.6 mg/l amphotericin B (Sigma-Aldrich, Saint-Quentin Fallavier, France) to prevent *Y. lipolytica* growth.

2.5 Quantification of *Y. lipolytica* in yogurts

Observation of yogurt surfaces during storage provided a first qualitative estimation of fungal growth. In addition, at the end of each storage period, yogurts were 10-fold diluted in sterile 0.1% peptone water and homogenized for 1 min using a Stomacher (Stomacher®_ Mix 1, AES Laboratoire, Combourg, France). One hundred microliters of each dilution were surface-plated on yeast extract glucose chloramphenicol (YEGC) agar in duplicate, and yeast colonies were enumerated after 48 h incubation at 25°C.

2.6 Experiment 1: assessment of the effects of various factors on antifungal activity using a fractional factorial design

The effect of five factors (initial sucrose percentage of 0 vs 10%, fermentation time, presence or absence of *L. harbinensis* K.V9.3.1Np and/or *L. rhamnosus* K.C8.3.1I, contamination rate of *Y. lipolytica*), and their interactions on antifungal activity were determined by plating and enumeration of *Y. lipolytica* after 6 weeks of storage at 10°C.

In this study, using all the combinations of levels of the five variables in a complete two-level factorial design 2^k would entail using $2^5 = 32$ runs. Instead, we used a fractional factorial design, where only a subset of the complete factorial design (2^{k-p} combinations) is

considered. Their construction is based on confounding some main effects and/or interactions (Box et al., 1978). This technique allows the building of one-half, one-quarter, or even one-eighth, of the complete design. The confounded effects cannot be separated. In the present study, 2^{5-1} (i.e 16) effects were determined: the mean value of the response, 5 main effects of factors (confounded with 4-factor interaction effects), and 10 2-factor interaction effects (confounded with 3-factor interaction effects). Five factors A, B, C, D and E and their levels were selected based on commonly applied yogurt process factors and through preliminary trials (data not shown). Five factors were studied: the initial sucrose concentration (**A**), the inoculation rate of *L. harbinensis* K.V9.3.1Np (**B**), the inoculation rate of *L. rhamnosus* K.C8.3.1I (**C**), the fermentation time (**D**) and the inoculation rate of *Y. lipolytica* (**E**). Each factor was studied at 2 levels (−1) and (+1) reported in Table 1. Additional central runs (coded at level 0 for quantitative factors B, C, D and E) were also included in the matrix. Levels (0) of factors in additional central runs are reported in Table 1. The matrix of the 2^{5-1} design is reported in Table 2. The 16 combinations of the 2^{5-1} factorial design (Table 2, block I) were duplicated (Table 2, block II) in order to evaluate the experimental variance and block effect (Table 2).

2.7 Experiment 2: assessment of *L. harbinensis* K.V9.3.1Np minimal inhibitory inoculation rate

The minimal inoculation rate of *L. harbinensis* K.V9.3.1Np providing a significant antifungal effect toward *Y. lipolytica* was sought. Nine concentrations of *L. harbinensis* K.V9.3.1Np: 0 (control yogurt), 5×10^1 , 5×10^2 , 5×10^3 , 5×10^4 , 5×10^5 , 1×10^6 , 5×10^6 and 5×10^7 cfu/ml of milk were tested. Yogurts were prepared and inoculated as described above and the fermentation was conducted at 42°C for 6 h. After cooling at 10°C, yogurts were contaminated with 100 ± 20 cells of *Y. lipolytica* and stored for 6 weeks at 10°C. *L. harbinensis* K.V9.3.1Np was

enumerated and pH values were measured immediately after yogurt inoculation, after fermentation and weekly during storage, whereas fungal enumeration was performed after 6 weeks of storage at 10°C, as described above. Experiments were done in triplicate for concentrations 5×10^4 , 5×10^5 and 5×10^6 cfu/ml and in duplicate for the others.

2.8 Experiment 3: assessment of the stability of the antifungal activity of *L. harbinensis* K.V9.3.1Np during storage

Stability of antifungal activity during storage was evaluated on yogurts inoculated with different *L. harbinensis* K.V9.3.1Np concentrations (5×10^4 , 5×10^5 , 1×10^6 and 5×10^6 cfu/ml), and contaminated with *Y. lipolytica* (100 cells/yogurt) at different stages (after 6h of fermentation, after one week and after 5 weeks of storage at 10°C). Following contamination, yogurts were stored for an additional 6 weeks at 10°C before enumeration of *Y. lipolytica* population as described above. Series of yogurts uncontaminated with *Y. lipolytica* were also prepared for pH measurements during storage and bacterial enumeration on semi-selective media as described above.

2.9 Sensory evaluation

In order to determine whether the presence of *L. harbinensis* K.V9.3.1Np had a significant impact on yogurt organoleptic properties (taste, flavor and texture), a sensory analysis was performed using a triangle test following the AFNOR NF V 09-013 guidelines. Three coded samples were presented to an untrained panel of 36 members. The assessors were then asked to indicate which sample was different from the two others. Yogurts containing *L. harbinensis* K.V9.3.1Np at 5×10^6 cfu/ml and control yogurts were obtained as described above and stored at 10°C for one week prior to sensory analyses.

2.10 Statistical analyses

Experimental design and statistical analyses were performed using the Statgraphics plus software (Statistical Graphics Corp., VA, USA).

3. Results

3.1 Experiment 1: effect of various factors on antifungal activity

Concentrations of starter bacteria, bioprotective cultures and *Y. lipolytica* after 6 weeks of storage at 10°C determined after the trials of the 2^{5-1} factorial fractional design are shown in Table 2. Since growth of *Y. lipolytica* in culture conditions corresponding to central runs (0,0,0) was null, neither a steepest ascent nor a lack-fit test could be performed in our experimental conditions. According to our data, no significant difference was found between block I and block II. Data from block I and block II were then pooled together to perform the ANOVA.

The coefficient of determination r^2 , which gives the variation percentage of the response due to factor variations and interactions, was high for each response considered ($r^2=81.6-86.8\%$, Table 3), except for *S. thermophilus* growth ($r^2=49\%$). This indicates that one or several factors and their interactions could significantly explain growth variations observed in the yogurt matrix. The two abiotic factors, initial sucrose concentration (A) and fermentation time (D) as well as their interaction (AD), had no significant impact on *Y. lipolytica* nor LAB growth. An initial contamination of 100 or 1000 *Y. lipolytica* cells (factor E) did not impact any of the studied responses, including *Y. lipolytica* final concentrations. Interactions AE, and DE had no significant impact on any of the 6 considered responses in this study (Table 3). Factors A, D and E had no effect on the growth of *L. harbinensis* K.V9.3.1Np and *L. rhamnosus* K.C8.3.1I. On the other hand, when *L. rhamnosus* K.C8.3.1I was inoculated, the final population level of *S. thermophilus* was significantly increased (P -value <0.01),

whereas the final population level of *L. bulgaricus* decreased when *L. rhamnosus* K.C8.3.1I or *L. harbinensis* K.V9.3.1Np were inoculated (Table 3). However, *L. bulgaricus* growth data have to be taken with caution due to the difficulties encountered in the enumeration of this strain.

Statistical analyses clearly revealed that only *L. harbinensis* K.V9.3.1Np addition (factor B), had a significant inhibitory effect (P -value $<10^{-4}$) on *Y. lipolytica* growth in yogurts (Table 3). Moreover, there was no significant interaction between factor B and the other factors, indicating that these factors did not affect the *L. harbinensis* K.V9.3.1Np antifungal activity, neither its growth. Indeed, *Y. lipolytica* growth was not detected in *L. harbinensis* K.V9.3.1Np inoculated yogurts (level + 1 of factor B), except for the trial number 36 (10% of initial sucrose, presence of *L. harbinensis* K.V9.3.1Np, presence of *L. rhamnosus* K.C8.3.1I, 6 hours of fermentation, contamination by 1000 cfu of *Y. lipolytica*), for which *Y. lipolytica* reached a concentration of 3.4 log₁₀ cfu/g (Table 2).

3.2 Experiment 2: assessment of *L. harbinensis* K.V9.3.1Np minimal inhibitory inoculation rate

In this experiment, initial *L. harbinensis* K.V9.3.1Np inoculation concentrations were 0 (control yogurt), 5×10^1 , 5×10^2 , 5×10^3 , 5×10^4 , 5×10^5 , 1×10^6 , 5×10^6 and 5×10^7 cfu/ml. After 6 weeks of storage at 10°C, *Y. lipolytica* grew (with a maximal concentration of 8.8 log₁₀ cfu/g) only in yogurts inoculated with less than 5×10^6 cfu/ml of *L. harbinensis* K.V9.3.1Np (Table 4). Therefore, the minimal inhibitory inoculation rate of *L. harbinensis* K.V9.3.1Np allowing a total inhibition of *Y. lipolytica* was comprised between 1×10^6 and 5×10^6 cfu/ml. In addition, whatever the initial inoculation rates of *L. harbinensis* K.V9.3.1Np, there were no differences in yogurts pH after fermentation and during storage (SD of mean pH ≤ 0.1) (data not shown) as already observed (Delavenne et al., 2013). This suggests that pH modifications were not

responsible for the inhibitory effect. Interestingly, *L. harbinensis* K.V9.3.1Np pursued its growth during storage. Indeed, with initial inoculation rates of 5×10^4 and 5×10^5 cfu/ml, *L. harbinensis* K.V9.3.1Np reached populations above 5×10^6 cfu/g during storage, but with no inhibitory effect in these yogurts. These results suggest that the antifungal activity of *L. harbinensis* K.V9.3.1Np was either dependent on the initial inoculation rate or on the population reached at the stage at which contamination with *Y. lipolytica* occurred.

3.3 Experiment 3: assessment of the stability of the antifungal activity of *L. harbinensis* K.V9.3.1Np during storage

Whatever the stage of contamination (after 6h of fermentation, after one week or after 5 weeks of storage at 10°C), the antifungal activity (total inhibition of *Y. lipolytica*) was maintained in yogurts initially inoculated with 5×10^6 cfu/ml (Figure 1). Moreover, in yogurts inoculated with 5×10^5 cfu/ml of *L. harbinensis* K.V9.3.1Np, no antifungal activity was observed when contaminations occurred immediately after fermentation, whereas total inhibition of *Y. lipolytica* was observed when contaminations occurred after 1 week (in trial 1 only) and after 5 weeks (trials 1 and 2) of storage (Figure 1). In these trials, it was found that *L. harbinensis* K.V9.3.1Np concentrations reached 2.5×10^6 cfu/g (trial 1) after 1 week of storage and 2×10^7 and 3×10^7 cfu/g (trial 1 and 2 respectively) after 5 weeks of storage. Similar results were observed in yogurts with an initial inoculum of 1×10^6 cfu/ml. Indeed, an antifungal activity was only observed when contamination occurred after 1 week of storage (*L. harbinensis* K.V9.3.1Np concentrations of 5×10^6 and 8×10^6 cfu/g, in trials 1 and 2 respectively) and after 5 weeks (*L. harbinensis* K.V9.3.1Np concentrations of 2.5×10^7 and 7.5×10^7 cfu/g in trials 1 and 2, respectively). In contrast, yogurts inoculated with 5×10^4 cfu/ml *L. harbinensis* K.V9.3.1Np showed no antifungal effect when its population was equal or

below 2.5×10^6 (trial 1) and 8×10^6 (trial 2) cfu/g whatever the stage of contamination. On the contrary, when *L. harbinensis* K.V9.3.1Np concentrations were below 2.5×10^6 (trial 1) or 8×10^6 (trial 2) cfu/g at the time of contamination no antifungal activity was found, whatever the contamination time (Figure 1). These data indicate that antifungal activity may appear after *L. harbinensis* K.V9.3.1Np growth and that it is determined by the concentration reached at the contamination time and not by the initial concentration of *L. harbinensis* K.V9.3.1Np. To be antifungal, *L. harbinensis* K.V9.3.1Np must, at the time of contamination, have reached a concentration greater than or equal to the threshold concentration comprised between 2.5 and 8×10^6 cfu/g.

3.4 Sensory analyses

Only 17 out of the 36 assessors indicated which yogurt was different from the two others, showing that there was not any significant difference ($P > 0.05$) in the organoleptic properties of control yogurts and yogurts inoculated with *Lb. harbinensis* K.V9.3.1Np.

4. Discussion

In a previous study, 11 strains isolated from raw milk and belonging to *Lactobacillus casei*, *L. rhamnosus*, *Lactobacillus paracasei*, *Lactobacillus zeae* and *L. harbinensis* species were evaluated for their antifungal activity in yogurt (Delavenne et al., 2013). Two strains, *L. harbinensis* K.V9.3.1Np and *L. rhamnosus* K.C8.3.1I, were identified as potential yogurt bioprotective cultures. In this context, the antifungal activity of these 2 strains was further investigated.

Technological factors such as initial sucrose concentration and fermentation time are likely to vary depending on the yogurt type desired. In yogurt production, sucrose can be added up to

10% (Béal and Sodini, 2003). However, it is known that such an initial sucrose percentage significantly decreases water activity and may negatively affect the growth and lactic acid production of LAB starters (Vinderola et al., 2002; Béal and Sodini, 2003). In this study, such supplementation had no effect on the acidification kinetics of LAB starters. After different storage periods, LAB starter and antifungal LAB strain concentrations were identical with or without sucrose. More importantly, sucrose had no significant effect on *L. harbinensis* K.V9.3.1Np antifungal activity. This finding may be particularly relevant from an industrial point of view because it indicates that sucrose addition could be performed prior to fermentation without disturbing the antifungal activity of this strain. In the tested conditions, fermentation time did not affect either the antifungal activity of *L. harbinensis* K.V9.3.1Np. In contrast to bacteriocinogenic LAB strains, which certain have been found to partially inhibit commercial starter LAB cultures (Oumer et al., 2001), *L. harbinensis* K.V9.3.1Np did not significantly affect the growth and acid production of the LAB starter cultures tested and did not negatively impact the sensory properties of yogurts. In addition, *L. harbinensis* K.V9.3.1Np did not lose its antifungal activity in the presence of other strain, e.g., *L. rhamnosus*, as previously observed for bacteriocinogenic cultures of *Lactobacillus plantarum* and *Enterococcus faecium* (Manzano and Jiménez-Díaz, 2012). Once again, this strain fulfills important criteria which are required for bioprotective cultures (Gálvez et al., 2007). It is worth mentioning that the antifungal activity of *L. harbinensis* K.V9.3.1Np was previously found to dramatically increase in the presence of yogurt starters but was neither related to pH nor to lactic and acetic acid concentrations (Delavenne et al., 2013). The reasons for this synergy remain unknown but we observed that the antifungal activity of *L. harbinensis* K.V9.3.1Np was maintained with other commercial starter cultures of *L. bulgaricus* and *S. thermophilus* (data not shown), suggesting that the synergy was not strain dependent. It would be interesting to further investigate this aspect in future work. As previously reported for

bacteriocin production (Di Cagno et al. 2010; Man et al., 2012), a cross-species induction may be responsible for the production of antifungal compounds by *L. harbinensis* K.V9.3.1Np via signaling molecules or because *L. bulgaricus* and/or *S. thermophilus* may produce intermediary compounds which can then be metabolized by *L. harbinensis* K.V9.3.1Np into compounds with antifungal activity.

In this study, a concentration ranging above 2.5 to 5×10^6 cfu/g of yogurt at the contamination time was necessary to achieve a total inhibition of *Y. lipolytica*. This suggests that the antifungal molecule(s) produced by *L. harbinensis* K.V9.3.1Np acted through an all-or-nothing mechanism with no inhibition below their respective MICs and total inhibition above it. This finding also indicates that the antifungal compounds, at concentrations below their respective MICs, do not or only slightly affect cell viability and growth. Indeed, *Y. lipolytica* populations were similar at the end of storage in control yogurts and yogurts containing a concentration of *L. harbinensis* K.V9.3.1Np below 2.5 to 8×10^6 cfu/g. Finally, because *Y. lipolytica* inhibition only occurred when population of *L. harbinensis* K.V9.3.1Np were above a certain concentration threshold, it may be hypothesized that the production of one or several antifungal compounds are controlled by a quorum sensing regulatory mechanism. Such regulatory mechanism is well documented for bacteriocins production by LAB (Gobbetti et al., 2007) and much less for production of antifungal compounds, although it has already been described for pyrrolnitrin in members of the genus *Burkholderia* (Schmidt et al., 2009).

In this study, it was clearly established that a minimal inoculation rate of 5×10^6 cfu/ml of milk, was sufficient to yield a total inhibition of *Y. lipolytica* throughout a 6-weeks storage period at 10°C . In recent studies, it was found that milk inoculation at 10^8 cfu/ml of *Lactobacillus plantarum* 16 was necessary to achieve a fungistatic effect against *Rhodotorula mucilaginosa* during yogurt storage at 4°C for 4 weeks (Crowley et al., 2012). And that $\sim 4 \times 10^7$ cfu/ml of *Lactobacillus casei* AST 18 allowed to inhibit the growth of a *Penicillium*

sp. isolate during yogurt storage at 4°C for 22 days (Li et al., 2013). While it may be hazardous to make a comparison between results obtained in our study and the latter studies, because different fungal strains and storage temperature were used, it can be concluded that *L. harbinensis* K.V9.3.1Np presented a high antifungal activity at a lower inoculation rate than in these studies. Moreover, we previously showed that milk inoculation at 5×10^6 cfu/ml was sufficient to inhibit *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Penicillium brevicompactum* and *Rhodotorula mucilaginosa* strains, indicating that the antifungal compounds produced by *L. harbinensis* K.V9.3.1Np possess a large activity spectrum (Delavenne et al., 2013). If this strain may be propagated at high concentration with a good survival rate to lyophilization, given the low inoculation rate required to exert an antifungal activity, the utilization of this strain in yogurt may be acceptable from an economic point of view.

In conclusion, *L. harbinensis* K.V9.3.1Np is a promising bioprotective strain, active against different fungal targets (Delavenne et al. 2013), that could be used as an alternative to chemical additives for preventing yogurt fungal contaminations without compromising its organoleptic properties. Moreover, the antifungal activity was maintained during storage and after modification of the technological processes. Yogurt and fermented milk spoilage often result from airborne contaminations, contaminated equipment or raw or processed ingredients involving a small number of fungal cells. The utilization of this strain could be interesting to decrease the risk of fungal spoilage associated with ingredients such as fruit preparations, like blueberries, which are extremely sensitive to fungal contaminations and stabilized using moderate heat treatments (Penney et al., 2004). Even if most species of the genus *Lactobacillus* are considered “generally recognized as safe” organisms, *L. harbinensis* K.V9.3.1Np has only been recently described in literature and it does not have any history of utilization in dairy products. We are currently evaluating its safety based on the qualified

presumption of safety (QPS)-procedure for LAB. We showed that this strain did not produce biogenic amines and that its antibiotic minimum inhibitory concentrations were below the breaking points as specified by the European Food Safety Agency (data not shown). Finally, an investigation of the antifungal compounds involved is currently carried out.

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Table 1. Levels of factors in the factorial fractional design 2^{5-1}

Factors	Levels		
	-1	0	+1
A Sucrose concentration (%)	0	-	10
B K.V9.3.1Np (cfu/ml)	0	2.5×10^6	5×10^6
C K.C8.3.1I (cfu/ml)	0	2.5×10^6	5×10^6
D Duration (h)	3	4.5	6
E <i>Y. lipolytica</i> (cells/ pot)	100	500	1000

A: Sucrose concentration (0 vs. 10% sucrose), B: inoculation rate of *L. harbinensis* K.V9.3.1Np, C: inoculation rate of *L. rhamnosus* K.C8.3.1I, D: duration of fermentation, E: inoculation rate of *Y. lipolytica*.

Table 2. Concentrations of starter bacteria, bioprotective cultures and *Yarrowia lipolytica* in yogurts after 6 weeks of storage at 10°C obtained after the assays of the 2^{5-1} factorial fractional design.

		Factors					Concentrations (log ₁₀ cfu/g)				
		A	B	C	D	E	<i>S. thermophilus</i>	<i>L. bulgaricus</i>	<i>L. harbinensis</i> K.V9.3.1Np	<i>L. rhamnosus</i> K.C8.3.1I	<i>Y. lipolytica</i>
Block 1	Trial	1	+1	+1	+1	+1	7.8	< 7 ^a	6.9	8.5	0 ^b
		2	-1	0	0	0	7.6	< 7	7.6	8.3	0
		3	-1	+1	+1	-1	7.9	< 7	7.7	8.3	0
		4	+1	0	0	0	8.1	< 7	7.4	8.4	0
		5	+1	-1	-1	+1	7.5	6.6	0	0	7.8
		6	-1	+1	-1	+1	6.4	< 7	8.1	0	0
		7	-1	+1	+1	+1	7.4	< 7	7.5	8.3	0
		8	-1	0	0	0	7.3	< 7	7.5	8.2	0
		9	+1	-1	-1	+1	7.5	6.9	0	0	7.9
		10	-1	-1	-1	-1	7.2	nd ^c	0	0	8.3
		11	+1	-1	+1	+1	7.1	< 7	0	8.5	6.5
		12	-1	-1	+1	+1	7.2	< 7	0	8.3	7
		13	-1	-1	+1	+1	6.6	< 7	0	8.5	7.6
		14	-1	-1	-1	+1	6.0	6.9	0	0	7.6
		15	+1	-1	+1	-1	6.8	< 7	0	8.8	7.3
		16	-1	+1	-1	+1	7.3	< 7	8.3	0	0
		17	+1	+1	-1	-1	6.8	< 7	8.6	0	0
		18	+1	0	0	0	7.5	< 7	7.5	8.3	0
		19	+1	+1	+1	-1	7.2	< 7	8.0	8.6	0
		20	+1	+1	-1	+1	6.8	< 7	8.1	0	0
Block 2	Trial	21	+1	0	0	0	8.0	< 7	7.3	8.6	0
		22	+1	-1	-1	+1	6.8	6.0	0	0	8
		23	+1	+1	+1	-1	7.5	< 7	7.8	8.6	0
		24	+1	-1	+1	+1	7.4	< 7	0	8.7	7.2
		25	-1	0	0	0	7.4	< 7	7.3	8.3	0
		26	-1	+1	+1	-1	7.8	< 7	8.0	8.5	0
		27	-1	-1	+1	+1	7.3	< 7	0	8.8	7.9

28	-1	0	0	0	0	8.0	< 7	7.2	8.4	0
29	-1	+1	-1	+1	+1	6.8	< 7	8.1	0	0
30	-1	-1	-1	+1	-1	7.0	6.5	0	0	8.5
31	+1	+1	-1	-1	+1	7.2	< 7	8.3	0	0
32	-1	+1	-1	-1	-1	7.8	< 7	8.3	0	0
33	-1	+1	+1	+1	-1	7.5	< 7	7.3	8.3	0
34	+1	-1	-1	-1	-1	6.3	6.4	0	0	7.9
35	+1	0	0	0	0	7.5	< 7	7.3	8.4	0
36	+1	+1	+1	+1	+1	7.1	< 7	7.2	8.6	3.4
37	+1	+1	-1	+1	-1	6.7	< 7	8.1	0	0
38	-1	-1	-1	-1	+1	6.0	6.5	0	0	8.2
39	-1	-1	+1	-1	-1	7.1	< 7	0	9.0	8.1
40	+1	-1	+1	+1	-1	7.7	< 7	0	8.8	6.8

Results are expressed in log₁₀ cfu/g of yogurt. Each yogurt trial is associated to a values combination (-1; 0; +1) of the 5 factors: A (sucrose concentration in milk), B (rate of inoculation of *L. harbinensis* K.V9.3.1Np), C (rate of inoculation of *L. rhamnosus* K.C8.3.1I), D (duration of fermentation) and E (contamination rate of *Y. lipolytica*). ^a*L. bulgaricus* could not be enumerated by the used method in yogurts supplemented with *L. harbinensis* K.V9.3.1Np and/or *L. rhamnosus* K.C8.3.1I;^b in the case of *Y. lipolytica* concentrations below the detection threshold of 2 log₁₀ cfu/g, the absence of fungal growth was confirmed by plating a whole yogurt on YEGC agar. ^c Not determined.

Table 3. Estimated main effects and interactions from factorial fractional 2^{5-1} design.

Factors and interactions	Effect of responses									
	Growth of lactic acid bacteria and yeast in yogurts after 6 weeks of storage at 10°C									
	<i>L. harbinensis</i> K.V9.3.1Np		<i>L. rhamnosus</i> K.C8.3.1I		<i>S. thermophilus</i>		<i>L. bulgaricus</i>		<i>Y. lipolytica</i>	
	<i>P</i> -values		<i>P</i> -values		<i>P</i> -values		<i>P</i> -values		<i>P</i> -values	
Mean values of the response	4.6 ± 0.3 ^a		5.10 ± 0.34		7.2 ± 0.07		1.1 ± 0.2		3.7 ± 0.3	
A : Sucrose concentration	-0.01	0.90	0.04	0.9	0.04	0.6	0.15	0.44	-0.03	0.91
B : inoculation of K.V9.3.1Np	3.94	<10⁻⁴	-0.05	0.89	0.13	0.13	-1.43	<10⁻⁴	-3.25	<10⁻⁴
C : inoculation of K.C8.3.1I	-0.17	0.60	4.28	<10⁻⁴	0.23	0.01	-1.43	<10⁻⁴	-0.10	0.75
D : fermentation duration	-0.11	0.72	-0.025	0.94	-0.04	0.63	0.19	0.37	<10⁻³	0.99
E : contamination rate	0.025	0.93	0.005	0.98	-0.1	0.26	0.21	0.33	0.052	0.87
AB	-0.01	0.98	0.024	0.94	-0.14	0.10	-0.18	0.39	0.19	0.56
AC	-0.03	0.92	0.03	0.93	-0.03	0.66	-0.18	0.39	0.02	0.95
AD	-0.03	0.92	0.005	0.98	0.09	0.30	-0.24	0.27	0.08	0.80
AE	-0.005	0.98	-0.01	0.97	0.08	0.31	-0.21	0.32	0.08	0.80
BC	-0.17	0.59	-0.05	0.89	0.04	0.60	1.43	<10⁻⁴	0.25	0.44
BD	-0.11	0.72	0.01	0.97	-0.16	0.08	-0.19	0.37	0.15	0.65
BE	0.025	0.93	0.01	0.96	0.045	0.60	-0.21	0.34	0.09	0.76
CD	-0.04	0.88	-0.02	0.94	0.08	0.33	-0.19	0.37	0.02	0.93
CE	0.038	0.90	0.005	0.98	-0.012	0.88	-0.21	0.34	0.10	0.75
DE	0.01	0.97	0.02	0.95	-0.06	0.48	-0.22	0.32	0.09	0.77
R² (%)^b	86.8		84.6		49.0		86.1		81.6	

A: sucrose concentration in milk, B: rate of inoculation of *L. harbinensis* K.V9.3.1Np, C: rate of inoculation of *L. rhamnosus* K.C8.3.1I, D: duration of fermentation and E contamination rate of *Y. lipolytica*. *P* values in bold are highly significant.

^a Standard error calculated from residual error

^b Coefficient of determination (% of bacterial or yeast explained by variations of significant factors and interactions in the polynomial model)

Table 4. Population of *Yarrowia lipolytica* determined after 6 weeks of storage at 10°C depending on *L. harbinensis* K.V9.3.1Np inoculation rates.

<i>L. harbinensis</i> initial concentrations (cfu/ml)	<i>Y. lipolytica</i> concentrations (log ₁₀ cfu/g)		
	Trial 1	Trial 2	Trial 3
0	8.5	8.0	7.9
5×10 ¹	8.7	nd ^b	nd
5×10 ²	8.4	nd	nd
5×10 ³	8.8	nd	nd
5×10 ⁴	8.2	8.8	8.0
5×10 ⁵	8.2	7.9	7.8
1×10 ⁶	nd	7.9	8.3
5×10 ⁶	< 2 ^a	< 2	< 2
5×10 ⁷	< 2	nd	nd

^a Concentrations < 2 log₁₀ cfu/g means that the population was under the detection threshold of the counting method. ^bNot determined.

Figure 1. Enumeration of *Yarrowia lipolytica* (cfu/g) in yogurts, according to the initial *L. harbinensis* concentration ([*L. harbinensis*]i) and the concentration reached at the time of contamination ([*L. harbinensis*]c) after fermentation (H6), after 1 week of storage (W1) or after 5 weeks of storage (W5). A: trial 1; B: trial 2; (■) *L. harbinensis* concentration at the time of contamination (axis on the left); (□) *Y. lipolytica* concentration 6 weeks after contamination (axis on the right).