

## ORIGINAL ARTICLE

# Development of a new method for the detection of lactic acid bacteria capable of protecting ham against Enterobacteriaceae

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## Keywords

Enterobacteriaceae, lactic acid bacteria, protective culture, screening, sequential culturing method.

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## Abstract

**Aims:** Challenge trials seem to be the best assessment approach to evaluate the potency of food protective cultures. However, this method is time consuming and often difficult to implement. Here, we describe the development of the 'sequential culturing method', a new method for the screening of strains as protective cultures.

**Methods and Results:** The sequential culturing method is based on the simulation, in a meat simulation medium (named BHI5L200), of the inhibition of Enterobacteriaceae by *Lactobacillus*, observed previously *in situ*. Results obtained with this sequential culturing method were in good agreement with those of the challenge test on sliced cooked ham and confirmed the antagonistic potency of *Lactobacillus*. The results obtained from the screening of 187 lactic acid bacteria (LAB) indicated that *Lactobacillus sakei*, *Lactococcus lactis diacetylactis* and *Carnobacterium* spp. were strong inhibitors of Enterobacteriaceae whereas *Pediococcus* spp., *Leuconostoc* spp., *Weisselia* spp. and other species of *Lactobacillus* and *Lactococcus*, did not possess the same inhibitory capacity.

**Conclusions:** Sequential culturing method appeared to be a useful tool to rapidly select LAB cultures which are good candidates for bioprotection of meat.

**Significance and Impact of the Study:** Sequential culturing method and simulating media could efficiently mimic challenge test experiments in the selection of potential protective culture for all types of food, on the condition to have the appropriate simulating media, corresponding to the food for which protective cultures were searched.

## Introduction

Meat and meat products constitute a favourable medium for the growth of micro-organisms. Refrigeration has been traditionally applied to extend the shelf life of such perishable foods. However, this most important technological development is controversial since it could contribute to the selection of psychrotrophic bacteria (Sorhaug and Stepaniak 1997; O'Sullivan *et al.* 2002). Many of these belong to the Enterobacteriaceae group and have been detected in several meat products, in which they were involved in food poisoning

(*Yersinia enterocolitica*) or responsible for reduction of the commercial quality of foodstuff (*Serratia* spp., *Hafnia alvei*) (Garcia de Fernando *et al.* 1995; Borch *et al.* 1996; Gamage *et al.* 1998; Samelis *et al.* 2000; Ellis and Goodacre 2001; Kang *et al.* 2002; Vermeiren *et al.* 2005). Moreover, some mesophilic Enterobacteriaceae species (*Salmonella* spp., pathogenic *Escherichia coli*) are capable of multiplying in slightly temperature-abused refrigerated foods (Garcia de Fernando *et al.* 1995; Bell 2002).

Consumers are drawn to natural foods with no or reduced chemical preservative contents. This perception

has stimulated research interest in biopreservation that depends on the use of antagonistic micro-organisms, or their antimicrobial products, to inhibit undesired micro-organisms in order to enhance safety and extend shelf life of fresh products (Holzapfel *et al.* 1995).

In meat, lactic acid bacteria (LAB) constitute a part of the initial microflora (Rantsiou and Coccolin 2006) which develop after meat processing. In general, conditions that favour their growth result in an extension of the storage life and enhance safety of chilled meats. Recent approaches in the preservation of meat products increase the use of LAB as protective microbiota to inhibit spoilage and pathogenic bacteria (Kotzekidou and Bloukas 1996; Bredholt *et al.* 1999, 2001; Pidcock *et al.* 2002).

When evaluating a bacterial strain for its biopreservation capacity, it is important to consider that meat products are complex systems with several factors influencing the microbial growth and subsequent metabolite production. Therefore, the influence of the medium formulation and fermentation technology on the performance of strains needs to be tested. In this way, challenge trials have been considered as the most appropriate assessment approach (Adams and Mitchell 2002). However, this method is time consuming and often difficult to implement.

In this study, we developed the new 'sequential culturing method' for screening LAB strains active against Enterobacteriaceae. The method was designed to reproduce the protective activity observed in challenge tests of three strains of LAB, namely *Lactobacillus sakei* L2512 and L110 and *Pediococcus acidilactici* P1521, in a medium simulating ham, named BHI5L200 (Hequet *et al.* 2007). Next, the sequential culturing method was used to screen a large number of LAB, isolated from food environment, for their antimicrobial activity.

## Materials and methods

### Bacterial strains, media and cultural conditions

The bacterial strains used in this work are listed in Table 1. LAB strains were grown at 30°C in

DeMan-Rogosa-Sharpe (MRS) broth (BD Difco, Le Pont-De-Claix, France). Enterobacteriaceae strains were grown at 30°C in Brain Heart Infusion (BHI) broth (BD Difco) without shaking. Tryptone salt medium is composed of 1 g l<sup>-1</sup> Tryptone (Biokar Diagnostics, Pantin, France) and 8.5 g l<sup>-1</sup> NaCl. The BHI5L200 medium (Hequet *et al.* 2007) is composed of BHI broth supplemented with 3 g l<sup>-1</sup> yeast extract (BD Difco), 3 g l<sup>-1</sup> glucose (Sigma, St Quentin Fallavier, France), 1 ml l<sup>-1</sup> Tween 80 (Sigma) and 200 g l<sup>-1</sup> Lab Lemco powder (Oxoid, Dardilly, France). Initial pH of BHI5L200 is 7.0.

Colony forming unit (CFU) monitoring was carried out by enumerating colonies present in 10 µl of a serial log dilutions of each sample spotted on MRS agar for LAB strains and Violet Red Bile Glucose (BD Difco) agar for Enterobacteriaceae strains and incubated at 37°C overnight.

### Challenge test experiments

An entire cooked ham was obtained from a local industrial food producer. It was sliced, under nonaseptic conditions, with a cutting device sprayed or not with LAB suspended in a 0.9% NaCl solution. LAB suspension was prepared in order to obtain approx. 10<sup>6</sup> cells per g of sliced ham. During the slicing, LAB suspensions were continuously sprayed on the cutting device to ensure the constant presence of 10<sup>6</sup> cells per g of sliced ham. After slicing, 36 cm<sup>2</sup> pieces were cut under aseptic conditions. Samples were incubated under vacuum at 4°C for the first week followed by 3 weeks at 8°C to stimulate temperature abuse. Packaging was carried out using Multivac A300/16 (Sepp Hagenmüller GmbH and Co., Wolfertschwenden, Germany). The permeability spectrum of the packaging film was: 40–50 cm<sup>3</sup> m<sup>-2</sup> for oxygen, 146 cm<sup>3</sup> m<sup>-2</sup> for CO<sub>2</sub> and 8 cm<sup>3</sup> m<sup>-2</sup> for nitrogen. For analysing the final products, the packaging material was removed and the sample was transferred aseptically into a volume of tryptone salt corresponding to 4 ml of broth per g of ham. After shaking at 230 rev min<sup>-1</sup> (2 × 1 min) with a stomacher (Seward, Worthington, UK) various bacterial

**Table 1** Bacterial strains used in this study

Bacterial strains	Relevant characteristics*	Source/reference
<i>Serratia marcescens</i> 152	Wild-type strain, cooked ham origin	Danisco collection
<i>Serratia liquefaciens</i> 6	Wild-type strain, cooked ham origin	Danisco collection
<i>Hafnia alvei</i> 1	Wild-type strain, cooked ham origin	Danisco collection
<i>Escherichia coli</i> ATCC8739	Wild-type strain, cooked ham origin	Danisco collection
<i>Lactobacillus sakei</i> L2512	Industrial wild-type strain, sakG <sup>+</sup>	Simon <i>et al.</i> (2002)
<i>Lactobacillus sakei</i> L110	Industrial wild-type strain	Danisco collection
<i>Pediococcus acidilactici</i> P1521	Industrial wild-type strain, ped <sup>+</sup>	Guyonnet <i>et al.</i> (2000)

\*sakG, sakacin G; ped, pediocin PA.1; +, producer.

populations were monitored by CFU counting. LAB (L2512, L110 and P1521) and Enterobacteriaceae counts were realized at 0, 1, 2, 3 and 4 weeks. Results of LAB counts confirmed the even distribution of the LAB on the pieces of ham (data not shown). Indeed, during all the period of storage, number of the three tested LAB strains was, at least,  $10^6$  cells per g of sliced ham.

Detected Enterobacteriaceae were not inoculated but occurred from initial contamination before packaging of ham pieces.

### Sequential culturing method

BHI5L200 medium was inoculated with  $10^5$ – $10^6$  CFU ml<sup>-1</sup> of each tested LAB strain and incubated for 48 h at 37°C. Bacteria were first removed by centrifugation (7500 g, 20 min) followed by filtration (Analytapore; pore size, 0.22 µm; Fischer Scientific, Illkirch, France) of the precultured supernatant. When necessary the pH of the LAB precultured BHI5L200 and control BHI5L200 was decreased to 5.8, corresponding to the pH of ham precultured with LAB (Vermeiren *et al.* 2004), with 6 mol l<sup>-1</sup> HCl solution prior to filtration. Resultant precultured media were stored at -20°C before using. Two hundred microlitres of BHI5L200 precultured or not was poured into five wells of a 96 microtitre plate. Three wells were then seeded ( $10^2$  CFU ml<sup>-1</sup>) with one of the indicator Enterobacteriaceae strains, *Serratia marcescens* 152, *Serratia liquefaciens* 6, *H. alvei* 1 and *Escherichia coli* ATCC8739, and two control wells were not inoculated. Microtitre plate was incubated for 7 days at 30°C. Twice a day, growth of the indicator strain was monitored at 595 nm (Sunrise Reader, Tecan). Growth rate in a precultured medium corresponds to the average of the OD<sub>595 nm</sub> of the three seeded wells minus the average of the OD<sub>595 nm</sub> of the two nonseeded wells.

To quantify the antagonism phenomenon, we calculated the Enterobacterial Inhibition Factor (EIF). EIF corresponds to the difference in time necessary to reach an OD<sub>595 nm</sub> of 0.1 between a strain grown in 'non-precultured medium' as opposed to the same strain in 'precultured medium'. The value of 0.1 for the OD was arbitrarily chosen. The growth of S152 was measured in the medium precultured with itself. We decided to consider as a reference value the EIF of 90 h measured for S152.

Each week, two 96 microtitre plates were 'tested'. The plates were filled with: water in external wells to reduce edge effect due to evaporation; five wells with BHI5L200 precultured with L2512 (positive control); five wells with BHI5L200 precultured with P1521 (negative control); five wells with BHI5L200 non-precultured (to calculate EIF). The other wells were filled with screened LAB strains. Thus, 21 new LAB strains were tested by sequential

culturing method each week. Consequently, 9 weeks have been necessary to screen the collection constituted of 187 LAB strains.

### Statistical analysis

All experiments were replicated three times. Data were pooled and the mean values and standard deviations were determined using Excel software.

## Results

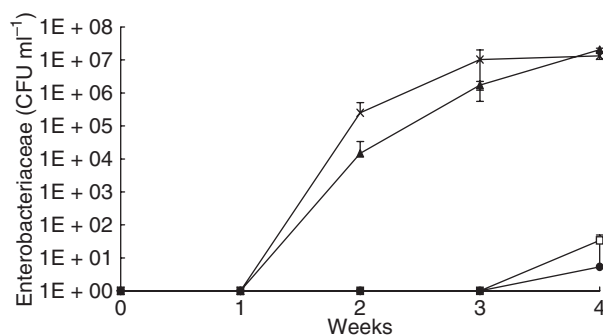
### Inhibition of Enterobacteriaceae by *Lactobacillus sakei* strains on sliced cooked ham in challenge tests

The ability of LAB to protect sliced cooked ham from Enterobacteriaceae spoilage was tested by challenge test. The growth of foodborne Enterobacteriaceae was evaluated via comparison of those obtained on sliced and nontreated ham pieces with those obtained with LAB sprayed on the slicing device. In these assays, no Enterobacteriaceae were inoculated but developed naturally on the ham which was not handled under aseptic conditions before cutting.

Results (Fig. 1) showed that the growth of Enterobacteriaceae was inhibited significantly by strain L2512 since <math>10^1</math> CFU g<sup>-1</sup> of spoilage bacteria was detected after 4 weeks whereas >math>10^5</math> CFU g<sup>-1</sup> were detected after 2 weeks for the control. These results (Fig. 1) showed that L110 inhibited the Enterobacteriaceae as well as L2512 whereas P1521 had no effect on the inhibition of spoilage bacteria.

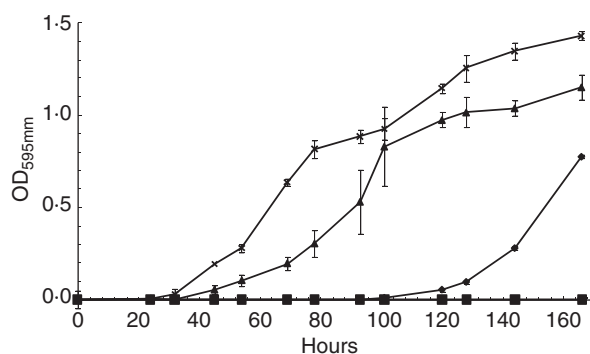
### Sequential culturing method

In a previous study (Hequet *et al.* 2007), we demonstrated that the simulation medium BHI5L200 was



**Figure 1** Growth of Enterobacteriaceae on sliced cooked ham (×) and after pulverization of a suspension containing *Lactobacillus sakei* L2512 (●), *L. sakei* L110 (□), *Pediococcus acidilactici* P1521 (▲) on the cutting device. Error bars denote standard deviation from the means values ( $n = 3$ ).

suitable for replacing food samples for challenge tests of LAB vs *Listeria*. The use of this liquid medium in co-culture experiments allowed us to conclude that such a system is a powerful tool for the selection of bacteriocinogenic protective cultures from meat. Consequently, we used this method to select LAB strains active against Enterobacteriaceae. Thus, the three LAB strains tested in challenge test experiments (L2512, L110 and P1521), were assayed for their antagonistic activity against four selected indicator strains of Enterobacteriaceae which were isolated from spoiled ham (Table 1). Unfortunately, none of the LAB strains showed inhibitory activity in BHI5L200 co-culture experiments (data not shown). In reality, the growth rate of Enterobacteriaceae in BHI5L200 is greater than those of LAB strains (data not shown), which could explain the aforementioned result. Thus, to reproduce the antagonism phenomenon observed in challenge test experiments, we developed the sequential culturing method in which the Enterobacteriaceae are inoculated in a medium previously modified by the growth of the LAB strain, or not for the control. The growth of the indicator strain *S. marcescens* 152 (S152) in media precultured with the three LAB strains are presented in Fig. 2. The growth of S152 in the control was detectable 24 h after inoculation. In the media precultured with either L2512 or L110, the indicator strain appeared unable to grow during the experiment (168 h). Moreover, after 168 h, S152 reached a concentration of  $3.8 \pm 0.3 \times 10^8$  CFU ml<sup>-1</sup> in the control whereas it was undetectable in BHI5L200 precultured with L2512 or L110. In the BHI5L200 media precultured with P1521, the concentration of S152 at 168 h was  $4.5 \pm 0.6 \times 10^8$  CFU ml<sup>-1</sup> and the OD<sub>595 nm</sub> increased in <36 h. The growth of S152 was measured in the medium precultured with itself (Fig. 2). As expected, growth of



**Figure 2** Growth of *Serratia marcescens* 152 at 30°C in BHI5L200 medium precultured with *Lactobacillus sakei* L2512 (●), *L. sakei* L110 (□), *Pediococcus acidilactici* P1521 (▲) and *S. marcescens* 152 (◆) or not precultured (×). Error bars denote standard deviation from the means values ( $n = 3$ ).

the target strain was inhibited with a lower extent than observed with *Lactobacillus*. This indicated that nutrient depletion of the medium was not the only factor implicated in the growth inhibition. We decided to consider as a reference value the EIF of 90 h measured for S152, which allowed us to give an inhibitor level to the tested LAB strains.

Thus, the calculated EIF for both *Lactobacillus*, superior to 130 h, corresponds to strong inhibitors, whereas the EIF factor 20 h indicates that *P. acidilactici* P1521 is a weak inhibitor (Table 2). These results were in good agreement with those of the challenge test on sliced cooked ham and confirmed the antagonistic potency of the *L. sakei* strains but not *P. acidilactici* strain.

Furthermore, similar results were obtained with three other foodborne Enterobacteriaceae, *S. liquefaciens* 6, *H. alvei* 1 and *E. coli* ATCC8739 (Table 2). Indeed, *L. sakei* L2512 stayed a strong inhibitor as it showed maximal EIF values against the three Enterobacteriaceae strains whereas *P. acidilactici* P1521 seemed to be a weak inhibitor as it presented small EIF values. Concerning *L. sakei* L110, results showed that it was strong inhibitor of *S. liquefaciens* 6 because it showed a maximal EIF value of >112 h. Moreover, *L. sakei* L110 seemed to be a medium inhibitor of *H. alvei* 1 and *E. coli* ATCC8739 as it presented intermediary EIF values of these obtained for *L. sakei* L2512 and *P. acidilactici* P1521, which were respectively strong and weak inhibitor. However, these results need to be confirmed on the one hand, by increasing the number of experiments and, on the other hand, by measuring the EIF values of *S. liquefaciens* 6, *H. alvei* 1 and *E. coli* ATCC8739 in the medium precultured with themselves, in order to confirmed the inhibitor level of the three LAB strains against these three Enterobacteriaceae strains.

Finally, this sequential culturing method appeared efficient to simulate the effect of protective cultures on ham as it was observed in challenge tests.

### Screening of Enterobacteriaceae-inhibiting LAB

Meat-borne LAB from the Danisco culture collection were screened for the inhibition of growth of S152 using the sequential culturing method. The 187 strains, from various food origins and covering the different species found in meat products were screened over a 9 week period.

Sixty-one LAB strains displayed EIF values superior to 130 h whereas the 126 remaining strains displayed EIF values inferior to 90 h (data not shown), which is the reference value for nutrient competition. The 61 strains with maximal EIF were finally considered as inhibitors. Interestingly, when the EIF values were related to the species of the tested strains, some inhibiting species were found to

**Table 2** Enterobacterial Inhibition Factor (EIF) corresponding to the inhibition of three other strains of Enterobacteriaceae in BHI5L200 precultured with LAB strains

	<i>Serratia marcescens</i> 152*	<i>Serratia liquefaciens</i> 6†	<i>Hafnia alvei</i> 1†	<i>Escherichia coli</i> ATCC8739†
<i>Lactobacillus sakei</i> L2512	>130 ± 0	>112	>128	>130
<i>Lactobacillus sakei</i> L110	>130 ± 0	>112	72	50
<i>Pediococcus acidilactici</i> P1521	21 ± 4	44	16	0

EIF corresponds to the difference in time necessary to reach an OD<sub>595 nm</sub> of 0.1 between a strain grown in 'non-precultured medium' as opposed to the same strain in 'precultured medium'.

\*EIF values correspond to means values obtained for calculated EIF of three different sequential culturing method experiment ( $n = 3$ ).

†EIF values correspond to calculated EIF of one sequential culturing method experiment ( $n = 1$ ).

**Table 3** Proportion of strains in each species with EIF >130 h detected from the Danisco LAB collection screened

Species	Number of tested strains	Percentage of strong inhibitors*
<i>Lactobacillus sakei</i>	21	<b>71</b>
<i>Lactobacillus curvatus</i>	14	14
<i>Lactobacillus plantarum</i>	4	25
Other <i>Lactobacillus</i>	4	0
<i>Pediococcus pentosaceus</i>	14	21
<i>Pediococcus acidilactici</i>	3	0
<i>Carnobacterium</i> spp.	4	<b>75</b>
<i>Lactococcus lactis lactis</i>	50	26
<i>Lactococcus lactis lactis</i> biovar <i>diacetylactis</i>	14	<b>71</b>
<i>Lactococcus lactis cremoris</i>	31	23
<i>Leuconostoc</i> spp.	4	0
<i>Weissella viridescens</i>	6	17
Not determined	18	33
Total	187	33

\*Bold type highlights the LAB species which have a high percentage of strong inhibitor.

be more effective (Table 3). Among the *Carnobacterium* spp., *L. sakei* and *Lactococcus lactis lactis* biovar *diacetylactis* screened strains, >70% displayed an inhibitory effect whereas <30% of the *Pediococcus* spp., *Leuconostoc* spp., *Weissella* spp. strains displayed an inhibitory effect.

## Discussion

Challenge tests, which are essential to validate the efficiency of a protective culture in food protection, are quite tedious and time consuming. Indeed as shown above (Fig. 1), 21 days were necessary to detect an antagonistic activity on sliced ham. Consequently this method is not used for the routine screening of bacterial libraries for their antagonistic activities. Classical screening technologies, based on growth inhibition of a target strain, are conducted in culture media that do not reflect the food environment which has to be protected. Thus, we developed a new method designed to mimic

the anti-enterobacterial activity of three strains of LAB (*L. sakei* L2512 and L110 and *P. acidilactici* P1521) in BHI5L200, a simulating ham medium (Hequet *et al.* 2007). Because co-culture experiments in BHI5L200 were unsuitable for this purpose, we developed the sequential culturing method. This method is reproducible, faster than challenge tests and capable of identifying LAB strains which are good candidates as protective cultures of meat from the others.

Nevertheless, the sequential culturing method showed that strains of *Carnobacterium* spp., *L. sakei* and *L. lactis lactis* biovar *diacetylactis* are more frequently found to be strong inhibitors of Enterobacteriaceae in comparison to other tested species. These results are in good agreement with others published works. Several studies have shown considerable interest in *L. sakei* for the bioprotection of meat products (Bredholt *et al.* 1999, 2001; Vold *et al.* 2000; Katikou *et al.* 2005). For example, Katikou *et al.* (2005) have shown that *L. sakei* CECT 4808 was more efficient than *Lactobacillus curvatus* CECT 904 to reduce the development of Enterobacteriaceae in refrigerated vacuum-packed sliced beef. Our screening results seem to reflect the same trend since the percentage of strong inhibitors of *L. sakei* was five times greater than those of *L. curvatus*. Moreover, according to the complete genome analysis, it was proposed that *L. sakei* could be used to control pathogens in meat because its metabolism is particularly well adapted to meat environment (Chaillou *et al.* 2005) and to the environmental conditions (refrigeration temperature, gas packaging) that prevail during manufacturing process (Marceau *et al.* 2003, 2004). Sequential culturing results showed that *diacetylactis* biovar strains are more efficient in Enterobacteriaceae bioprotection of meat than *L. lactis lactis*. To our knowledge, no studies have described the use of *Lactococcus lactis* strain in a meat bioprotection context. However, recent works have shown that a *diacetylactis* biovar strain was more efficient than *L. lactis lactis* to control the development of *E. coli* and *Salmonella enteritidis* strains in dairy products (Mufandaedza *et al.* 2006). Even if results indicate that the screened *Carnobacterium* strains could

potentially be used for the bioprotection of meat, the number of tested strains is insufficient to generalize this trend to the entire genus.

Preliminary results, concerning the characterization of the *Lactobacillus* antagonistic activity, showed that acetic acid was probably the main molecules implicated in the anti-enterobacterial effect. The experiment consisted in addition of various amounts of acetic acid, as well as lactic acid, in non-precultured BHI5L200 before inoculation of S152. Growth monitoring of the enterobacteria showed that it was inhibited (EIF >130 h) by 25 mmol l<sup>-1</sup> of acetic acid but not with 50 mmol l<sup>-1</sup> of lactic acid (EIF ≈ 30 h).

In conclusion, the medium simulating ham as well as the sequential culturing method were successfully used to detect inhibitory strains. This method allows one to rapidly select LAB cultures which are potential candidates for bioprotection of meat products. In this way, *L. sakei* L2512, which was demonstrated to protect sliced cooked ham from *Listeria* by producing sakacin G (Hequet *et al.* 2007), could be a particularly efficient strain to prevent meat contaminations by spoilage and pathogenic bacteria.

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