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Anti-listerial potential of food-borne yeasts in red smear cheese

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ABSTRACT

Screening of 175 yeasts in an agar plate co-cultivation assay revealed that five out of 31 species reduced *Listeria monocytogenes* by 4–5 log units, one exceptionally active *Pichia norvegensis* reduced *Listeria* by 7 log units. To test the anti-listerial activity of this *Pichia* strain on cheese, Tilsit cheese and smeared acid curd cheese (Harzer) were prepared. The Tilsit cheese surface was inoculated with a 3%-NaCl brine containing *Brevibacterium linens*, *Microbacterium gubbeenense*, *Corynebacterium casei*, *Staphylococcus equorum*, *Debaryomyces hansenii*, *P. norvegensis* and *L. monocytogenes*. Ripening was done at 13 °C and >95% relative humidity. On the Tilsit, but not on the Harzer cheeses, a decrease of listerial cell numbers by 1–2 log units was observed. The difference between high inhibition in agar plate co-cultivation versus cheese is probably due to a decreased expression of the unknown inhibitory substance due to lactate, but not by the low pH.

1. Introduction

The risk of contamination by *Listeria monocytogenes* is associated with a wide variety of food (Farber & Peterkin, 1991). Adequate methods are needed to protect consumers from *Listeria* contamination. Since food production without any chemical preservatives is more and more preferred, biological preservation by, e.g., protective cultures has been of substantial interest (Cleveland, Montville, Nes, & Chikindas, 2001; Holzapfel, Geisen, & Schillinger, 1995; Leistner & Gorris, 1995). In many cases lactic acid bacteria (LAB) are used as protective cultures since they have been traditionally involved in many food fermentations, such as dairy, meat, vegetables and bakery products (Abee, Krockel, & Hill, 1995; Bredholt, Nesbakken, & Holck, 2001; Loessner, Guenther, Steffan, & Scherer, 2003). Also, an inhibitory action of coryneform bacteria and staphylococci found in the complex surface microbial ripening consortia of red smear cheeses towards *L. monocytogenes* has been reported (Carnio, Eppert, & Scherer, 1999; Eppert, Valdes-Stauber, Götz, Busse, & Scherer, 1997; Valdés-Stauber, Götz, & Busse, 1991). This is important because cheeses are often contaminated with *Listeria* spp., in particular with the pathogenic species *L. monocytogenes* (McLauchlin, Mitchell, Smerdon, & Jewell, 2004; Ryser & Marth, 1991).

Listeria cell counts on contaminated cheeses commonly range between 10 and 10^5 cfu cm^{-2} (Rudolf & Scherer, 2001). Studies of Terplan, Schoen, Springmeyer, Degle, and Becker (1986) indicated a higher contamination frequency in red smear cheeses compared with other dairy products. The growth of smear-ripening cultures on the cheese surface rises the pH in the cheese rind rapidly which causes ideal conditions for listerial multiplication (Krämer, 1992). Because of the pH gradient, *Listeria* are found to be especially localized on the cheese surface (Farber & Peterkin, 1991).

Growth of coryneform bacteria is dependent on the deacidification of the cheese surface by yeast populations (Brennan, Cogan, Loessner, & Scherer, 2004; Eliskases-Lechner & Ginzinger, 1995; Prillinger, Molnar, Eliskases-Lechner, & Lopandic, 1999). Thus, secretion and inhibitory effect of bacterial anti-listerial substances is also delayed at the beginning of the cheese ripening process. *L. monocytogenes*, on the other hand, is able to survive in low pH environments and to even grow at pH values of around 5.0 (Farber & Peterkin, 1991). Hence, listerial growth on the cheese surface is only repressed after a certain ripening time. Due to their acid tolerance, yeasts dominate the microflora of smeared cheeses during the early stages of ripening (Eliskases-Lechner & Ginzinger, 1995). Therefore, yeasts expressing an anti-listerial activity could be effective natural antagonists of *L. monocytogenes* on cheese.

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There is only one report about anti-listerial potential of yeasts (Goerges, Aigner, Silakowski, & Scherer, 2006). The aim of the present study was to collect information on anti-listerial yeasts to assess the potential within these microorganisms to serve as protective cultures. For this purpose, 175 yeasts were screened for their anti-listerial activity using a co-cultivation assay. The most interesting yeast, a *Pichia norvegensis* strain, was then included in a ripening culture and was tested in artificially contaminated smear cheese model systems. Its suitability as protective culture against *L. monocytogenes* on cheese is discussed.

2. Materials and methods

2.1. Microbial strains

A total of 175 yeasts (Table 1) were selected from various sources, with a focus on yeast species relevant for the production of smeared cheeses. For details see Supplementary data (Table S1). Two *L. monocytogenes* indicator strains were used. Strain WSLC 1685, commonly known as Scott A (Fleming et al., 1985), was used in co-cultivation assays at cheese ripening temperature as well as in the presence of cheese ripening bacteria. Strain WSLC 1364, a representative from the red smear cheese Vacherin Mont d'Or outbreak in 1987 (Bille, 1989), was used in cheese ripening experiments and in all other experiments. The following cheese ripening bacteria and one additional yeast strain have been used: *Arthrobacter arilaitensis* WS 4549, *Brevibacterium aurantiacum* WS 4550, *Brevibacterium linens* WS 4574, *Debaryomyces hansenii* WSYC 772, *Corynebacterium casei* WS 4552, *Microbacterium gubbeenense* WS 4553 and *Staphylococcus equorum* WS 4554. All microbial strains used are deposited in the Weihenstephan culture collection.

Table 1
Overview of the *L. monocytogenes* WSLC[a] 1364 reduction of the screened yeast strains.

	No. of isolates	Listerial reduction ($\log cm^{-2}$)								
		n.d.[b]	0	1	2	3	4	5	6	7
Candida catenulata	2	0	0	2	0	0	0	0	0	0
Candida colliculosa	1	0	0	0	0	1	0	0	0	0
Candida glabrata	1	0	0	1	0	0	0	0	0	0
Candida lipolytica	2	0	0	2	0	0	0	0	0	0
Candida parapsilosis	3	0	0	3	0	0	0	0	0	0
Candida rugosa	1	0	0	1	0	0	0	0	0	0
Candida sake	1	0	0	0	1	0	0	0	0	0
Candida sp.	1	0	0	1	0	0	0	0	0	0
Candida tenuis	2	0	0	2	0	0	0	0	0	0
Candida zeylanoides	1	0	0	1	0	0	0	0	0	0
Clavispora lusitaniae	4	0	0	2	1	1	0	0	0	0
Debaryomyces hansenii/ *Candida famata*	77	0	2	71	3	1	0	0	0	0
Galactomyces geotrichum/ *Geotrichum candidum*	7	2	0	4	1	0	0	0	0	0
Issatchenkia orientalis/ *Candida krusei*	24	0	0	3	1	3	12	5	0	0
Kluyveromyces lactis	2			0	2	0	0	0	0	0
Kluyveromyces marxianus	18	0	0	2	5	6	5	0	0	0
Pichia anomala	1	0	0	0	1	0	0	0	0	0
Pichia cactophila	1	0	0	1	0	0	0	0	0	0
Pichia deserticola/ *Candida ethanolica*	2	0	0	1	0	1	0	0	0	0
Pichia fermentans	1	0	0	0	0	1	0	0	0	0
Pichia galeiformis	1	0	0	0	0	0	1	0	0	0
Pichia guilliermondii	1	0	0	1	0	0	0	0	0	0
Pichia norvegensis/ *Pichia cactophila*	1	0	0	1	0	0	0	0	0	0
Pichia norvegensis	1	0	0	0	0	0	0	0	0	1
Pichia sp.	1	0	0	1	0	0	0	0	0	0
Pichia triangularis	2	0	1	1	0	0	0	0	0	0
Saccharomyces cerevisiae	4	1	0	0	0	2	1	0	0	0
Torulaspora delbrueckii	3	0	0	0	1	1	1	0	0	0
Trichosporon asahii	1	0	0	1	0	0	0	0	0	0
Trichosporon ovoides	5	0	1	3	1	0	0	0	0	0
Yarrowia lipolytica	3	0	0	3	0	0	0	0	0	0
Number of strains	175	3	4	108	17	17	20	5	0	1
		3		146			25		1	
% of Strains	100	2		83			14		1	

[a] WSLC, Weihenstephan *Listeria* Collection.
[b] n.d., Not determined.

2.2. Co-cultivation assay on solid and in liquid medium

The co-cultivation of the yeast and *L. monocytogenes* strains on agar media were performed as described by Goerges et al. (2006). Briefly, a 100 μL aliquot of a diluted 17 h culture of the *L. monocytogenes* indicator strain was mixed with a yeast suspension prepared by centrifuging 300 μL of the yeast main culture at $2500 \times g$ for 5 min, discarding the supernatant, and re-suspending the pellet in 100 μL of ¼ Ringer's solution. The *Listeria*–yeast mixture was then spread onto yeast extract glucose agar, resulting in 10^5–10^6 yeast cells and ~6 *Listeria* cells per cm^2, respectively, and incubated for 24 h at 27 °C. Cell lawns from 3.5 cm^2 agar pieces were removed, serially diluted, and appropriate dilutions were plated in duplicate on yeast extract glucose chloramphenicol bromophenol blue (YGCB) agar or Oxford agar (Oxoid, Wesel, Germany) for determining yeast or *Listeria* counts.

Listeria titres from 6 cfu cm^{-2} up to a maximum of $\sim 6 \times 10^5$ cfu cm^{-2} were used for the assay to represent a wide range of realistic contamination rates on smear ripened cheese and to check for the yeasts' inhibition limit. pH values during co-cultivation were determined using a flat surface pH electrode. The absence of *L. monocytogenes* in co-cultivation experiments was tested by an enrichment procedure according to the European standard DIN EN ISO 11290-1:1996 + A1:2004 (EN ISO, 2005).

2.2.1. Co-cultivation at ripening temperature

To adapt the procedure to cheese ripening conditions the temperature was reduced to 14 °C. The co-cultivation using the yeast strains *P. norvegensis* WSYC 592 and *Pichia* sp. WSYC 623 was performed as described above. A *L. monocytogenes* WSLC 1685 inoculum of 10^6–10^7 cfu mL^{-1}, resulting in 10^5–10^6 cfu cm^{-2} after streaking on the agar plate, was used. The listerial cell counts were determined every day over a 6 day period.

2.2.2. Co-cultivation with sodium chloride

The influence of sodium chloride was tested in liquid medium. Both yeasts, WSYC 592 and 623, and the *Listeria* strain WSLC 1364 were grown and their inocula determined as described by Goerges et al. (2006). Five milliliters of a 24 h main liquid yeast culture was inoculated with an aliquot of a diluted 17 h culture of the *L. monocytogenes* indicator strain resulting in *Listeria* cell counts of 10^5–10^6 cfu mL^{-1} in the co-cultivation assay. The *Listeria*–yeast-liquid culture was then adjusted to a 3% final NaCl concentration (~500 mM) by adding an adequate aliquot of a 5 M filter-sterilized NaCl stock solution (NaCl from Fluka, Munich, Germany). The listerial inhibition was checked after 2, 6 and 24 h by plating appropriate dilutions in duplicate on Oxford agar (Sifin, Berlin, Germany).

2.2.3. Co-cultivation in the presence of cheese ripening bacteria

A yeast cell pellet obtained by centrifuging 300 μL of the yeast main culture was re-suspended with a 100 μL aliquot of a suspension containing 10^6–10^7 ripening bacterial cells per mL or 10^5–10^6 cells cm^{-2}, respectively, after streaking on agar plate. The bacterial suspension was prepared as follows: one loop (2 mm in diameter) high-piled with cells from a 3 to 4 days old ripening

bacterium culture grown on tryptic soy agar containing 0.1% glucose (TSG) [TSA (Carl Roth, Karlsruhe, Germany), 10.0 g D (+)-glucose (Fluka)] was inoculated in 5 mL of brain heart infusion (Merck, Darmstadt, Germany) and incubated at 30 °C for 24 h at 180 rpm using a shaker (CERTOMAT® MO, B. Braun Biotech International, Melsungen, Germany). Two milliliters of this culture were centrifuged at 2500 × g for 7 min, the supernatant discarded and the pellet re-suspended in 1 mL ¼ Ringer's solution. The cell counts used in the co-cultivation experiment were checked on TSG agar plates incubated at 30 °C for 3–4 days. According to the *L. monocytogenes* WSLC 1685 cell density desired on the agar plate used in the co-cultivation experiment (6×10^1 or 6×10^4 cfu cm^{-2}) a 100 μL aliquot of a diluted 17 h-*Listeria* culture was added to the yeast–ripening bacterium-mixture. Further steps followed the standard co-cultivation procedure described in Goerges et al. (2006).

2.2.4. Co-cultivation with lactate as carbon source

To cause more cheese like conditions for the co-cultivation experiments, the glucose content of the standard medium (2%; w/v) was replaced by 2% (w/v) of a 50% sodium lactate solution (Merck) which ensured growth for both organism groups comparable to that under standard conditions.

2.3. Cheese ripening experiments

Typical Tilsit green cheeses (pasteurised milk, ca. 4.5 kg, 12 × 12 × 40 cm, 45% fat in dry matter) were obtained from a local producer after cooling in tap water (10 °C, 2 h) before brining. Cheeses were transported to the lab in brine solution (prepared fresh with pasteurised tap water, >18% sodium chloride, pH 5.2, ca. 15 °C) and further incubated over 24 h. The brine was inoculated with *B. linens* WS 4574, isolated from Tilsit cheese, *M. gubbeenense* WS 4553, *C. casei* WS 4552 (10^4 cfu mL^{-1} each), *D. hansenii* WSYC 772 and *S. equorum* WS 4554 (10^6 cfu mL^{-1} each). Cultures were produced in modified plate count medium (Hoppe-Seyler, Jaeger, Bockelmann, & Heller, 2000), concentrated 10 fold by centrifugation, and stored at 4 °C before use (0.5% peptone, 0.5% casein hydrolysate, 0.5% glucose, 2% sodium chloride, pH 7).

For experiments, cheeses were cut into 3 pieces; the middle section was used for cell count determination after brining and then discarded; both end sections were placed on a plastic shelve, the ends facing up, protecting the cut surface from mould contamination. The smear solution was prepared by diluting the concentrated suspension with autoclaved 3% sodium chloride solution to give final concentrations for *D. hansenii* and *P. norvegensis* of 2×10^6 cfu mL^{-1} each and for bacteria of $5–10 \times 10^7$ cfu mL^{-1} each. The smear was applied with an autoclaved sponge, which was discarded after use. To avoid excessive ventilation the shelves with cheeses were placed in a 60 L glass container with a non-tight fitting lid (Bockelmann, Hoppe-Seyler, Jaeger, & Heller, 2000). The container was placed in heavily ventilated ripening chamber at 13 °C and >95% relative humidity. During the first week smearing was repeated twice, until the surface pH was above pH 7 measured with a flat surface electrode. Then a final smear was applied containing additional 5×10^6 cfu mL^{-1} *L. monocytogenes* WSLC 1364 resulting in a starting contamination on the cheese surface of 1.9×10^3 cfu cm^{-2}, which was several log units below the smear and yeast counts (total >10^8 cfu cm^{-2}). No further treatment was applied except sampling for surface cell count determination (Hoppe-Seyler et al., 2000).

Acid curd cheeses, e.g., Harzer cheese, are typical red smear cheeses produced in Germany, Austria and Czech Republic. They are produced from acid curd (synonym quarg), coagulated with a thermophilic starter culture without rennet. By pressing, the dry mass is set to >32%. After storage and transport in plastic bags, the acid curd is mixed with salts, small cheese discs are formed, and the surface is sprayed with smear cultures. Details about the microbiology of production and ripening were described by Bockelmann (2002).

For the experimental (contaminated) cheese batches several changes had to be applied. The presence of the yeast species *Kluyveromyces marxianus* and *Candida krusei* in the cheese milk and consequently the acid curd is essential for ripening. Replacement of *C. krusei* with the anti-listerial *P. norvegensis* WSYC 592, a species with a very similar metabolic profile (API 32C, BioMérieux, Nürtingen, Germany), in the cheese milk (batch 1) was not successful, because of untypical ripening of quarg and cheese. In batch 2, *P. norvegensis* was used (a) as third yeast inoculated into the cheese milk together with *K. marxianus* and *C. krusei* and (b) only for surface treatment. Since application of *P. norvegensis* via smearing leads to better smear development and cheese ripening (no mould contamination), these conditions were used for cheese batch 3. Smearing was not performed by spraying to avoid *Listeria* containing aerosols. Instead, cheeses were dipped into smear solution containing *B. linens* WS 4574, *C. casei* WS 4552 and *S. equorum* WS 4554 (ca. 10^7 cfu mL^{-1} each), *P. norvegensis* WSYC 592 (6×10^5 cfu mL^{-1}) and *L. monocytogenes* WSLC 1364 (3×10^3 cfu mL^{-1}). The initial concentrations of *P. norvegensis* and *L. monocytogenes* on the cheese surface were 5.6×10^5 cfu cm^{-2} and 42 cfu cm^{-2}, respectively.

To count cells, yeasts were plated on yeast extract glucose chloramphenicol (YGC) agar (Merck). Coryneforms and staphylococci were plated on modified milk agar (Hoppe-Seyler et al., 2000) which favours colour development and facilitates selective counting. When necessary (<1% of total bacterial counts), staphylococci were additionally plated on the selective SK agar (Merck). In addition, typical colonies were subjected to phase contrast microscopy.

3. Results and discussion

3.1. Screening of yeasts for anti-listerial potential by a co-cultivation assay

Within the screening, 175 yeasts were co-cultivated with 6 cfu cm^{-2} *L. monocytogenes* WSLC 1364 as an indicator strain. As shown in Table 1, a total of 83% of the screened yeasts had no or no significant anti-listerial potential. They were able to reduce listerial growth by a maximum of 3 log units cm^{-2} compared with the control in which *Listeria* grew up to 10^7 cfu cm^{-2} (data not shown). In most of these cases, *Listeria* cell counts were diminished by 1 log unit cm^{-2}, which is probably due to competition for nutrients. While 14% of the yeasts were able to inhibit *L. monocytogenes* by 4 and 5 log units (cfu cm^{-2}), only a single strain caused an extraordinary reduction of 7 log units (cfu cm^{-2}) compared with the *Listeria* control without any yeast present (Table 1). It is also known from smear cheese bacteria that only a minority of strains are efficient antagonists of *Listeria* (Carnio et al., 1999; Ryser, Maisnier-Patin, Gratadoux, & Richard, 1994; Valdés-Stauber et al., 1991).

In the present study 77 *D. hansenii* strains were tested since this is one of the most prevalent and frequently found yeasts on red smear cheese (Bockelmann & Hoppe-Seyler, 2001; Goerges et al., 2008; Mounier et al., 2006; Rea et al., 2007) and other cheese varieties (Addis, Fleet, Cox, Kolak, & Leung, 2001; Fadda, Mossa, Pisano, Deplano, & Cosentino, 2004; Pereira-Dias, Potes, Marinho, Malfeito-Ferreira, & Loureiro, 2000). Besides, *D. hansenii* is known to be a salt tolerant yeast (Petersen, Westall, & Jespersen, 2002; Suzuki, Yamada, Okada, & Nikkuni, 1989) that is found in cheese brines (Kammerlehner, 2003; Petersen & Jespersen, 2004; Seiler & Busse, 1990). Therefore, an anti-listerial *D. hansenii* strain could be a useful candidate for a wide field of applications. However, most

representatives of *D. hansenii* only caused a marginal reduction of *Listeria*. A single *D. hansenii* strain, isolated from a rinser located in a dairy, expressed a small anti-listerial potential with a 3 log units reduction (Table 1). While there exists a considerable number of *D. hansenii* strains producing killer toxins (Santos, Marquina, Barroso, & Peinado, 2002; Suzuki et al., 1989) which act towards the same or closely related yeast species (Magliani, Conti, Gerloni, Bertolotti, & Polonelli, 1997; Schmitt & Breinig, 2002), a significant anti-listerial activity was not found in our screening experiment.

At a first glance, a strong anti-listerial activity of yeasts seems to be restricted to certain species. A high number of *Issatchenkia orientalis*/*C. krusei* strains as well as *K. marxianus* isolates showed a remarkable effect on *L. monocytogenes*. Only one yeast, *P. norvegensis* WSYC 592, was able to eradicate *L. monocytogenes*, i.e., no listeriae could be recovered by enrichment. This *P. norvegensis* strain was isolated from a milk product. Initially it was identified as *C. krusei* by API 20C AUX (BioMérieux), however, after sequence analysis of the 26S rDNA gene and identification by FTIR spectroscopy (Buchl, Wenning, Seiler, Mietke-Hofmann, & Scherer, 2008) the identification had to be revised. *Pichia* species are common on various smeared and non smeared cheeses (Corsetti, Rossi, & Gobbetti, 2001; Laurencík et al., 2008; Prillinger et al., 1999; Rantsiou, Urso, Dolci, Comi, & Cocolin, 2008) although they are not among the most important species for cheese ripening (Corsetti et al., 2001). Some *P. norvegensis* strains were isolated from fermented milk products (Rohm, Eliskases-Lechner, & Bräuer, 1992). An isolate from Spanish blue-veined cheese was identified as *P. norvegensis*, though by phenotypic methods. Using molecular tools this yeast was assigned to *Pichia dubia* (Álvarez-Martín, Flórez, López-Díaz, & Mayo, 2007). Species of the genus *Pichia*, among them *P. norvegensis*, are known to be associated with LAB in sourdough (Fleet, 2007; Gobbetti, 1998). It is well-known that yeasts interact not only with surface ripening bacteria but also with LAB (Corsetti et al., 2001; De Freitas, Pinon, Maubois, Lortal, & Thierry, 2009; Ferreira & Viljoen, 2003).

3.2. Anti-listerial potential of P. norvegensis WSYC 592 in experimental cheese ripening

According to the co-cultivation experiments described above, the yeast strain *P. norvegensis* WSYC 592 expressing the strongest anti-listerial effect was considered to be a promising candidate to be applied on cheese surfaces and was therefore chosen for in situ experiments using two different cheese varieties, i.e., Tilsit and smeared acid curd cheese.

Previously, two experimental Tilsit cheese batches were separately produced with initial *L. monocytogenes* WSLC 1364 concentrations of 11 and 140 cfu cm^{-2}. Reduced listerial counts were observed over the ripening period of 2 weeks. However, there were no differences compared with control cheeses ripened without the anti-listerial *P. norvegensis* (data not shown). Ripening could not be extended because of problems with mould growth due to the difficult handling of cheeses during the ripening process (e.g., a typical brush could not be used for surface treatment of contaminated cheeses). The development of *L. monocytogenes* WSLC 1364 on Tilsit cheese with an inoculum of 1.9×10^3 cfu cm^{-2} was studied over a period of about 4 weeks. While *Listeria* in the control cheese without anti-listerial yeast (Fig. 1a) grew from initial 1.9×10^3 cfu cm^{-2} up to 6.3×10^6 cfu cm^{-2}, it reached cell numbers of 3.5×10^5 cfu cm^{-2} when cheeses were additionally smeared with *P. norvegensis* WSYC 592 (Fig. 1b). This corresponds to a listerial reduction of about 1.5 log units (cfu cm^{-2}).

Compared with the results obtained under controlled co-cultivation conditions (see above), the anti-listerial effect on Tilsit cheese was much lower than expected. Modification of the time point at which the cheeses were contaminated with *L. monocytogenes* did not improve the results (data not shown). The presence of *P. norvegensis* did not significantly influence any other ripening organisms. Both cheeses were of comparable appearance after 4 weeks of ripening. Eppert et al. (1997) also reported a listerial growth reduction of only 1–2 log units when a single-strain-culture of a linocin producing *B. linens* strain was applied to model cheese. From bacteriocin research it is known that their efficacy is often decreased in food matrices since there are various factors affecting the production or effectiveness of the anti-microbial substance (Gálvez, Abriouel, López, & Omar, 2007; Schillinger, Geisen, & Holzapfel, 1996).

Within ripening experiments on acid curd cheese, the initial *L. monocytogenes* WSLC 1364 concentration of batch 2 was at 4.4×10^3 cfu cm^{-2}. During 2 weeks, *L. monocytogenes* grew to 2.7×10^8 cfu cm^{-2} in spite of the presence of *P. norvegensis* (2×10^6 cfu cm^{-2}). Batch 3 was performed with more realistic listerial concentrations of 42 cfu cm^{-2}. After 2 weeks of ripening, cheeses produced with anti-listerial *P. norvegensis* showed only marginal inhibition of *Listeria*: 7.0×10^6 cfu cm^{-2} versus 1.2×10^7 cfu cm^{-2} in control cheeses without *P. norvegensis*. Since no clear inhibition of *L. monocytogenes* by *P. norvegensis* was observed no further cheese trials were performed. In contrast to Tilsit cheese, ripening of acid curd cheese was slightly decelerated when *P. norvegensis* was used as addition in the smear culture.

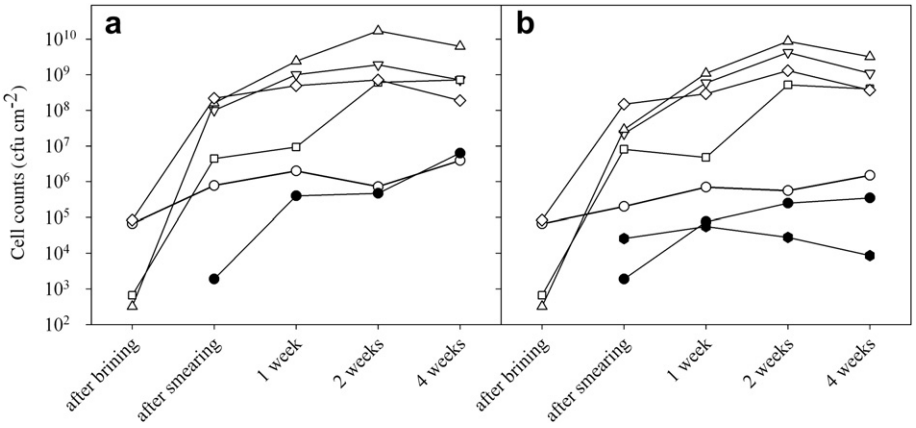

Fig. 1. Development of the surface ripening microorganisms of experimental Tilsit cheeses. *Debaryomyces hansenii* (○), *Staphylococcus equorum* (◇), *Corynebacterium casei* (△), *Microbacterium gubbeenense* (□) and *Brevibacterium linens* (▽) were used for brining and smearing of cheeses, artificially contaminated with *Listeria monocytogenes* WSLC 1364 (●); (a) control cheeses, (b) cheese ripened in the presence of anti-listerial *Pichia norvegensis* (⬢).

3.3. Potential causes for a reduced anti-listerial activity of P. norvegensis *WSYC 592 on smeared cheese*

Co-cultivation experiments on solid medium with different *L. monocytogenes* WSLC 1364 and yeast cell numbers (data not shown) showed that a yeast inoculum of 10^5 cfu cm^{-2} is necessary to guarantee an effective reduction of *Listeria* titres commonly found on smear ripened cheese (Rudolf & Scherer, 2001). The reduced anti-listerial activity may in part be due to the fact that the anti-listerial yeast was not able to increase its initial cell counts over the ripening period in Tilsit cheese (Fig. 1b). However, in studies on acid curd cheese *L. monocytogenes* WSLC 1364 grew up to 10^6 cfu cm^{-2} although the required yeast counts were reached. Therefore, this may not be the main reason for the failure of anti-listerial yeast to inhibit *Listeria*. To find other potential causes for the reduced anti-listerial potential of *P. norvegensis* WSYC 592 in a cheese model system, a set of parameters which might have an influence were studied.

3.3.1. Influence of the ripening temperature

The temperature of the co-cultivation assay was decreased from initially 27 °C, which is an ideal temperature for the cultivation of yeast, to 14 °C, a commonly used ripening temperature for smear ripened cheese. It is known that the temperature might have a significant influence on the bacteriocin production and/or their efficacy (Allende et al., 2007; Anthony, Rajesh, Kayalvizhi, & Gunasekaran, 2009; Gálvez et al., 2007; Krier, Revol-Junelles, & Germain, 1998). Kinetics of listerial and yeast cell counts were determined over a 6 days period (Fig. 2). At day 3, the *Listeria* numbers of three independently performed experiments ranged between 4×10^5 cfu cm^{-2} and zero. Nevertheless, all experiments resulted in the complete killing of the *L. monocytogenes* WSLC 1685 strain from day 4 on (Fig. 2). McAuliffe, Hill, and Ross (1999) reported that a Lacticin producing *Lactococcus lactis* strain used as a protective culture against *L. monocytogenes* in cottage cheese reduced 10^4 cfu of *L. monocytogenes* Scott A per gram to zero. We conclude that in our experiments temperature is not a critical factor for the reduction of *Listeria* on cheese.

3.3.2. Influence of sodium chloride

A liquid co-culture assay was performed to test whether NaCl has an influence on the anti-listerial properties of *P. norvegensis* WSYC 592 against *L. monocytogenes* WSLC 1364. Using a final concentration of 3% NaCl, which is a salt concentration in accordance with those applied on cheese surfaces (Brennan et al., 2004; Feurer, Vallaeys, Corrieu, & Irlinger, 2004; Mounier et al., 2006; Rea et al., 2007; Valdés-Stauber, Scherer, & Seiler, 1997) or in smear water (Bockelmann, 2002), did not result in a decrease of the inhibitory activity of the yeasts.

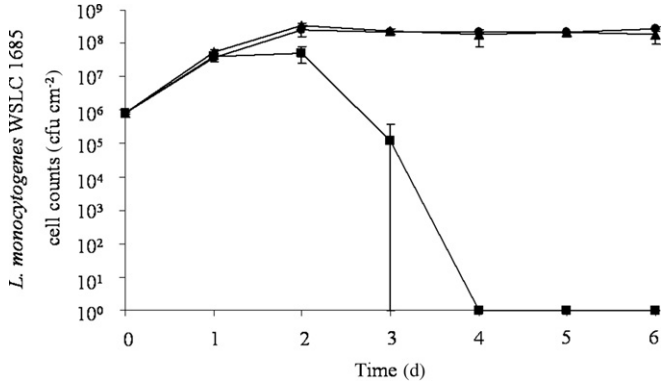

Fig. 2. Inhibition of *Listeria monocytogenes* WSLC 1685 by the yeast strain *Pichia norvegensis* WSYC 592 at 14 °C during a co-cultivation assay on solid medium (■). *Pichia* sp. WSYC 623 (▲) showed no inhibition. As a control *L. monocytogenes* was cultivated without yeasts (●). Inoculation level: ca. 10^5–10^6 cfu *L. monocytogenes* cm^{-2}. Cell counts are given as mean values of two to three independent experiments with range error bars.

3.3.3. Influence of cheese ripening bacteria

The *P. norvegensis* strain added to the ripening cultures of acid curd cheese caused a delay in ripening whereas no significant influences were observed for its application in Tilsit cheese. It is known that complex interactions exist between yeast and bacteria in cheese microbial consortia which are, however, not completely understood (Corsetti et al., 2001; Mounier et al., 2008). To clarify whether ripening bacteria present on cheese surfaces might be responsible for the decreased potential of the *P. norvegensis* strain WSYC 592 to inhibit *L. monocytogenes* WSLC 1685, a selection of ripening bacteria (see above) were included in the co-cultivation experiments. While the coryneform bacteria did not influence the yeast's anti-listerial activity, the *S. equorum* strain partially abolished the anti-listerial effect (Table 2) so that the indicator strain was kept at about its inoculation level of 7×10^4 cfu cm^{-2}. In both controls *Listeria* grew up to 10^8 cfu cm^{-2}. The reason for the interference by the *S. equorum* is unknown and was not further studied. However, this has to be taken into account when application of *P. norvegensis* is considered, since *S. equorum* constitutes an essential component of red smear cultures of several surface-ripened cheeses (Bockelmann, Willems, Neve, & Heller, 2005).

3.3.4. Influence of the carbon source

In the co-cultivation assays, glucose was used as a carbon source. While a substitution of glucose by sodium lactate did not impair growth of *Pichia* or *Listeria*, the anti-listerial effect of the *P. norvegensis* strain WSYC 592 was strongly inhibited. Only a reduction of *L. monocytogenes* WSLC 1364 by 1 log unit (cfu cm^{-2}) compared with the control was observed (Fig. 3). We noticed that, during the 24 h co-cultivation which started with an initial pH of 6.6, the pH decreased to 4.6 in the presence of glucose while it increased to a pH value above 8 when lactate was the carbon source (data not shown). However, an inhibition of *L. monocytogenes* induced by low pH can be excluded since the pH was similar after the co-cultivation in the presence of the inhibitory *P. norvegensis* WSYC 592 and the non-inhibitory control yeast, *Pichia* sp. WSYC 623, showing pH values of 4.58 ± 0.10 and 4.76 ± 0.13, respectively. Also, *Listeria* can grow at moderately low pH. It is known from bacteriocin research that even slight changes of environmental

Table 2
Influence of cheese ripening bacteria on the inhibition of *L. monocytogenes* WSLC[a] 1685 by *Pichia norvegensis* WSYC[b] 592.

Cheese ripening bacteria strains	Listerial cell counts (cfu cm^{-2}) ± SD[c] after 24 h of co-cultivation, in the presence of cheese ripening bacteria	
	Without *P. norvegensis*	In the presence of *P. norvegensis* WSYC 592
Arthrobacter arilaitensis	$3 \pm 1.7 \times 10^8$	0[d]
Brevibacterium aurantiacum	$2 \pm 0.4 \times 10^9$	0
Brevibacterium linens	$2 \pm 0.05 \times 10^9$	0
Corynebacterium casei	$3 \pm 1.8 \times 10^8$	0
Microbacterium gubbeenense	$4 \pm 1.2 \times 10^8$	0
Staphylococcus equorum	$8 \pm 1.2 \times 10^7$	$2 \pm 1.7 \times 10^5$

[a] WSLC, Weihenstephan *Listeria* Collection.
[b] WSYC, Weihenstephan Yeast Collection.
[c] Cell counts are given as mean values ± standard deviation of two independent experiments.
[d] Enrichment for *L. monocytogenes* WSLC 1685 was positive in one out of two experiments.

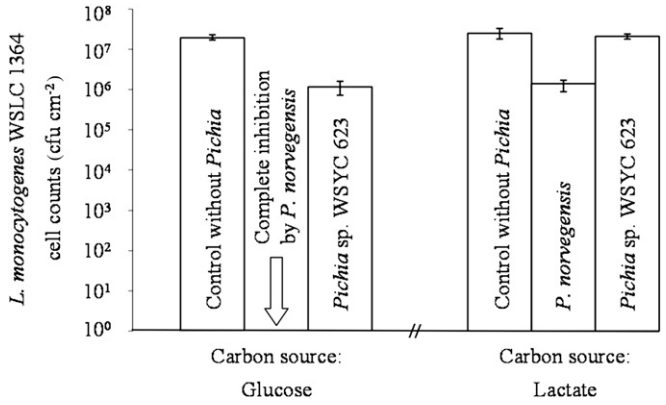

Fig. 3. Influence of glucose (2%, w/v) and lactate (2%, w/v) on the inhibition of *Listeria monocytogenes* WSLC 1364 by the yeast *Pichia norvegensis* WSYC 592 and *Pichia* sp. WSYC 623. As a control, *L. monocytogenes* was grown without yeasts. *L. monocytogenes* inoculation level was 7 cfu cm^{-2}. Cell counts are given as mean values of two to three independent experiments with range error bars.

conditions may influence the production of anti-microbial substances (Delgado et al., 2007; Leal-Sánchez, Jimenez-Diaz, Maldonado-Barragan, Garrido-Fernandez, & Ruiz-Barba, 2002; Mataragas, Drosinos, Tsakalidou, & Metaxopoulos, 2004). Delgado et al. (2007) reported on a slight decrease of bacteriocin production by lactate. We suggest that either lactate or glucose act on the regulation governing the expression of the unknown inhibitory substance.

4. Conclusion

According to the co-cultivation experiments used as cheese model system a minority of yeasts show an excellent capability to kill *L. monocytogenes*. The application of the most potent yeast *P. norvegensis* WSYC 592, which inhibited *L. monocytogenes* strongly in a co-cultivation assay on agar plates, as a part of a defined microbial red smear cheese ripening consortium did not result in a satisfactory inhibition of *Listeria* in artificially contaminated smear cheeses. Probably, this was due to a down regulation of the expression of the inhibitory substance mediated by lactate or an up-regulation mediated by glucose. Since next to nothing is known about such inhibitory effects produced by yeasts, the unknown molecular basis of the inhibitory effect must be elucidated to optimize application in complex food ecosystems.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.idairyj.2010.08.002.

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