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Antifungal effect of dairy propionibacteria—contribution of organic acids

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Abstract

Large amounts of food and feed are lost every year due to spoilage by moulds and yeasts. Biopreservation, i.e. the use of microorganisms as preservatives instead of chemicals, has gained increased interest. Lactic acid bacteria and propionibacteria might be particularly useful due to their important role in many food fermentations. Knowledge of the antifungal effects of the organic acids produced by these bacteria is necessary to understand their inhibitory activity. We evaluated the antifungal activity of the type strains of five dairy propionibacteria, *Propionibacterium acidipropionici*, *P. jensenii*, *P. thoenii*, *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* against eight food- and feedborne moulds and yeasts. A dual culture system assayed the inhibitory activity on three different agar media, sodium lactate (SL), de Man Rogosa Sharp (MRS) and MRS without acetate (MRS-ac). The amounts of organic acids produced during growth of propionibacteria in liquid SL, MRS and MRS-ac were also determined. The minimal inhibitory concentration (MIC) values of propionic, acetic and lactic acid were established for all fungi at pH 3, 5 and 7. Propionic acid, followed by acetic acid, was the most potent antifungal acid. Inhibition at pH 7 generally required concentrations above 500 mM for all three acids, at pH 5 the MIC values for propionic and acetic acids were 20–120 mM and above 500 mM for lactic acid. At pH 3, the MIC values were, with one exception, below 10 mM for both propionic and acetic acid and above 160 mM for lactic acid. The yeast *Pichia anomala* was the fungus most resistant to organic acids. The propionibacteria exhibited a pronounced species variation in antifungal activity on MRS (±acetate) agar, with *P. thoenii* being the most potent. Four of the five propionibacteria species produced more propionic and acetic acid in liquid SL medium than in MRS (±acetate) broth. However, when SL agar was used as the growth medium, none of the propionibacteria inhibited fungal growth.

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1. Introduction

Consumer demands for natural preservatives have caused an interest in using the preserving capacity of bacteria naturally occurring in food and feeds. Lactic

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acid bacteria, used in dairy products, sausages, beverages and silage, have preserving features, not only related to the production of lactic and acetic acid. We have previously identified antifungal activity from a large number of lactic acid bacteria (Magnusson et al., 2003) and isolated several compounds with antifungal activity, e.g. proteinaceous compounds (Magnusson and Schnürer, 2001), phenyllactic acid and cyclic dipeptides (Ström et al., 2002) and hydroxylated fatty acids (Sjögren et al., 2003). Merry and Davies (1999) have earlier suggested that propionibacteria can be used to prevent aerobic spoilage of silage, i.e. growth of moulds and yeasts.

In the present study, the antifungal effects of the five most common dairy propionibacteria *Propionibacterium acidipropionici*, *P. jensenii*, *P. thoenii*, *P. freudenreichii* subspecies *freudenreichii* and *P. freudenreichii* subspecies *shermanii* were investigated. Propionibacteria gain energy through fermentation of lactate and sugars to propionate, acetate and carbon dioxide (Piveteau, 1999). Cabo et al. (2002) recently suggested that antifungal effects observed when certain lactic acid bacteria are grown on MRS is due to a synergistic effect of the lactate formed and the 35 mM acetate from the substrate. We have assayed the antifungal effect of propionibacteria grown on MRS, both with and without acetate, as well as on sodium lactate (SL) agar. Previous publications have revealed the antimicrobial activity of lactic and acetic acid against enterobacteria and *Listeria* (Östling and Lindgren, 1993), clostridia (Jonsson, 1989) and *Saccharomyces cerevisiae* (Narendranath et al., 2001b) and the effect of propionic acid against *Aspergillus*, *Fusarium* and *Penicillium* species (Higgins and Brinkhaus, 1999; Paster et al., 1999; Razavi-Rohani and Griffiths, 1999; Rusul et al., 1987). Inhibition of yeasts by lactate, acetate and propionate and their mixtures was investigated by Moon (1983). To be able to use these data as background information during search for new antifungal compounds, a determination of MIC values for our specific target organisms at relevant pH is necessary. The fungal species were selected among relevant food and feed spoilage organisms, and the pH levels for the MIC assay were held at a range representative of food and feed environments. The two objectives of this study were (1) to establish a compilation of MIC values and (2) to determine the antifungal effects of propionibacteria grown on different substrates.

2. Materials and methods

2.1. Microorganisms

The propionibacteria used in this study were the type strains of each of the dairy propionibacteria species, obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen): *P. acidipropionici* DSMZ 4900, *P. freudenreichii* subsp. *shermanii* DSMZ 4902, *P. freudenreichii* subsp. *freudenreichii* DSMZ 20271, *P. thoenii* DSMZ 20276 and *P. jensenii* DSMZ 20535. The fungi used in the overlay assay and the MIC determinations of organic acids were the yeasts *Pichia anomala* J121, *Rhodotorula mucilaginosa* J350 (CFSQE 63) and *Kluyveromyces marxianus* J367 (CBS 1555) and the moulds *Aspergillus fumigatus* J9, *Aspergillus nidulans* J283 (FSGC A4 wt), *Penicillium commune* J238 (IBT 12400), *Penicillium roqueforti* J268 (IBT 6754), J229 (IBT 3877), J231 (IBT 6366), J282 (SVA 2815/96), J284 (J5A SVA A432/88) and *Fusarium sporotrichioides* J304 (ITEM 168). All fungi are kept in the culture collection of the Department of Microbiology, Swedish University of Agricultural Sciences.

2.2. Media and growth conditions

Cultures of propionibacteria were grown on modified SL medium (1% sodium lactate, 1% tryptone, 0.5% yeast extract and 0.5% KH_2PO_4) for up to 10 days at 30 °C in anaerobic jars (GasPak System, Becton, Dickinson and Sparks, USA) under CO_2+N_2 atmosphere (GasPak Plus, Becton, Dickinson and Sparks). Yeasts were cultured in malt extract (ME) broth (Difco Laboratories, Detroit, USA) at 25 °C over night and moulds were cultured on malt extract agar (MEA) slants (Oxoid, Basingstoke, England) at 25 °C for 3 days. For the overlay assay (see below), three different media were evaluated: SL, de Man Rogosa Sharp (MRS) (Oxoid) and MRS without acetate (MRS-ac) (10 g/l peptone, 8 g/l "Lab-Lemco" powder, 4 g/l yeast extract, 20 g/l

glucose, 2 g/l K_2HPO_4, 2 g/l $C_6H_8O_7 \times 2NH_3$, 0.2 g/l $MgSO_4 \times 7H_2O$ and 0.05 g/l $MnSO_4 \times 4H_2O$). The bacteria were inoculated on each plate and incubated for 3 days at 30 °C under anaerobic conditions (see above). Then soft agar (0.05% malt extract (Difco Laboratories) and 1% agar base (Oxoid)) containing fungal cells or spores/conidia were overlaid on the bacterial plates, which were further incubated at 30 °C for 2 or 3 days. For metabolite production analysis, broths of the same three media (SL, MRS and MRS-ac) as in the overlay assay were used. Ten milliliters of each medium was inoculated with each of the strains of propionibacteria and incubated as still cultures at 30 °C for 3 days before sampling.

2.3. Antifungal overlay assay

The antifungal activity of the propionibacteria on different media was investigated with an overlay assay. On each agar plate of SL, MRS and MRS-ac, two 2-cm lines of the propionibacteria were inoculated. Plates were then incubated at 30 °C under anaerobic conditions for 3 days. Ten milliliters of 45 °C soft agar, containing 10^4 yeast cells or mould spores/conidia per milliliter, was then poured onto the agar plates and incubated under aerobic conditions at 30 °C for 2 or 3 days (depending on fungal growth rate). The degree of inhibition was measured as the area of inhibited growth in relation to the total area of the petri dish and the scale was the following: −=no visible inhibition, (+)=weak inhibition in the soft agar above the bacterial growth, +=inhibition area per bacterial line 0.1–3.0%, ++=inhibition area per bacterial line 3.0–8.0% or +++=inhibition area per bacterial line >8.0% (Magnusson and Schnürer, 2001).

2.4. Metabolite production analysis

Broth cultures of SL, MRS and MRS-ac were inoculated with propionibacteria at 10^7 bacteria/ml (counted with a Petroff-Hausser cell counter (Hausser Scientific Partnership, Horsham, UK)). The cultures were incubated at 30 °C for 3 days before harvest. Cell-free supernatants were obtained by centrifugation (4500 rpm, 10 min), followed by filtration through a 0.45-μm filter. HPLC analysis of all supernatants, including samples of sterile medium, was performed on a cation exclusion column (HC-75, 7.8×305 mm, Hamilton, Reno, USA) at 60 °C. As mobile phase 5 mM H_2SO_4, at a flow rate of 0.6 ml/min, was used and the eluate was monitored with a refractive index detector (Agilent 1100 Series, Agilent Technologies, Germany) at 40 °C. Standard solutions of glucose, lactate, acetate and propionate at 10, 25, 50, 75 and 100 mM were included in the analysis. Samples showing concentrations above 100 mM for any substance were diluted twofold and reanalysed.

Each sample was also analysed for bacterial viable count after the 3 days of incubation. An aliquot was diluted in peptone water and spread on an SL agar plate to determine bacterial viable counts. After 10 days of incubation under anaerobic conditions at 30 °C, the colonies on the viable count plates were counted.

2.5. Determination of MIC values

The MIC values of propionic, acetic and lactic acid for a number of target fungi were determined with a microtitre plate assay. Solutions of each acid at pH 3, 5 and 7 (adjusted with NaOH and HCl) were prepared. Dilutions of the acids, with final concentrations of 0, 10, 50, 100, 200 and 500 mM, were then distributed in a 96-well microtitre plate together with ME broth and yeast cells or mould spores/conidia at a final concentration of 5×10^4/ml. The MIC was defined as the lowest concentration where no growth could be observed in the well. When the MIC results were between 0 and 500 mM, further dilutions were done. All solutions and media used in the assay were sterile and pH adjusted with NaOH or HCl. The plates were incubated at 30 °C in plastic bags, supplemented with a moist paper tissue to maintain humidity, and monitored at 630 nm in a microtitre plate reader (Microplate Autoreader, Bio-Tek Instruments, Winooski, USA) every second day for up to 10 days.

2.6. Proportion undissociated acid and pH of solutions of organic acids

The following information is given as background information to aid the interpretation of observed antifungal effects. For pH 3, 4, 5, 6 and 7, respectively, the proportions of undissociated acid

were calculated from the Henderson-Hasselbach equation $(\mathrm{p}H = \mathrm{p}K + \log([\mathrm{A}^-]/[\mathrm{HA}]))$:

Propionic acid (pK_a 4.87)	99%, 88%, 43%, 6.9%, 0.74%
Acetic acid (pK_a 4.76)	98%, 85%, 37%, 5.4%, 0.57%
Lactic acid (pK_a 3.86)	88%, 42%, 6.8%, 0.72%, 0.07%

The pH values of the three organic acids in water were determined at concentrations reflecting those of the growth substrates and the observed production by propionibacteria:

	10 mM	50 mM	100 mM
Propionic acid	3.2	2.9	2.8
Acetic acid	3.2	2.9	2.8
Lactic acid	2.8	2.4	2.3

3. Results

3.1. Influence of the media on fungal inhibition

The antifungal activity of the propionibacteria was analysed with the overlay assay on SL, MRS and MRS-ac, and the results are summarised in Table 1. Generally, *Propionibacterium thoenii* had the most pronounced antifungal effect. However, the yeast *Kluyveromyces marxianus* was only slightly inhibited on MRS and not at all on SL or MRS-ac and was thus not further investigated (n.d., Table 1). When grown on MRS agar, the different propionibacteria showed large variations in the degree of fungal inhibition. *P. thoenii* had a "++"-inhibitory effect on *A. fumigatus*, while *P. acidipropionici*, *P. freudenreichii* subsp. *shermanii* and *P. jensenii*, displayed ++ and *P. freudenreichii* subsp. *freudenreichii* only inhibited growth of *A. fumigatus* to the "+"-level (Fig. 1). Similar results were obtained when *P. roqueforti* was used as target organism. Inhibition assays on the other two media did not give the same result. When the assay was performed on MRS-ac, the inhibition pattern mostly followed the pattern from MRS, but both stronger and weaker inhibition could be seen (Fig. 1). On SL agar the inhibition, if at all present, was only visible as a thinner layer of fungal growth on top of the bacterial lines, corresponding to (+). Fig. 2 shows the results from four indicator fungi, assayed with *P. thoenii* on all three media. The two yeast species tested were weakly inhibited on MRS (±acetate), but not at all on SL agar. *P. roqueforti* was equally inhibited on MRS and MRS-ac, but *A. fumigatus* was more strongly inhibited on MRS, as also shown in Fig. 1. The pH modulates the inhibiting effect of

Table 1
Inhibitory effects of the five propionibacteria against four indicator fungi in the overlay assay with three different substrates (SL, MRS and MRS-ac)

Inhibiting bacteria	Medium	Indicator fungus			
		R. mucilaginosa	*K. marxianus*	*P. roqueforti*	*A. fumigatus*
P. acidipropionici	SL	–	n.d.[a]	–	(+)
	MRS	+	n.d.	++	++
	MRS-ac	+	n.d.	++	++
P. freudenreichii shermanii	SL	–	n.d.	–	–
	MRS	+	n.d.	+	++
	MRS-ac	+	n.d.	+	+
P. freudenreichii freudenreichii	SL	–	n.d.	–	–
	MRS	(+)	n.d.	+	+
	MRS-ac	(+)	n.d.	+	+
P. thoenii	SL	–	–	–	(+)
	MRS	++	(+)	++	+++
	MRS-ac	+	–	++	+
P. jensenii	SL	–	n.d.	–	–
	MRS	+	n.d.	+	++
	MRS-ac	+	n.d.	+	+++

[a] n.d.: not determined.

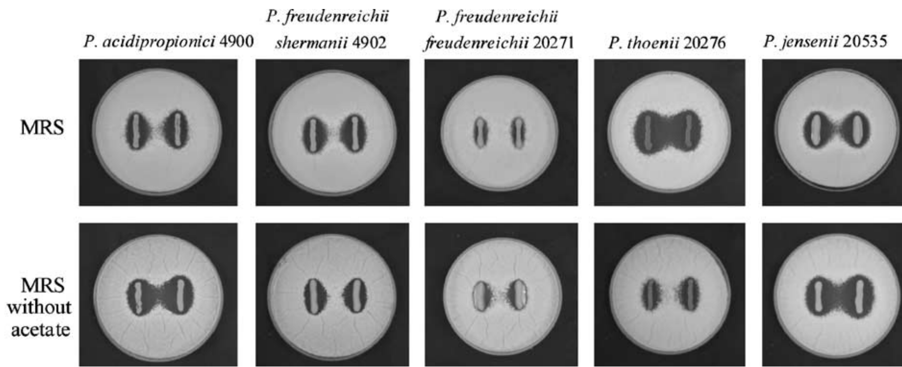

Fig. 1. Overlay assay with *P. acidipropionici*, *P. freudenreichii* subsp. *shermanii*, *P. freudenreichii* subsp. *freudenreichii*, *P. thoenii* and *P. jensenii* on agar of MRS and MRS-ac and with *A. fumigatus* as target fungus.

organic acids and a comparison of pH values in the different media at different stages of bacterial and fungal growth was done (Table 2). Before the inoculation, the three media had fairly similar pH (5.6–6.1), but after growth of propionibacteria the pH in SL was higher, suggesting a stronger buffering capacity (Table 2). After growth of *A. fumigatus* in the overlaid soft agar for 3 days, the pH in the inhibited zone varied considerably, from pH 4.6 up to pH 8.0.

3.2. Metabolite production

The five species of propionibacteria were cultured for 3 days in broth of the three different media to investigate substrate effects on the production of organic acids (Table 3). In all but one case, the propionibacteria multiplied strongly in all three media. In MRS and MRS-ac, the decrease in glucose could be related to the increase in acetate and propionate, while in SL medium the lactate played

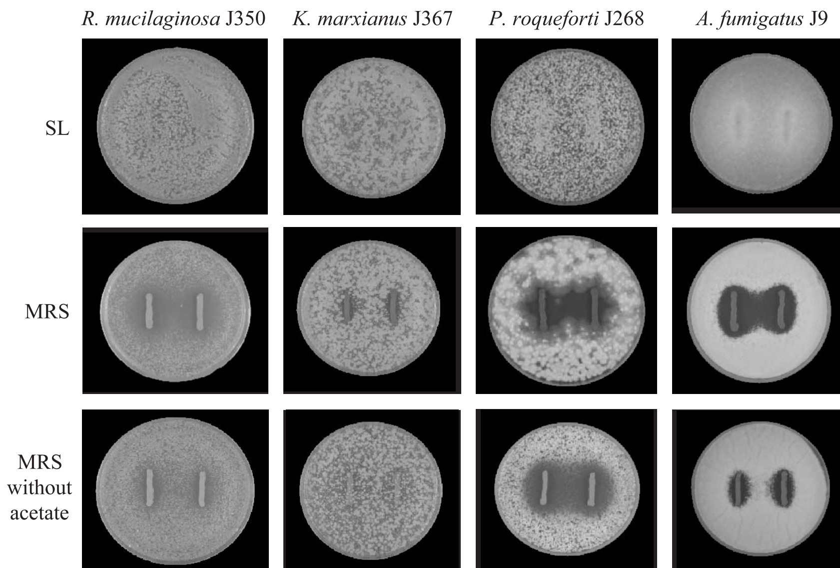

Fig. 2. Inhibitory effects of *P. thoeni* against the yeasts *R. mucilaginosa* and *K. marxianus* and the moulds *A. fumigatus* and *P. roqueforti*, in an overlay assay with three different agar substrates (SL, MRS and MRS-ac).

Table 2
Effects of propionibacteria and soft agar overlay with *A. fumigatus* on the pH of agar of SL, MRS and MRS-ac

	Medium		
	SL	MRS	MRS-ac
Uninoculated	5.6	6.1	6.1
Growth of *P. acidipropionici*[a]	6.0	4.8	4.6
Growth of *P. thoenii*[a]	6.0	4.7	4.6
P. acidipropionici overlaid with *A. fumigatus*[b]	n.d.	8.0	5.3
P. thoenii overlaid with *A. fumigatus*[b]	n.d.	5.5	7.1

The pH was measured in the inhibition zone of the overlaid soft agar, which itself had pH 5.5.
n.d.: not determined on SL agar because the fungus overgrew the plates, leaving no inhibition zone where the pH could be measured.
[a] The bacteria were inoculated as in the overlay assay and incubated anaerobically for 3 days before sterile soft agar was poured on top and the plates were incubated further 3 days before the pH was measured.
[b] The bacteria were inoculated as in the overlay assay and incubated anaerobically for 3 days before soft agar with *A. fumigatus* spores was poured on top and the plates were incubated further 3 days before the pH was measured.

the corresponding role. *P. thoenii* grew weakly in SL, but all the other propionibacteria produced the highest amounts of both propionate and acetate when grown in this medium. The viable number of bacteria after incubation was close to 10^9/ml for the complete set of data, apart from *P. jensenii* in MRS-ac, where only weak growth was detected (Table 3).

3.3. MIC values of organic acids

Three of the moulds and one yeast species did not grow at pH 3 (Table 4). The others were inhibited at concentrations between 4 and 30 mM of propionic and acetic acid, while a concentration of 160 mM or more of lactic acid was required for total inhibition. At pH 5, all fungi were inhibited at 60 mM or less of propionic acid, 120 mM or less of acetic acid, but lactic acid concentrations above 500 mM were required for inhibition of most species. The exception was *P. roqueforti*, inhibited already at 70 mM. At pH 7, only 0.74% of the propionic acid is found in the undissociated form. For most indicator fungi, this gave total inhibition only at concentrations of 200–500 mM, but *P. roqueforti* was inhibited already at 50 mM. For both acetic and lactic acid, more than 500 mM were required at pH 7 for total inhibition of all fungi, except for *P. roqueforti*. Due to the unexpected

Table 3
Concentrations of glucose, organic acids, pH values and viable cell counts in the supernatant of SL, MRS and MRS-ac inoculated with 10^7 bacteria/ml and incubation for 3 days at 30 °C

Supernatant	Medium	Content in mM of:				pH	cfu/ml
		Glucose	Lactic acid	Propionic acid	Acetic acid		
Uninoculated	SL	0	168	0	0	5.7	–
	MRS	89	0	0	36	5.7	–
	MRS-ac	89	0	0	0	6.1	–
Inoculated with							
P. acidipropionici	SL	0	30	85	33	5.9	9.2
	MRS	56	0	63	40	4.7	8.5
	MRS-ac	55	0	73	17	4.4	8.5
P. freudenreichii shermanii	SL	0	11	106	35	6.1	9.4
	MRS	65	0	29	33	5.2	9.1
	MRS-ac	62	0	46	17	4.7	9.4
P. freudenreichii freudenreichii	SL	0	11	97	31	6.0	9.2
	MRS	74	0	26	35	5.3	9.4
	MRS-ac	57	0	40	16	4.6	9.6
P. thoenii	SL	0	123	35	0	5.7	9.1
	MRS	59	0	51	36	4.6	9.4
	MRS-ac	75	0	34	<10	4.8	8.8
P. jensenii	SL	0	<10	102	30	6.1	9.3
	MRS	53	0	54	41	4.8	9.4
	MRS-ac	84	0	0	0	6.1	7.9

All values are means of duplicate determinations.

Table 4
MIC of propionic acid (pK_a 4.87), acetic acid (pK_a 4.76) and lactic acid (pK_a 3.86) acid at pH 3, 5 and 7 for three yeasts and five mould species

Indicator fungus	Propionic acid			Acetic acid			Lactic acid		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
Yeasts									
P. anomala	20	60	>500	30	100	>500	>500	>500	>500
R. mucilaginosa	8	30	500	20	50	>500	160	>500	>500
K. marxianus	–[a]	60	500	–[a]	80	>500	–[a]	>500	>500
Moulds									
A. fumigatus	8	50	500	20	80	>500	250	>500	>500
P. roqueforti	–[a]	40	50	–[a]	80	50	–[a]	70	70
P. commune	–[a]	40	200	–[a]	50	>500	–[a]	>500	>500
A. nidulans	10	50	>500	4	120	>500	200	>500	>500
F. sporotrichoides	–[a]	20	500	–[a]	30	>500	–[a]	>500	>500

[a] No growth detected at pH 3.

acid sensitivity of *P. roqueforti* J268, four other strains of this species (J229, J231, J282 and J284) were analysed. The results were all in agreement with the results from strain J268 (data not shown). Regardless of pH, the yeast *P. anomala* was the fungus most tolerant to organic acids.

4. Discussion

We have compared the antifungal effect of five species of propionibacteria on three different growth media. When the propionibacteria were grown on the SL medium, no activity against any of the fungal strains tested could be detected, although this is the recommended medium for propionibacteria (Cummins and Johnson, 1992). Both the bacteriocins Jenseniin G (Grinstead and Barefoot, 1992) and Propionicin T1 (Faye et al., 2000) have been isolated from culture filtrates of propionibacteria grown in the lactate based SL. Culturing propionibacteria in the glucose based medium MRS instead may induce formation of other active metabolites.

P. thoenii was the most potent fungal inhibitor on MRS medium, but the other four type strains did also have a visible inhibitory effect on fungal growth. To exclude the possibility that the effects were caused by a synergistic effect with the substrate acetate, the dual culture assay was also performed on MRS-ac. When the mould *A. fumigatus* was evaluated against *P. jensenii*, it was more strongly inhibited when the propionibacteria were cultured on MRS-ac. Opposite results were found for all fungi assayed against *P. thoenii*, while the antifungal activity of *P. acidipropionici* and *P. freudenreichii freudenreichii* did not differ between the two substrates. The results suggest that the statement by Cabo et al. (2002) about the influence of acetate on the inhibitory effect of lactic acid bacteria may not be fully relevant for propionibacteria.

The pH of all three agar media, as well as the overlay soft agar, was fairly similar at start. After 3 days of incubation with bacteria, the pH value remained high on SL, while it was reduced 1.5 pH units on both MRS and MRS-ac. This indicates a similar acid production with these two media, as indeed also was found in liquid substrate. The increased pH observed after growth of *A. fumigatus* is likely to be caused by assimilation of organic acids, a metabolic capability, common to many moulds (Pitt and Hocking, 1999). Our results confirm that propionibacteria can grow equally well on glucose and lactate (Vorobjeva, 1999). Four of the five propionibacteria produced higher amounts of propionic and acetic acid in liquid SL medium, but no inhibition of any of the indicator fungi was observed on SL agar. This apparent contradiction could be explained by the higher end pH of the SL medium, thus having only a small proportion of the more active undissociated acid. Alternatively, the production of antifungal compounds could be more pronounced with glucose than with lactate.

When investigating possible antifungal compound production by dairy propionibacteria, background knowledge about the inhibition caused by the organic acids produced during the fermentation, or already present in the medium, is essential. Scattered information on the MIC of organic acids for fungi, in particular *S. cerevisiae*, can be found in the literature. In contrast, we determined the MIC of propionic, acetic and lactic acid at three different pH values, for three non-*Saccharomyces* yeasts and five moulds, all of relevance in food or feed ecosystems. Narendranath et al. (2001a) investigated the tolerance of *S. cerevisiae* to acetic and lactic acid. While the observed tolerance to acetic acid was comparable with our results on three other yeasts, the non-*Saccharomyces* yeasts can withstand much higher amounts of lactic acid. We found that *P. anomala* was the only fungus evaluated that could tolerate more than 500 mM lactic acid at pH 3. We have previously observed a high resistance of *P. anomala* also to antifungal lactic acid bacteria (Magnusson et al., 2003). The inhibition of acid tolerant yeast species by acetate, lactate, propionate, as well as mixtures of these acids, was investigated by Moon (1983). She found the inhibition to be linear with concentration for acetate and lactate but quadratic for propionate. Although the methodology used is not identical, our results for single organic acids generally agree with those of Moon (1983).

MIC value determinations rely on discrimination of growth or none growth of the target organism. The initiation of growth is influenced by the inoculum size (Jensen et al., 1987; Fernandez-Garayzabal and Genigeorgis, 1990). We used 5×10^4 cells or spores/conidia per milliliter in the examination of MIC of propionic, acetic and lactic acid. This level was, based on previous experience, considered a suitable inoculum size with regard to experimental reproducibility. Another inoculum size may have given slightly different results, which is important to remember when the MIC are referred to in future projects.

Aspergillus, *Penicillium* and *Fusarium* moulds are common spoilage organisms in feed and food storage systems and Higgins and Brinkhaus (1999) used a disk diffusion method to study organic acid inhibition of isolates of these genera. Unfortunately, as the pH values were not given and the fungi were not identified to species levels, meaningful comparisons are difficult to make. *P. roqueforti* is usually able to grow on substrates with high levels of acetic and propionic acid (Samson et al., 2002). Unexpectedly, we found that *P. roqueforti* was the fungus most sensitive to organic acids. Four other *P. roqueforti* strains were then assayed, giving similar results as with strain J268. One reason for the unexpectedly low organic acid tolerance of the *P. roqueforti* strains could be that we used a very sensitive assay. If a disk diffusion method had been used instead, this would probably not have exposed the fungal cells/conidia directly to the acid, as was now the case with the microtitre plate assay.

Mixtures of acetate, lactate and propionate have a synergistic inhibitory effect on the indicator strains (Moon, 1983). We have observed inhibition from propionibacteria that only partly can be explained by the presence of organic acids. Synergistic effects between the organic acids present are however, likely to be important. The pH reduction by the acids will also influence results by affecting the degree of dissociation of the acids. All three acids thus reduced pH to about 3 in unbuffered water, even at concentrations as low as 10 mM. The concentration of propionic acid produced by the dairy propionibacteria (30–100 mM) in liquid culture inhibits growth of most of the tested fungal species. The acetic acid levels produced would also be high enough to prevent growth of some of the tested species. Magnusson et al. (2003) observed that the level of lactic acid produced by a number of lactic acid bacteria could reach 100 mM when grown in MRS medium for approximately 24 h. The commensalistic interactions observed between propionibacteria and lactic acid bacteria (Liu and Moon, 1982; Perez Chaia et al., 1995; Piveteau et al., 1995) enables research on the synergistic antifungal properties of cocultures. In a recent study by Miescher Schwenninger and Meile (2004), the inhibitory effect of mixed cultures of propionibacteria and lactic acid bacteria against food spoilage yeasts was evaluated. The selected strains showed only weak inhibitory activities separately, but mixed cultures were highly effective against spoilage yeasts and did not have any influence on the quality properties of the food. Further studies should now focus on elucidating such fungal inhibitory phenomena.

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