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Microbial re-inoculation reveals differences in the leavening power of sourdough yeast strains

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Abstract

A method based on microbial re-inoculation, or the so-called backslopping and subsequent proofing of rye bread dough simulating commercial one-stage sourdough process, was used for the screening of the leavening capacity of sourdough yeast strains. Two yeast strains were initially tested with seven *Lactobacillus* strains. Thereafter, 17 yeast strains, mostly of sourdough origin, were tested with a backslopping procedure with heterofermentative *Lactobacillus brevis* as an acidifying lactic acid bacteria (LAB). The highest leavening capacity was found in sourdoughs containing *Candida milleri*, in particular when it was accompanied by obligately homofermentative *Lactobacillus acidophilus* or facultatively heterofermentative *Lactobacillus plantarum* when it acted homofermentatively. The leavening capacity of the reference strain *Saccharomyces cerevisiae* was about half that of *C. milleri* in all sourdoughs tested. The re-inoculation procedure increased the differences found in the leavening capacity of the tested yeast strains during final proofing of rye bread dough. The backslopped sourdoughs containing a heterofermentative *Lactobacillus* strain were more suppressive than those containing a homofermentative strain. The highest leavening capacity was found in *C. milleri* strains. The use of one backslopping cycle before assaying the leavening capacity of a laboratory sourdough is recommended since it helps to differentiate between yeast strains to be tested for their leavening power in the final bread dough.

Keywords: Yeast; Sourdough; *Candida milleri*; *Lactobacillus*; Rye bread; Leavening; Backslopping

1. Introduction

Sourdough is a system originally used for dough leavening and is composed microbiologically of lactic acid bacteria (LAB) and yeast. Consecutive microbial re-inoculation, often called "backslopping", of the micro-organisms from a previous batch is used to maintain the microbial flora, which is adapted and selected to the process applied. Many different recipes and processes are used for sourdough preparation worldwide, aiming at both technological and product improvements. Historically, endogenous sourdough yeasts were used for proofing bread until yeast from breweries or distilleries became available. Although the availability of baker's yeast has diminished the use of sourdough for leavening of bread dough, local specialities including some rye breads are still leavened exclusively by sourdough. Today, some bakeries are interested in raising their own consumer profile by exploiting endogenous leavening based on their own bakery-specific sourdoughs. Thus the use of endogenous yeast for leavening has again become an attractive feature in sourdoughs.

The yeast species most frequently found in sourdoughs is *Candida milleri* (Kurtzmann & Fell, 1998; Meroth, Hammes, & Hertel, 2003; Vernocchi et al., 2004; Yarrow, 1978). Strains assigned to species *Saccharomyces cerevisiae*, *Saccharomyces exiguus* and *Candida holmii* are also common (for review, see Hammes & Gänzle, 1998; Hansen, 2004; Lönner & Ahrné, 1995). *C. milleri* yeast is genetically close to *S. cerevisiae* (Granström, Aristidou, Jokela, & Leisola, 2000; Mäntynen et al., 1999). An even closer genotypic relationship may exist between a recently presented sourdough yeast *Candida humilis* and *C. milleri*

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(Pulvirenti, Caggia, Restruccia, Gullo, & Guidici 2001), but reports on phylogenic difference are not conclusive (Middlehoven & Kurtzman, 2003). Physiologically, the two yeasts express common features by both being maltose negative and are of sourdough origin. Predominant LAB strains in sourdoughs, such as *Lactobacillus sanfranciscensis*, *Lactobacillus brevis* and *Lactobacillus plantarum*, are characterised by either homolactic or heterolactic fermentation patterns (Axelsson, 2004). Sourdough yeasts differ in their sensitivity to the acetic acid produced by heterofermentative LAB (Suihko & Mäkinen, 1984).

The leavening is the slow and rate-limiting step in any baking process and especially when the leavening relies solely on the endogenous yeast. The yeast activity is usually described by plate count techniques even if yeast content in a sourdough is not necessarily equivalent to the leavening activity found in bread dough. Studies on the gassing power of sourdough yeast have normally focused on the fermentation of a first-day sourdough (Brandt, Hammes, & Gänzle, 2004; Gänzle, Häusle, & Hammes, 1997; Gobbetti, Corsetti, & Rossi, 1995; Martínes-Anaya, Pitchard, Bayari, & Benedito de Barber, 1990) and the backslopping procedure has been neglected, probably because it is considered laborious. In this study we used a process that simulated a one-stage bakery sourdough process, to give a more realistic insight into the role of the leavening performance of different sourdough yeast strains during proofing of the final bread dough.

We studied sourdough yeast strains in combinations with selected LAB strains and measured the leavening capacity in sour bread dough made with sourdough that was started with the strains to be tested. The one-stage fermentation procedure developed was used to study the leavening capacity of 17 sourdough yeast strains.

2. Materials and methods

2.1. Strains used and growth conditions

In the first part of the study, yeast stains *C. milleri* C232 and *S. cerevisiae* C236 (Table 1) were tested separately with seven single lactobacilli. The LAB strains, previously isolated from Finnish rye sourdough, and identified by API (Salovaara & Katunpää, 1984), were *Lactobacillus acidophilus* E611, *L. brevis* E612, *L. sanfranciscensis* E613, *Lactobacillus casei* E614, *Lactobacillus fermentum* E615, *L. plantarum* E617 and *L. plantarum* E618. For comparison, a sourdough inoculated with only the yeast strain but no LAB was made. In the second part, leavening capacity of 17 different sourdough yeast strains (Table 1) was studied using the heterofermentative *L. brevis* E612 strain as acidifying LAB in a 2nd-day sourdough.

Each sourdough was always started from pure culture stored on plates at 4 °C. The yeast strains were cultured in YM broth (Difco) at 25 °C with 120 rpm shaking to obtain a final cellular suspension of 10^8 cfu/ml. The *Lactobacillus* strains were cultured in MRS broth (Difco) at 30 °C in 5% CO_2 atmosphere up to the late logarithmic phase to obtain a final cellular suspension of 10^9 cfu/ml.

Table 1
Yeast strain used in this study

Species	Strain code (abbreviation)	Origin of the strain	Ref.
Candida milleri	VTT C-95232 (C232)	Sourdough, Finland	[a]
Saccharomyces cerevisiae	VTT C-95236 (C236)	Sourdough, Finland	[a]
Candida milleri	VTT C-96250	Sourdough, Finland	[a]
Candida milleri	VTT C-87175	Sourdough, Finland	[a]
Candida milleri	ATCC 60592	Sourdough, USA	
Candida milleri	ATCC 62655	Sourdough, USA	
Candida humilis	CBS 2664	Olive mill waste, Spain	
Candida humilis	CBS 7541	Bread, Morocco	
Saccharomyces exiguus	CBS 379	Unknown	
Saccharomyces exiguus	CBS 135	Buttermilk, Netherlands	
Baker's isolate	A2	Sourdough, Finland	[b]
Baker's isolate	B3	Sourdough, Finland	[b]
Baker's isolate	C4	Sourdough, Finland	[b]
Baker's isolate	D5	Sourdough, Finland	[b]
Baker's isolate	D52	Sourdough, Finland	[b]
Baker's isolate	E6	Sourdough, Finland	[b]
Baker's isolate	E62	Sourdough, Finland	[b]

[a] Salovaara and Savolainen (1984).
[b] Mäntynen et al. (1999).

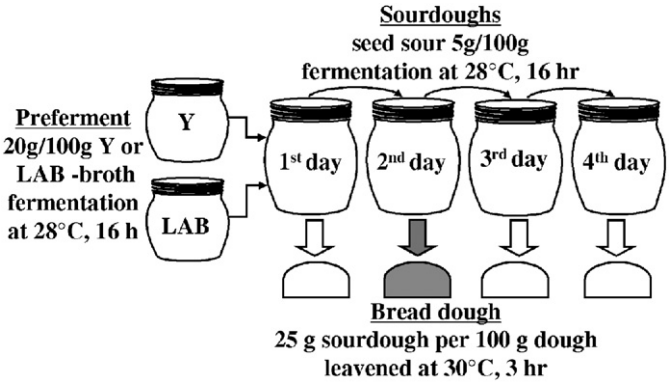

Fig. 1. Schematic representation of the backslopping system, where *Y* = yeast strain and LAB = *Lactobacilli* strain used.

2.2. Preparation of the sourdough

The backslopping procedure and the one-stage sourdough including bread dough making is shown in Fig. 1. A preferment (dough yield: DY 350) of each strain was made with 34 g whole rye flour, 65 g water and 20 g culture suspension. The 1st-day sourdough was prepared by mixing 180 g whole rye flour, 270 g tap water and 25 g of one yeast and one LAB preferment. The 2nd-, 3rd- and 4th-day sourdough were made mixing 189 g whole rye flour, 286 g tap water and 25 g of the previous days'

sourdough. The inoculation level for all sourdoughs was 10^6 cfu/g for yeast and 10^7 cfu/g for lactobacilli, respectively. Both preferment and all sourdoughs (DY 250) were fermented in water bath at 28 °C for 16 h. Whole meal rye flour (ash content 1.7 g, protein content 10.3 g moisture 11.8 g per 100 g flour and falling number 180 s) was used. Seed sour for the next days sourdough was stored at 4 °C for 8 h. Sourdoughs were made and analysed in duplicate.

2.3. Leavening capacity of sourdoughs

Fully fermented, i.e. mature sourdoughs (28 °C, 16 h) were used for studying leavening capacity of sourdough yeasts in bread dough. Rye bread dough was made from 100 g sourdough, 162 g rye flour, 134 g water and 4 g salt. The dough yield was 200 and temperature 30 °C. The gas production was measured with Risograph (Rdesign, Pullman, WA, USA). From each dough three 100 g portions were placed into Risograph jars that were closed, put into the water bath and pipes connected. Leavening was tested at 30 °C for 180 min. Gas production was reported as total carbon dioxide evolved after 180 min of leavening as average of three parallel measurements. Yeast content of the bread dough was calculated from the yeast content (cfu/g) of the sourdough used. Standard deviation was calculated as the root mean square deviation of the values from their arithmetic mean. The sample size minus one was used. Standard error was calculated by dividing the standard deviation with the root square of sample size minus one.

2.4. Analytical measurements

Samples for yeast and LAB content measurements were taken from the mature sourdoughs at the end of 16 h fermentation. Counts of LAB were made by plating appropriate dilution of the sample on MRS agar (Difco) supplemented with 0.02 ml/l natamysin to inhibit yeast growth, and incubated at 30 °C for 72 h in 5% CO_2 atmosphere. The yeast was counted on YGC-agar (Difco) and the plates were incubated at 25 °C for 48 h.

Samples for titratable acidity (TTA) and pH determination were taken from sourdoughs after 16 h fermentation, samples were stored at −20 °C, thawed at 4 °C and analysed on an aliquot of 10 g sourdough mixed with 100 ml distilled water. The pH of the suspension expresses sourdough pH. Titration of the suspension with 0.1 mol/l NaOH to a final pH of 8.5 expresses TTA as the amount (ml) of NaOH used. In addition, the pH-decrease during sourdough fermentation was recorded using a Consort pH-controller R301.

Lactic acid and acetic acid concentrations were analysed by Hewlett Packard LC1090 (HPLC) using an Aminex HPXH-240972 column at 50 °C with 0.003 M sulphuric acid as mobile phase, which also was used for extraction. The extraction was carried out by adding 80 ml of 0.003 M sulphuric acid to 5 g of sour dough samples in 100 ml volumetric flasks, which were incubated in a 80 °C water bath for 15 min. The flasks were shaken manually once per minute during incubation. After incubation, the flasks were cooled and filled to the mark with 0.003 M H_2SO_4 and mixed. The solutions were centrifuged at +5 °C for 20 min at 10,000 rpm. The supernatants were collected for HPLC analysis.

3. Results

3.1. Influence of LAB on yeast-leavening capacity

In general, the leavening capacity of the *C. milleri* yeast strain C232 in a bread dough made from a 1st-day sourdough was 280–380 ml during 3 h proofing of the bread dough, or 1.5 times higher than that of the *S. cerevisiae* 236 strain giving 170–210 ml, respectively (Fig. 2). In most cases, incorporation of *Lactobacillus* strains to the *C. milleri* sourdoughs reduced CO_2 production by 10–20% on average. *C. milleri* showed the highest leavening capacity with obligatory homofermentative *L. acidophilus* and lowest with heterofermentative *L. sanfranciscensis*. The leavening capacity of the *S. cerevisiae* yeast strain decreased by 20–35% depending on the LAB strain present (Fig. 2). At the beginning of the 3-h leavening test, the bread doughs with *C. milleri* sourdough contained $1.2–3.0 \times 10^7$ yeast cells and with *S. cerevisiae* $8.0 \times 10^6–2.3 \times 10^7$.

The viable cell count of *C. milleri* yeast in fully fermented sourdough was about 10^8 cfu/g (Table 2). The counts of *S. cerevisiae* were somewhat lower than those of *C. milleri*. The effect of the prevailing yeast on *Lactobacillus* content (about 10^9 cfu/g) of the mature sourdough was not consistently related to the homo- or heterofermentatively acting groups of LAB, but was rather a strain-specific feature. The acidity levels of the mature sourdough were in the normal range, pH 3.8–4.0, titratable acidity 13.6–15.8 depending on the LAB strain used. In general, sourdoughs containing *C. milleri* showed slightly lower TTA values

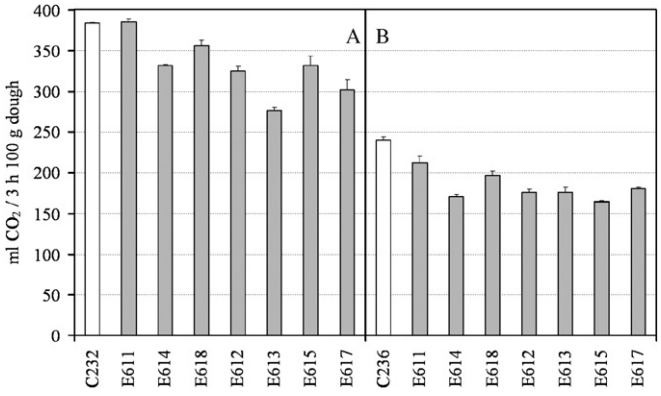

Fig. 2. Leavening capacity of bread dough containing 25 g/100 g of 1st-day sourdough and yeast fermented 16 h at 28 °C. (A) *C. milleri* C232 and (B) *S. cerevisiae* E236. LAB strains used *L. acidophilus* E611, *L. brevis* E612, *L. sanfranciscensis* E613, *L. casei* E614, *L. fermentum* E615, *L. plantarum* E617 or *L. plantarum* E618. C232 or C236 represents dough with only the yeast component inoculated.

Table 2
Chemical, physical and microbiological characteristics of the sourdoughs fermented with one yeast and one lactobacilli[a] strain

	Homofermentative LAB			Heterofermentative LAB			
	E611	E614	E618	E612	E613	E615	E617
C. milleri C232							
LAB = 10^9 cfu/g	2.1	1.9	3.7	2.5	0.5	1.0	3.2
$Y = 10^7$ cfu/g	12.0	11.0	11.0	9.3	4.8	8.6	7.2
pH	3.7	3.8	3.8	3.9	3.9	4.0	4.0
TTA	13.9	14.8	14.8	14.2	14.0	14.0	13.6
Lactic acid, g/100 g	1.31	1.38	1.67	1.04	1.05	0.90	1.12
Acetic acid, g/100 g	<0.05	<0.05	<0.05	0.14	0.18	0.13	0.17
S. cerevisiae C236							
cfu LAB 10^9	2.2	2.3	1.7	3.0	0.7	1.0	3.9
cfu Y 10^7	9.1	8.3	6.3	3.5	3.2	5.2	4.4
pH	3.8	3.8	3.8	3.9	3.9	3.9	3.8
TTA	14.8	15.8	15.8	14.7	14.8	14.7	14.6
Lactic acid, g/100 g	1.61	1.78	1.61	1.10	1.05	0.92	0.95
Acetic acid, g/100 g	<0.05	<0.05	<0.05	0.12	0.18	0.20	0.14

[a] *L. acidophilus* E611, *L. casei* E614, *L. brevis* E12, *L. sanfransiscensis* E613, *L. fermentum* E615 and facultatively heterofermentative *L. plantarum* E617 and E618.

than those with *S. cerevisiae*. The acid production of facultatively heterofermentative *L. plantarum* was strain-dependent: the strain E618 acted homofermentatively while E617 acted heterofermentatively and produced acetic acid.

3.2. Backslopping of sourdoughs

For studying potential differences in leavening capacity further we simulated traditional bakery sourdough making by backslopping the 1st-day sourdough. For the development of a backslopping procedure, *C. milleri* yeast strain C232 was selected as the yeast in the sourdough due to its high leavening capacity as shown in Fig. 2. Backslopping sourdoughs with obligate heterofermentative *Lactobacillus* suppressed the leavening capacity of the *C. milleri* in bread dough by about one third when the sourdough had been backslopped once (Fig. 3), whereas in a sourdough containing obligate homofermentative *Lactobacillus* strains the leavening remained relatively unaffected. The effect of leavening on backslopping with facultatively heterofermentative *Lactobacillus* was dependent on whether the strain acted heterofermentatively or homofermentatively in the sourdough. The suppressive effect of backslopping was observed in the 2nd-day sourdoughs ($p<0.01$). Simultaneously, acidity development during sourdough fermentation, measured as pH-decrease, was slower in the 1st-day sourdough than in the 2nd-, 3rd- and 4th-day sourdoughs (Fig. 4). The exposure time to low pH level, below 4.0, was significantly ($p<0.01$) shorter in the 1st-day sourdoughs, i.e. 14 h compared to 8–10 h in the backslopped sourdoughs. Thus a 2nd-day sourdough, backslopped with a heterofermentative *L. brevis* E612, was selected for testing the leavening capacity of different yeast strains. This procedure seemed to show most of the variations.

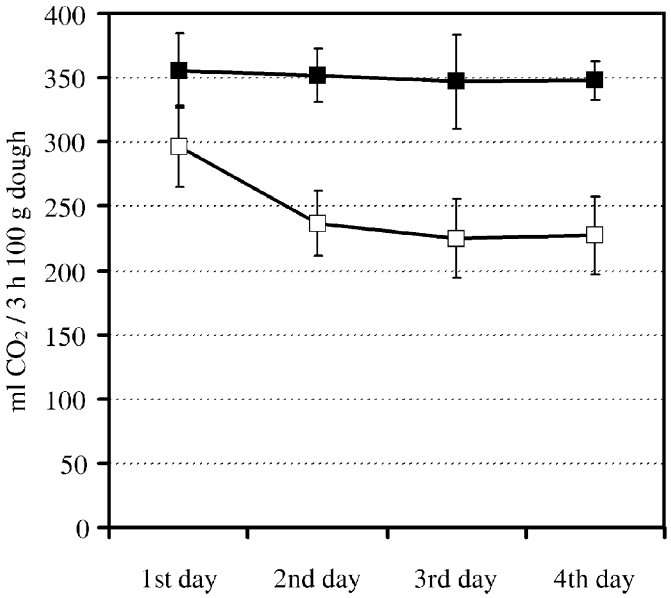

Fig. 3. Effect of backslopping on the leavening capacity of bread dough containing 25 g sourdough/100 g dough. Proofing temperature 30 °C, time 3 h. Sourdough with ■ homofermentative LAB and □ heterofermentative LAB. Standard deviation added. Yeast strain *C. milleri* C232.

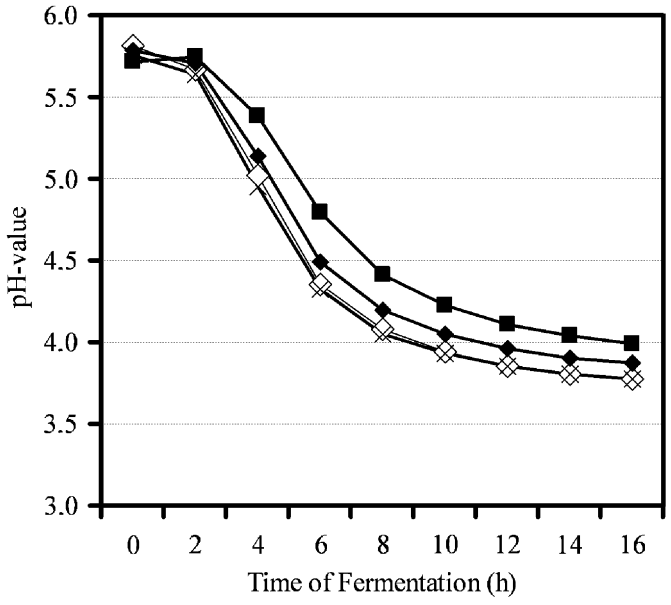

Fig. 4. Effect of backslopping on pH during sourdough fermentation at 28 °C for 16 h. ■, 1st day; ◇, 2nd day; ✖, 3rd day; ◆, 4th-day sourdoughs. Standard error was 0.2.

3.3. Leavening capacity of 17 yeast strains

The main idea of this study was to show relevant differences in the carbon dioxide production of sourdough yeast strains from different sources. The backslopping procedure described above was used to test 17 yeast strains for leavening capacity (Fig. 5). The yeast strains showed significant ($p>0.01$) differences in gas production. The

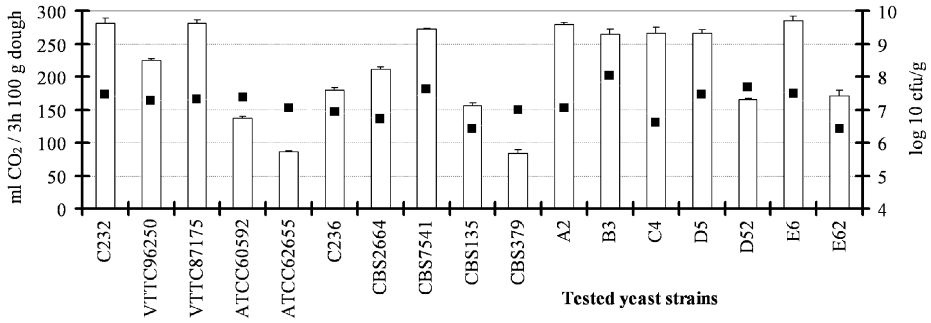

Fig. 5. Leavening capacity of 17 sourdough yeasts with associated *L. brevis* E612. Yeast content of the bread dough ■ is also given. Tested yeast strains were *C. milleri* C232, VTT C-96250, VTT C-87175, ATCC60592, ATCC62655; *S. cerevisiae* C236 *C. humilis* CBS2644, CBS7541; *S. exiguus* CBS379, CBS135 and yeast strains isolated from Baker's sourdoughs A2, B3, C4, D5, D52, E6, E62. Leavening was measured at 30 °C for 3 h from a bread dough containing 25 g per 100 g 2nd-day sourdough fermented at 28 °C for 16 h.

carbon dioxide produced during a 3 h dough proofing varied between 87 and 290 ml. This difference was only weakly supported by the yeast contents, which were on the same level 10^7 cfu/g in the bread doughs. Also, the yeast growth in the sourdough was on normal level 10^{7-8} cfu/g sourdoughs. A change in the prevailing yeast strain did not significantly affect the acidity produced by *L. brevis* E612. Final pH was at 3.9 and TTA at 14.2–14.8 ml. The origin of the yeast strains is shown in Table 1. The yeasts originating from Finnish sourdough had the highest leavening capacity. Variation in leavening capacity was greater than that expected from yeast content (cfu/g).

4. Discussion

The objective of the first part of the study was to compare the leavening capacity of sourdough yeast strains in backslopped sourdough acidified with selected LAB strains during final proofing. The results showed that *C. milleri* C232 strain had a higher leavening capacity than the *S. cerevisiae* strain used, also originating from a sourdough. This is in line with our earlier findings (Salovaara, 1995) that yeast strains isolated from Finnish sourdoughs and belonging to the *C. milleri* species have a good tolerance to the acidic environment and especially to the acetic acid level of the mature rye sourdough, which shows a high leavening capacity of the final bread dough. The acetic acid contents formed in our sourdoughs were lower than the reported levels for inhibition of yeast growth (Gänzle, Ehmann, & Hammes, 1998; Suihko & Mäkinen, 1984). The leavening capacity was affected by accompanying LAB strain and their acid-production pattern. This appeared to be a strain-specific property, which is affected by competition for substrates and adaptation to environmental stress. Other workers have also reported that *C. milleri* has a high tolerance of acetic acid with respect to growth or gas production during sourdough fermentation (Brandt et al., 2004; Gänzle et al., 1997; Hansen, Lund, & Lewis, 1989; Röcken, Rick, & Reinkemeier, 1992). In addition to acetic acid, the lactobacilli may also produce other substances that inhibit yeast growth and leavening capacity (Messens & De Vuyst, 2002). In general, it is considered that *C. milleri* (or *S. exiguus*) is fairly tolerant to high acidity whereas *S. cerevisiae* is more sensitive (Suihko & Mäkinen, 1984). Gobbetti et al. (1995) found that the production of carbon dioxide by *S. exiguus* was not comparable to the high gassing power of *S. cerevisiae* during wheat sourdough fermentation, whereas in our study an *S. cerevisiae* strain showed a substantially lower leavening capacity in a sour bread dough than the *C. milleri* strain.

Relatively high cfu/g for LAB and for yeast in particular were obtained in this study in comparison with earlier reports on the *C. milleri* content in sourdoughs (Brandt et al., 2004; Gobbetti et al., 1995; Hansen et al., 1989). The high yeast and lactobacilli contents are consistent with the acidity values found and to the relatively long fermentation time. The use of whole-meal rye flour, with its high nutritive content, contributed to the higher microbial count in comparison to wheat sourdoughs. The high yeast count was also reflected in the lactobacilli/yeast ratio, which in this study was approximately 10:1. The slightly lower cfu/g of *L. sanfranciscensis* E613 strain may be an expression of the heterogeneity among *L. sanfranciscensis* strains reported by Böcker, Stolz, and Hammes, (1995); Stolz, Böcker, Hammes, and Vogel (1995) and Kitahara, Sakata, and Benno (2005). The facultatively heterofermentative *L. plantarum* strains previously isolated from commercial sourdoughs showed a different acidity-development profile, indicating significant technological diversity of these species, also reported by Pepe et al. (2004).

We used a one-stage backslopping procedure for the screening of sourdough yeasts for their leavening properties. In backslopping the sourdough flora adapts to the environment of the sourdough system including accompanying microorganisms. Since there appears to be interest in baking without added yeast relying merely on the leavening capacity of the endogenous sourdough yeasts, we wanted to have an insight in the potential of some of the yeast strains available. Preliminary tests (results not shown) indicated that the direct testing of gassing power in test tubes of pure cultures might give different results

compared to the more challenging conditions in a backslopped system. As expected, backslopping affected leavening of the bread dough. The sourdoughs were grouped according to the acid production pattern of the accompanying LAB strain. Backslopping once with a heterofermentatively acting LAB the leavening capacity was suppressed whereas it remained on the same level when a homofermentatively acting LAB was used. The differences between subsequent backsloppings were small, thus single backslopping was used for screening, to keep the test simple. The backslopping procedure applied simulates a commercial one-stage sourdough process. The use of a relatively high amount of seed sour 5 g/100 g effectively controls the growth of endogenous flour-based microflora, supports a high ratio of yeasts to lactobacilli and keeps the microbes stable through backslopping. The initial and final microbial counts of yeast and lactobacilli were those typically present in commercial sourdoughs. The faster acidification found in the first backslopping, i.e. in the 2nd-day sourdough onwards, indicates that inoculated microbes are adapted to the system.

When the leavening capacity of 17 yeast strains was tested using the backslopping procedure, *L. brevis* E612 was selected, because heterofermentative LAB increases differences in the leavening capacity in this backslopping method. *L. brevis* is also common in industrial sourdoughs. As expected, most of the *C. milleri* strains originating from local rye sourdoughs possessed a higher leavening capacity in the sour rye bread dough than strains originating from other sources and culture collections. Interestingly, the *C. humilis* CBS7541 strain originating from a Moroccan wheat sourdough also showed high leavening capacity. Sourdough microbes are usually well adapted to the commercial sourdough process applied. Most isolated bakery strains proved to be strong carbon dioxide producers. These isolated strains, referred to as Baker's isolates (Table 1), were genetically closely related to known *C. milleri* strains (Mäntynen et al., 1999). The leavening capacity of sourdough yeast seems to be a strain-related property. Since the conditions in the sourdough for yeast growth and activity gradually deteriorate due to acidification, selection of yeast strains with high acid tolerance is crucial for good proofing of bread dough. A careful selection of yeast strains will provide a procedure for sour rye bread making without added baker's yeast. However, a substantially longer leavening time is presumably needed to achieve a satisfactory gas formation in the doughs and an acceptable texture of the bread when sourdough is used as a sole leavening agent.

5. Conclusion

The results of the study support the hypothesis that a sourdough containing the right strain of yeasts can potentially leaven bread dough without added baker's yeast. Substantial differences occur in the leavening capacity of yeast strains isolated from different sources. In general, the *C. milleri* strain isolated from Finnish rye sourdoughs showed a high carbon dioxide production rate during proofing of sour rye bread dough. The leavening capacity was reduced by the accompanying *Lactobacillus* strain. Acetic acid produced by heterofermentative lactobacilli suppressed leavening capacity. We propose a simple one-stage backslopping procedure for screening of the leavening capacity of sourdough yeast strains, since this method revealed differences in gas production of the tested yeast strains. The leavening potential of endogenous sourdough yeast suggests further work on this topic.

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