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ORIGINAL PAPER

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Effects of process parameters on growth and metabolism of *Lactobacillus sanfranciscensis* and *Candida humilis* during rye sourdough fermentation

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Abstract The effects of temperature, pH, inoculum level, and NaCl on the growth and metabolism of *Lactobacillus sanfranciscensis* and *Candida humilis* in rye sourdough were determined. The temperature optima for growth of *C. humilis* and *L. sanfranciscensis* were 28 and 32 °C, respectively. Yeast growth was inhibited at 35 °C. The pH did not affect yeast growth in the range 3.5–5.5, whereas growth of *L. sanfranciscensis* was inhibited at pH 4.0. A NaCl concentration of 4% (flour base) inhibited growth of *L. sanfranciscensis* but not *C. humilis*. The effects of the process parameters on the formation of lactate, acetate, ethanol, and CO_2 by the organisms were generally in agreement with their effects on growth. However, decreased formation of acetate by *L. sanfranciscensis* was observed at 35 °C although lactate and ethanol formation were not affected. In conclusion, the study provides a rationale for the stable persistence of *L. sanfranciscensis* and *C. humilis* in traditional sourdoughs and will facilitate the optimisation of sourdough fermentations in traditional and new applications.

Keywords Rye sourdough · *Lactobacillus sanfranciscensis* · *Candida humilis* · Modelling

Introduction

Sourdough fermentation is employed to improve the flavour, texture, and shelf life of baked goods. In specific applications, the production of carbon dioxide by the sourdough microflora contributes to dough leavening, and the acidification of the dough is essential when rye flour is used for baking. The effects of sourdough fermentation on bread quality result from the metabolic properties of the sourdough microflora, the activity of enzymes of the flour, and, indirectly, the effects of acidification on the solubility of flour constituents and enzyme activities. For example, cereal proteinases liberate amino acids that are converted to flavour volatiles by the metabolic activities of sourdough yeasts and lactobacilli, or through thermal reactions during baking [1, 2, 3]. Furthermore, lactic and acetic acids affect the taste and the aroma of breads, respectively. The solubilisation of pentosans during fermentation, the degradation of the gluten macropolymer by cereal enzymes, and the formation of exopolysaccharides by lactobacilli affect dough rheology and bread texture [4, 5, 6, 7]. The formation of organic acids as well as specific antimicrobial metabolites delays the growth of spoilage microorganisms during bread storage [8, 9, 10]. Because the metabolic properties of the fermentation microflora are species- or strain-specific, the optimisation of bread quality through sourdough fermentation necessitates the control of the composition and metabolic activity of the sourdough microflora.

Sourdoughs are traditionally maintained by continuous propagation as in-house sourdoughs. In these doughs (type I doughs) occur yeasts and lactobacilli with *Candida humilis* (syn. *Candida milleri*), *Saccharomyces exiguus*, *Lactobacillus sanfranciscensis*, and *L. pontis* being the predominant species [11, 12]. The microflora of type I doughs can be remarkably stable over several decades, although the bakery environment is loaded with all types of contaminating microorganisms [13]. The stable maintenance of sourdoughs with a complex microflora, and the control of the metabolic activity of the respective microorganisms, requires knowledge of the effect of process parameters on the growth and metabolic activity of sourdough yeasts and lactobacilli.

Previously, a model was proposed to predict the effect of temperature, pH, organic acids, and NaCl concentra-

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tion on the growth of *L. sanfranciscensis* and *C. humilis* [13]. This model was based on the growth in laboratory media of *C. humilis* and *L. sanfranciscensis* in a single culture. It was the aim of this work to validate the model predictions for growth and metabolism of *L. sanfranciscensis* and *C. humilis* in co-culture in rye sourdoughs. The parameter range was chosen to cover practical needs rather than the complete growth range of the organisms.

Materials and methods

Strains and growth conditions

C. humilis LTH H198 (syn. *C. milleri* LTH H198) and *L. sanfranciscensis* LTH 2581 were cultured at 30 °C in mMRS4 medium [14] containing 10 g L^{-1} maltose and 5 g L^{-1} each of glucose and fructose. These strains have been shown to prevail for two decades in a commercial sourdough starter [13, 15].

Sourdough fermentation

To prepare the sourdough inoculum, 0.5 mL overnight culture each of *L. sanfranciscensis* and *C. humilis* were mixed with 160 g rye flour (ash content 1.15 g/100 g, 14.1% moisture) and 109 g tap water. The dough was incubated for 14 h at 28 °C to obtain a sourdough inoculum with a pH ranging between 3.7 and 3.9, and cell counts of 1–3×10^7 cfu g^{-1} *C. humilis* and 2–4×10^9 cfu g^{-1} *L. sanfranciscensis*. For the preparation of sourdoughs, flour and water were adjusted to the desired fermentation temperature prior to mixing. Flour, water, and sourdough inoculum were mixed to achieve a dough yield (DY) of 200, and incubated in a water bath at the temperature indicated. Samples were taken directly after mixing and over 24 h of fermentation. For the adjustment of the initial pH, flour and 50% of the water were mixed and the pH was measured. Over a period of 30 min, 1 M HCl was gradually added to obtain the desired pH. Unless otherwise specified, the parameters were as follows: NaCl content 2% (flour base), fermentation temperature 28 °C, and addition of 5% sourdough inoculum, resulting in an initial pH of 5.5. Fermentations were carried out at various concentrations of NaCl (0–4% flour base), preferment levels of 0.1 to 50%, and temperatures ranging from 21 to 35 °C.

Cell counts and pH values, and calculation of the growth rates

For the selective enumeration of lactobacilli, appropriate dilutions of dough samples in peptone water were plated on mMRS4 agar containing 0.1 g L^{-1} cycloheximide, and plates were incubated at 30 °C in an atmosphere of 76% N_2, 4% O_2, and 20% CO_2. For selective enumeration of yeasts, yeast–glucose–chloramphenicol agar was used [16], and plates were incubated aerobically at ambient temperature. For the calculation of the maximum growth rate (μ_{max}) and the maximum cell yield (A), the cell counts were fitted to the logistic growth curve as described by Zwietering et al.[17].

Determination of organic acids, ethanol, and carbon dioxide

For the determination of the concentrations of organic acids and ethanol in dough, 2 g of dough was centrifuged at 13,000 *g* and the proteins were precipitated overnight at 4 °C in the presence of 3.5% perchloric acid. The precipitate was removed by centrifugation and the concentrations of lactate, acetate, and ethanol were analysed by HPLC as described by Hamad et al. [18].

To measure the volume of carbon dioxide produced during fermentation, 10 g of dough was placed in an airtight vessel and the displaced volume of a saturated NaCl solution was recorded during fermentation [19]. The gas volumes obtained were corrected to standard conditions (25 °C, 1,013 mbar).

Statistical treatment of the data

Fermentations at reference conditions (5% preferment, 2% NaCl, 28 °C) were carried out in triplicate. The determinations of cell counts, growth rates, and concentrations of metabolites had an experimental error of less than 25, 15, and 5%, respectively.

Results

Kinetics of growth and formation of metabolites during sourdough fermentation

In all fermentations, the dynamics of microbial growth and production of metabolites in rye doughs inoculated with a standardised sourdough inoculum were determined. Samples were taken over 24 h or until microbial growth and metabolism ceased. A typical time course of fermentation is shown in Fig. 1. A good fit of the logistic growth curve to the cell counts determined experimentally was obtained for all experiments. As an example, the experimental data and the predicted values for the fermentation carried out at 28 °C and 2% NaCl are depicted in Fig. 1. Thus, the parameters of the logistic growth curve—growth rate μ_{max}, maximum population density A, and lag phase λ—can be considered representative for the kinetics of growth of *L. sanfranciscensis* and *C. humilis* in dough. The production of lactate and ethanol correlated to the growth of the organisms.

A shift in the fermentation quotient (FQ, molar ratio of lactate to acetate) during incubation was observed in all fermentations (Fig. 1, data not shown). In the first stage of fermentation, lactate and acetate were formed in equimolar amounts, resulting in an FQ of 1. After production of

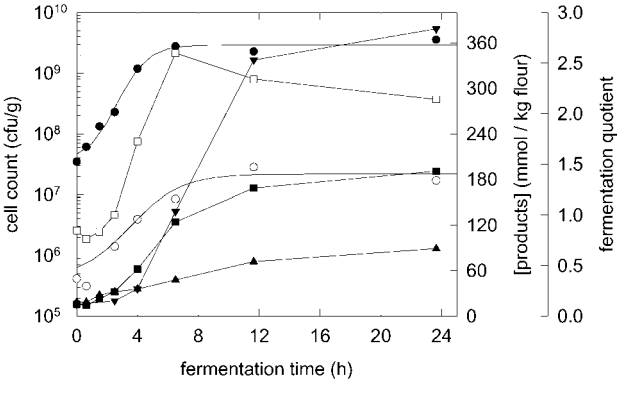

Fig. 1 Kinetics of growth and metabolism during a rye sourdough fermentation (DY 200, 28 °C, 2% salt). The process was started with *L. sanfranciscensis* LTH 2581 and *C. humilis* LTH H198 and the viable cell counts of *L. sanfranciscensis* (●) and *C. humilis* (◯) were determined. The *lines* represent the cell counts predicted with the logistic growth curve. Also shown are the concentrations of lactic acid (■), acetic acid (▲), ethanol (▼), and the fermentation quotient (❑)

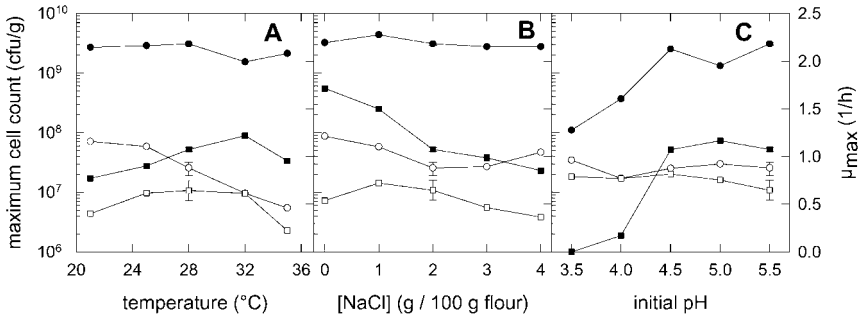

Fig. 2a–c Effect of temperature (**a**), salt content (**b**), and initial pH (**c**) on μ_{max} (■) and maximum cell count (●) of *L. sanfranciscensis* LTH 2581 (*closed symbols*) and *C. humilis* LTH H198 (*open symbols*) in a rye sourdough fermentation

10–15 mmol g^{-1} each of acetate and lactate, lactate formation exceeded that of acetate as *L. sanfranciscensis* produced ethanol instead of acetate. Correspondingly, the FQs increased rapidly to values between 2 and 3.

Effect of temperature, pH, and NaCl on growth of the sourdough microorganisms

In bakery practice, the dough temperature is an important parameter to control growth of lactobacilli and yeasts in sourdough. The growth rates and maximum population densities of *L. sanfranciscensis* and *C. humilis* at various temperatures are shown in Fig. 2a. The fermentation temperature did not affect the maximum population density of *L. sanfranciscensis* and the maximal growth rate was observed at 32 °C. Growth of *C. humilis* was optimal between 25 and 28 °C, and the maximum population density was reduced by an order of magnitude when the temperature was increased from 25 to 35 °C.

The effect of NaCl on microbial growth is depicted in Fig. 2b. The salt concentration did not affect substantially the growth rate and the maximum population density of *C. humilis*. In contrast, the growth rates of *L. sanfranciscensis* decreased linearly with increasing salt concentration, and addition of 4% NaCl reduced the μ_{max} by more than 50%.

The effect of the initial pH, adjusted with HCl, on microbial growth is shown in Fig. 2c. Whereas the growth of the yeast was not affected in the pH range tested, the growth rate of *L. sanfranciscensis* was maximal at pH values above 5.0 and at pH 4.0, the μ_{max} was decreased by 90% with a concomitant decrease of the final cell counts. The growth was inhibited at a pH of 3.5. Thus, acidification of the dough resulted in a major shift of the microbial association in doughs and yeast cell counts accounted for more than 30% of total cell counts.

Effect of inoculum size on growth of the sourdough microflora

Different from the chemical adjustment of pH, in practice the pH course of the fermentation is controlled by the buffering capacity of the flour used and the inoculum size. It is conclusive that a small inoculum results in a greater proportion of the fermentation occurring at a high pH, whereas inoculation with 20% or more of the sourdough inoculum results in an initial pH of 4.5 or below and the fermentation takes place predominantly at low pH values. The multiplication factor of *L. sanfranciscensis* and *C. humilis* as a function of the amount of sourdough inoculum used to start the fermentation is shown in Fig. 3. The multiplication factor was defined as final cell counts/initial cell counts. When different organisms are present in a sourdough resulting from repeated inoculation, a stable microflora over extended periods of time is only achieved when the multiplication factors of the individual organisms are equal. Low amounts of inoculum favoured growth of lactobacilli over yeast growth (Fig. 3). An inoculum size between 2 and 5% resulted in comparable multiplication factors for lactobacilli and yeasts, and thus the ratio of lactobacilli to yeasts remained stable. When the fermentation was started with 50% inoculum, corresponding to an initial pH of 4.1, the growth of lactobacilli was inhibited whereas yeasts multiplied by a factor of 3.5.

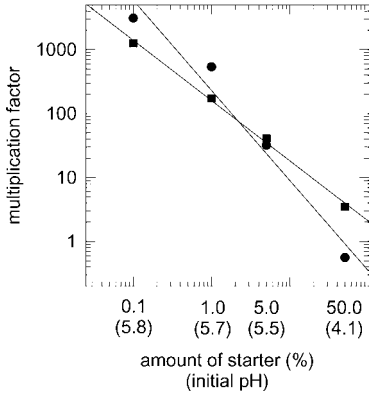

Fig. 3 Effect of the amount of sourdough inoculum on the multiplication factor of *L. sanfranciscensis* LTH 2581 (●) and *C. humilis* LTH H198 (■) during sourdough fermentation. The multiplication factor was calculated as the ratio of final cell counts to initial cell counts

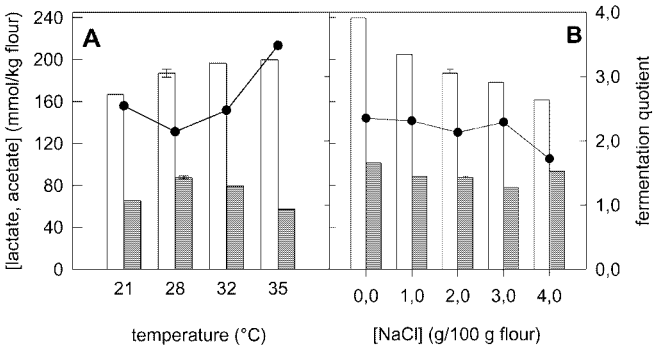

Fig. 4a,b Effect of NaCl and temperature on lactate and acetate formation in dough, and on the resulting fermentation quotient (FQ). Depicted are the concentrations of lactate (❑) and acetate (■), and the resulting FQ (●) after 24 h of a rye sourdough fermentation at various temperatures (**a**) or NaCl concentrations (**b**)

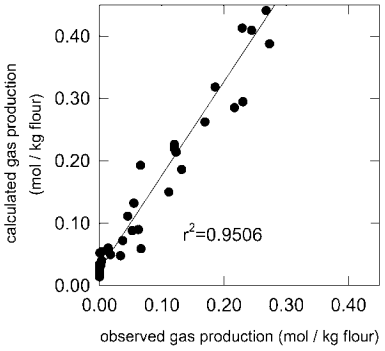

Fig. 5 Correlation of gas production in rye sourdoughs determined experimentally with the theoretical CO_2 production calculated from the concentrations of lactate, acetate, and ethanol

Effect of temperature and NaCl on metabolism of *L. sanfranciscensis*

The study of microbial growth described above needs to be supported by data on the metabolic activity of the microflora, as it is this property which finally affects the quality attributes of bread. Major products of the carbohydrate metabolism of lactobacilli are lactic acid, acetic acid, and ethanol. Lactate and acetate in dough originate exclusively from the metabolism of lactobacilli, whereas ethanol is also produced by yeasts. The effect of temperature on the final concentrations of lactate and acetate is shown in Fig. 4a. An increase of the fermentation temperature from 21 to 28 °C resulted in an increase of lactate and acetate concentrations and a slight decrease of the FQ. On a further increase to 35 °C, a decrease of the acetate concentration was observed, resulting in an increased FQ. Whereas 87 mmol g^{-1} acetate was formed at 28 °C, 57 mmol g^{-1} only was formed after fermentation at 35 °C. Remarkably, the acetic acid production in dough correlates to the growth rate of *C. humilis* (Fig. 2a) although acetate is not a product of yeast metabolism.

The effect of NaCl on the production of organic acids and the FQ are shown in Fig. 4b. Increasing salt contents of dough resulted in decreased lactate concentrations, whereas the acetate concentration remained essentially unaffected. Correspondingly, a decrease in FQ was obtained in doughs with enhanced NaCl levels.

Determination of carbon dioxide production in doughs

CO_2 produced by sourdough lactobacilli and yeasts contributes to dough leavening. The CO_2 levels produced by the sourdough microflora were determined and compared to the theoretical CO_2 production that was additionally calculated based on the concentrations of lactate, acetate, and ethanol in dough based on the following considerations. During heterofermentative metabolism by *L. sanfranciscensis*, one mole of CO_2 and one mole of (acetate+ethanol) per mole of lactate are formed. Thus, the amounts of CO_2 and ethanol from *L. sanfranciscensis* ($[CO_2]_{lsf}$ and $[ethanol]_{lsf}$, respectively) can be derived:

$$[\mathbf{lactate}] = [CO_2]_{lsf} = [\mathbf{acetate}] + [ethanol]_{lsf} \quad (1)$$

C. humilis produces one mole of CO_2 per mole of ethanol. Thus, CO_2 from *C. humilis* $[CO_2]_{ch}$ can be calculated:

$$[\mathbf{acetate}] + [\mathbf{ethanol}] - [\mathbf{lactate}] = [ethanol]_{ch} = [CO_2]_{ch} \quad (2)$$

The metabolite concentrations printed in bold are derived from the HPLC analysis where the concentration of the metabolites at time zero was subtracted. Metabolite concentrations printed in italics are calculated values.

In order to verify the suitability of the approach to calculate the total CO_2 production based on the concentration of other metabolites, the calculated total amounts of CO_2 were compared to the gas production determined experimentally (Fig. 5). The good correlation of experimental to theoretical values (r^2=0.95) confirms the suitability of this approach. It has to be taken into account that the calculated CO_2 production is generally greater than the measured gas volume because dissolved CO_2 and gas compressed inside the dough were not measured.

Table 1 shows the contribution of *L. sanfranciscensis* LTH 2581 and *C. humilis* LTH H198 to the production of CO_2 in sourdoughs fermented under various conditions. Under standard conditions, both organisms contributed equally to the overall formation of CO_2. The formation of CO_2 was correlated to the growth of the organisms, as was observed for the formation of lactate and ethanol. The CO_2 formation by *L. sanfranciscensis* was highest at those parameter levels favouring its growth, i.e. a high pH, a low amount of inoculum, a low NaCl concentration, and a temperature of 32 °C. The CO_2 formation of *C. humilis* was optimal at 28 °C and 0% NaCl. In accordance with the strong and weak effects of temperature and NaCl, respectively, on yeast growth, an increase of the fermentation temperature and salt content decreased CO_2 for-

Table 1 Production of CO_2 by *L. sanfranciscensis* LTH 2581 and *C. humilis* LTH H198 after 24 h of fermentation. The data were calculated from the concentrations in dough of lactate, acetate, and ethanol as described in the text

CO_2 (mmol/kg flour)		Amount of starter (%)			Temperature (°C)			[NaCl] (g/100 g flour)			Initial pH			
	Std.[a]	0.1	1	50	21	32	35	0	1	4	5.0	4.5	4.0	3.5
CO_2 from *L. sanfranciscensis*	167	204	202	111	157	187	183	221	205	136	134	154	90	53
CO_2 from *C. humilis*	219	156	242	143	121	178	101	302	219	261	194	163	171	180
Total CO_2	386	361	444	254	278	365	284	523	424	397	328	318	260	234
Contribution of *L. sanfranciscensis* (%)	43	57	46	44	56	51	64	42	48	34	41	49	35	23

[a] Standard condition (5% starter, 28 °C, 2% NaCl, initial pH of 5.5)

mation. Furthermore, a decreased pH moderately lowered CO_2 formation by *C. humilis*.

Discussion

We determined the effect of temperature, NaCl, and pH on the growth and metabolism of *C. humilis* and *L. sanfranciscensis*, which are two representative microorganisms of the sourdough microflora, in combined culture in rye sourdoughs. The experiments were designed to evaluate the suitability in bakery practice of a mathematical model for growth of these organisms [13]. This mathematical model described the growth of *L. sanfranciscensis* and *C. humilis* in laboratory media as single cultures. Here, model predictions were validated for growth in rye doughs in co-culture. The effects of temperature and pH on the growth rates in sourdough observed in this study were in very good qualitative and quantitative agreement with the model predictions. The in vitro data of μ_{max} obtained by Gänzle et al. [13] and the in situ data described here had a correlation coefficient r^2 of 0.78 (data not shown). Growth of *C. milleri* and *L. sanfranciscensis* was optimal at 28 and 32 °C, respectively, and the growth of *L. sanfranciscensis* was optimal above pH 5.0 and inhibited below pH 4.0. Comparable effects were observed when the dough pH was adjusted by using high or low amounts of preferment to start the fermentation, or when the dough pH was adjusted with HCl. This is in agreement with the hypothesis that dough pH rather than the level of organic acids is inhibitory to *L. sanfranciscensis* [13]. A quantitative comparison of the effect of NaCl in dough with those data obtained previously is not feasible, since minerals originating from flour increase the ionic strength in addition to NaCl levels. Nevertheless, a good qualitative agreement with the model predictions concerning NaCl effects on growth of *L. sanfranciscensis* and *C. milleri* was observed.

The effects of temperature, NaCl, and sucrose on the growth of a complex microflora consisting of several yeasts and four *Lactobacillus* species was investigated in wheat sourdoughs by Simonson et al. [20]. These authors reported that a variation of the temperature in the range of 15–27 °C did not induce major shifts in the ratio of yeasts to lactobacilli, comparable to results described here. Whereas in our studies 4% NaCl was inhibitory to *L. sanfranciscensis*, Simonson et al. reported that 4% NaCl had no inhibitory effect on the growth of lactobacilli. It was shown that salt-tolerant homofermentative lactobacilli present in low numbers became dominant in sourdough at high NaCl levels [20]. Likewise, *L. sanfranciscensis* is replaced by acid-tolerant or thermophilic lactobacilli through the continuous use of a high inoculum or fermentation temperatures above 35 °C [21].

We took into account the effects of process parameters on the metabolic activity of the sourdough microflora in addition to the effects on the microbial growth. Generally, the effect of process parameters on metabolites from *C. humilis* and *L. sanfranciscensis* was correlated with the growth of the organisms. However, a basic metabolic activity was also observed at conditions where growth was inhibited. For example, metabolic activity of *L. sanfranciscensis* and *C. humilis* was apparent at a pH of 3.5 and 35 °C, respectively, although growth was fully inhibited under those conditions.

NaCl and temperature affected the ratio of acetate to lactate formed by *L. sanfranciscensis*. Based on the biochemical pathway employed by *L. sanfranciscensis* for hexose utilisation, acetate formation by *L. sanfranciscensis* in dough is mainly dependent on the presence of fructose [22]. In the presence of fructose, acetate is the major metabolite and only after depletion of suitable electron acceptors is ethanol formed instead [14, 23]. Consequently, at the early stages of sourdough fermentation where fructose is available, the FQ is close to or equal to 1 and is increased only later during fermentation after fructose and other electron acceptors are depleted (this study). The preferential formation of acetate by *L. sanfranciscensis* also provides an explanation for the decreased FQ at high NaCl levels in dough. The high salt concentrations apparently reduced the pH tolerance of *L. sanfranciscensis*, resulting in decreased lactate and ethanol formation; however, acetate formation in the early stages of fermentation was unaffected.

The fermentation temperature does not affect the FQ when *L. sanfranciscensis* is grown in a single culture. However, decreased acetate levels were observed when sourdough was fermented at high temperature. This observation is in agreement with the results of previous studies on metabolite formation in sourdoughs with a

complex microflora [24]. In wheat and rye flours, the levels of fructose are low and fructose is released during fermentation from sucrose and fructose-oligosaccharides (FOS) through the activity of yeast invertase. Because *L. sanfranciscensis* is unable to hydrolyse FOS and many strains, including *L. sanfranciscensis* LTH 2581, are unable to hydrolyse sucrose, acetate formation in dough depends on yeast invertase activity [25]. Therefore, temperatures inhibiting yeast growth reduce acetate levels in dough and result in an increased FQ.

Although *C. humilis* contributed to only 3% of the total cell counts in fermentations under standard conditions, about 50% of the total carbon dioxide formed in dough originated from the yeast metabolism. This observation can be attributed to the fact that two moles of CO_2 per hexose are formed by *C. humilis*, compared to one CO_2 per hexose from *L. sanfranciscensis,* and that the metabolic activity is proportional to the cell surface area rather than to cell counts. The surface area of a cell of *C. humilis* can be estimated to exceed the surface area of a cell of *L. sanfranciscensis* approximately 20 times [26].

In conclusion, the data presented here and by Gänzle et al. [13] provide a useful tool to control the composition and metabolic activity of the microflora present in type I sourdoughs through the deliberate choice of the process parameters. As a complex microflora rather than two defined strains are present in commercial sourdoughs, the continuous use of conditions inhibitory to either *L. sanfranciscensis* or *C. milleri* will induce shifts in the sourdough microflora, favouring growth of other *Lactobacillus* species more adapted to the altered process conditions.

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