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Biodiversity and lifestyle of *Fructilactobacillus sanfranciscensis* and its interaction with yeasts in sourdough

Esther Rogalski

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“Without bread all is misery.” – William Cobbett, British journalist

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1 Abstract

Sourdough is a fermented mixture of flour and water. Lactic acid bacteria and yeast drive the fermentation enabling baked goods with superior sensorial properties and a long shelf live. A competitive key species in traditional sourdoughs is *Fructilactobacillus (F.) sanfranciscensis*. The strain-specific dominance in the sourdough as well as their interaction with yeasts like *Saccharomyces (S.) cerevisiae* and *Kazachstania (K.) humilis* are still unresolved. To follow strain-specific microbiota dynamics, a strain-specific differentiation system based on the clustered regularly interspaced short palindromic repeats (CRISPR) locus length polymorphism of the *F. sanfranciscensis* strains was developed. To discover the intra-species behavior of *F. sanfranciscensis* in rye sourdough fermentations and the influence of the presence/absence of yeasts competitive trials were performed in the sourdough. Eight different strains were sorted into two different strain sets. These strain sets were inoculated in rye sourdough without yeasts, with *S. cerevisiae* TMW 3.1064 and with *K. humilis* TMW 3.1034. It was possible to sort the eight *F. sanfranciscensis* strains into three different groups according to their behavior:

Group A: Three of the strains were dominant in the fermentation independently of the yeast inoculation.

Group B: Three strains were dominant in the fermentation depending on the yeast inoculation. *F. sanfranciscensis* TMW 1.392 was only dominant in the absence of yeasts and TMW 1.907 and TMW 1.2137 were dominant together with *S. cerevisiae* TMW 3.1064.

Group C: Two strains could not reach dominance under any condition.

Comparative genomics and metabolic studies of the *F. sanfranciscensis* strains revealed that the main differences are related to the metabolism of carbohydrates and the usage of external electron acceptors. These differences were determinative for the strain-specific behavior in the sourdough fermentation. These results show that a rather commensal or competitive interaction of *F. sanfranciscensis* with the yeasts *K. humilis* or *S. cerevisiae,* respectively, should be assumed than a mutualistic one. Furthermore, genetic analysis of the *F. sanfranciscensis* strains referred their putative origin to a maltose/sucrose/fructose-rich oxic environment, as it can be found on flowers and in insects. The elucidation of the mechanisms behind the interactions of these *F. sanfranciscensis* strains and the yeasts is decisive for product development, as these mechanisms determine a stable interaction of the sourdough microbiota. Ultimately, a stable sourdough microbiota leads to a stable sourdough product.

2 Zusammenfassung

Sauerteig ist eine fermentierte Mischung aus Mehl und Wasser. Die Fermentation mit Milchsäurebakterien und Hefe ermöglichen die Herstellung von Backwaren mit einem mild-saurem Geschmack und langer Haltbarkeit. Ein wettbewerbsfähiger Schlüsselorganismus in traditionellen Sauerteigen ist *Fructilactobacillus (F.) sanfranciscensis*. Die Dominanz einzelner Stämme dieser Spezies im Sauerteig sowie deren Wechselwirkung mit Hefen wie *Saccharomyces (S.) cerevisiae* und *Kazachstania (K.) humilis* sind noch ungeklärt. Zur Verfolgung der mikrobiellen Dynamik auf Stammebene wurde ein stammspezifisches Differenzierungssystem entwickelt, das auf dem CRISPR-Locus-Längenpolymorphismus der *F. sanfranciscensis*-Stämme basiert. Um das speziesinterne Verhalten von *F. sanfranciscensis* bei Roggensauerteigfermentationen sowie den Einfluss von Hefen zu untersuchen, wurden kompetitive Experimente im Sauerteig durchgeführt. Acht verschiedene Stämme wurden in zwei verschiedene Stammsätze sortiert. Diese Stammsets wurden in Roggensauerteig ohne Hefen mit *S. cerevisiae* TMW 3.1064 und mit *K. humilis* TMW 3.1034 inokuliert. Die acht *F. sanfranciscensis*-Stämme konnten nach ihrem Verhalten in drei verschiedene Gruppen sortiert werden:

Gruppe A: Drei Stämme dominierten die Fermentation unabhängig der Anwesenheit von Hefen.

Gruppe B: Drei Stämme dominierten die Fermentation in Abhängigkeit der Hefeinokulation. *F. sanfranciscensis* TMW 1.392 war nur in Abwesenheit von Hefen durchsetzungsfähig und TMW 1.907 und TMW 1.2137 waren zusammen mit *S. cerevisiae* TMW 3.1064 durchsetzungsfähig.

Gruppe C: Zwei Stämme waren nicht durchsetzungsfähig.

Vergleichende Genomik und metabolische Studien der *F. sanfranciscensis*-Stämme zeigten, dass die Hauptunterschiede mit dem Metabolismus von Kohlenhydraten und der Verwendung externer Elektronenakzeptoren zusammenhingen. Diese Unterschiede bestimmten das stammspezifische Verhalten bei der Sauerteigfermentation. Basierend auf diesen Ergebnissen wird eine kommensale bzw. kompetitive Wechselwirkung von *F. sanfranciscensis* mit den Hefen *K. humilis* und *S. cerevisiae* mehr als eine mutualistische Wechselwirkung angenommen. Darüber hinaus deutet die genomische Analyse der *F. sanfranciscensis*-Stämme auf einen Ursprung aus einer Maltose/Saccharose/Fructose-reichen, oxischen Umgebung, wie er auf Blumen und Insekten zu finden ist. Die Aufklärung der Mechanismen zu erforschen, die hinter den Wechselwirkungen von *F. sanfranciscensis* und den Sauerteighefen stehen, ist eine

entscheidende Grundlage für die Produktentwicklung, da diese Mechanismen eine stabile Wechselwirkung der Sauerteig-Mikrobiota bestimmen. Letztlich ist eine stabile Sauerteig-Mikrobiota Voraussetzung für ein stabiles Sauerteigprodukt.

3 Introduction

3.1 Sourdough today

3.1.1 Facts about sourdough

Bread is one of the few foods, which is common in many societies (Lönner and Ahrné, 1995; Neysens and De Vuyst, 2005). In the European Union (EU) two million people are employed in the bakery sector. With more than 190.000 small manufacturing entrepreneurs and 2.200 large companies up to 79 billion EUR were earned in the EU in 2014. The average EU citizen consumed 50 kg bread per year in 2014 (Bread Initiative, 2016). In Germany, private households bought 1.681.000 tons of bread with a turnover of 4.28 billion Euro. There is a broad variability in bread types with up to 3163 approved bread specialties in Germany. 73.5% of them were baked with sourdough (Deutsches Brotinstitut e.V., 2016; Brandt, 2019). Sourdough can be used for the preparation of a variety of cereal foods including breads, cakes, crackers, and pizza (Ottogalli *et al.*, 1996; Foschino *et al.*, 1999; Neysens and De Vuyst, 2005). In Italy sweet baked products out of sourdough are common in many traditional festivities like panettone cake, pandoro and Milanese cake or bisciola and more than 30% of the baked products in general are made with sourdough (Ottogalli *et al.*, 1996; De Vuyst and Neysens, 2005). The production of sourdough differs according to its purpose and the recipes for sourdough are part of the cultural and geographical identity with a broad regional variety (De Vuyst and Neysens, 2005). In Mediterranean countries, the San Francisco bay or South America wheat sourdoughs for wheat bread and cake are more common whereas in Germany, Central and Eastern Europe and Scandinavia rye, wheat, barley or mixed flours are used for sourdough (De Vuyst and Neysens, 2005).

3.1.2 Development of sourdough over the time

Fermented foods like milk, meat and cereals have a century-long history (Neysens and De Vuyst, 2005). The domestication of cereals in the Natufian cultures interleaved with the use of lactic acid bacteria for cereal fermentation less than 15,000 years ago (Hayden *et al.*, 2013; Gänzle and Ripari, 2016). The origin of sourdough is assumed in Egypt in the pharaonic period approx. 3100 to 3320 B.C. or in Switzerland where it was found 3700 B.C. (Samuel, 1996; Neysens and De Vuyst, 2005; Brandt and Gänzle, 2006; Gobbetti *et al.*, 2018). Furthermore, in Mesopotamia (today Iraq) 4000 B.C. the oldest oven was excavated as well as illustrations

about beer brewing were delivered around the same time. Making bread out of sourdough and beer brewing are strongly connected and there are discussions, which was invented first (Brandt and Gänzle, 2006). There is no doubt that fermentation with lactic acid bacteria (LABbelongs to the oldest, most traditional, and natural preservation method. Furthermore, it improves the value and nutrition of the fermented food for human diet. This fact is also true for sourdough (Gobbetti *et al.*, 2018). With the invention of the baker`s yeast in the 19th century the usage of sourdough decreased. The Baker´s yeast was able to leaven the bread faster and more efficiently than the sourdough, and freshly active sourdough is cost and time consuming (Brandt and Gänzle, 2006). In 1990 the research on sourdough began and the positive technology effects of it on shelf life (delay of stalling, spoilage prevention), flavor and rheology of baked goods as well as the complex microbiota of sourdough were investigated (Gobbetti *et al.*, 2018). These advantages of sourdough like the prolonged shelf life and the sensory quality of the baked goods leads to it`s come back (Decock and Cappelle, 2005). Furthermore, the consumers trend towards clean labels, natural and organic products and a clever marketing strategy for traditional baking, sourdough is raising again (De Vuyst and Neysens, 2005; Gänzle *et al.*, 2008; Brandt, 2019).

In traditional bakeries the sourdough fermentation is still used, and the metabolically active sourdough was propagated over decades. Moreover, sourdough baking is still practiced in private households (De Vuyst and Neysens, 2005). During the Corona pandemic in 2020 sourdough was getting more attention in private households as the yeast was sold out (Delap, 2020). In artisanal bakeries sourdough is coming back. In artisanal bakeries the sourdough can be made freshly by continuous propagation which is time consuming and effort for the companies as the sourdough needs to be propagated also during the night. Although, a commercial sourdough starter can be applied. The production of a commercial wheat sourdough starter was invented in the San Francisco Bay Area about 150 years ago. For rye sourdough the Böcker-Reinzucht-Sauer starter was developed >100 years ago (Kline and Sugihara, 1971; Böcker *et al.*, 1990). Often bakeries have one or two different homemade sourdoughs mostly wheat and rye sourdoughs, but the requirements of the consumers are increasing regarding a higher selection of baked goods and clean labels. To serve these requirements the bakeries buy ready-to-use sourdough from specialized companies, which leads to a broad variety of sourdoughs with different properties, tastes and values (Brandt, 2019). Although, all these products have the problems of stability of the microbiome and its re-activation upon long-term storage.

3.1.3 Properties of sourdough

The fermentation of flour and water with LAB and yeasts leads to the product sourdough. The metabolic end products of the fermentation with the LABs decreases the pH and results finally in a sour taste of the product. Freshly started sourdough begins with a pH between 5.0 to 6.2 and ends at a pH between 4.0 to 3.2, depending on the fermentation conditions. The fermented product is now used as mother sponge to propagate the microbiota into the freshly produced flour water mixture. This propagation with a mother sponge can be done over a long time. As mother sponge, old sourdoughs or a defined starter culture can be used (De Vuyst and Neysens, 2005; Brandt, 2019). The decrease of the pH is mainly related to the concentration of lactic acid and/or acetic acid produced by heterofermentative or homofermentative LABs (De Vuyst and Neysens, 2005). Whereas the homofermentative LABs produce mainly lactic acid, the production of acetic acid depends on the presence or absence of external electron acceptors and their utilization by heterofermentative LABs (Hammes *et al.*, 1996; Neysens and De Vuyst, 2005; D'Alessandro and De Pergola, 2014). Up to 200 mmol/liter of lactate and up to 60 mmol/liter of acetate are produced during a sourdough fermentation process (Gänzle *et al.*, 1998). The molar ratio between lactic and acetic acid is determined by the fermentation quotient (FQ). The FQ of a sourdough is influenced by the dough yield and the fermenting microbiota. Furthermore, the aroma of the baked product is influenced by the FQ. The optimal ratio in rye bread is considered between 2.0 and 2.7. Whereas in traditional Italian (wheat) sourdoughs the FQ is between 3.3 to 5.6 (Hammes and Gänzle, 1998; D'Alessandro and De Pergola, 2014). At the beginning of the sourdough fermentation the FQ is rather low due to the available fructose and oxygen. These substances can be used by heterofermentative LAB as external electron acceptors to produce acetate instead of ethanol. During the fermentation, while fructose and oxygen are depleted, the FQ increases and ethanol is produced instead of acetate (Hammes *et al.*, 1996; Neysens and De Vuyst, 2005). The whole niche sourdough is influenced by the decrease of the pH. Especially in rye bread the low pH of the sourdough leads to the activation of the rye pentosans and hence to the formation of a pentosan network in the rye bread. This is the reason why sourdough fermentation is particularly popular in northern countries with a lot of rye baked products (Brandt and Gänzle, 2006; Gänzle and Zheng, 2019). The low pH has an impact on many different factors and different proteolysis activities are triggered by the fermentation (Gänzle *et al.*, 2008). The sourdough microbiota influences the sourdough and baked goods quality (De Vuyst and Neysens, 2005). It leads to several important factors, which influence the dough and the baked goods. With the help of sourdough, the dough machinability of the bread is improved, which leads to a faster manufacturing and kneading of the dough.

Furthermore, the storage including the shelf life, texture and the stalling of the breads are increased with sourdoughs. The LABs produce antibacterial compounds, antifungal substances, and exopolysaccharides, which lead to antiropiness activities (Hammes and Gänzle, 1998; Korakli *et al.*, 2001; Korakli *et al.*, 2003; De Vuyst and Neysens, 2005). In addition, the organoleptic properties of the bread are influenced, for example by a higher bread volume, better crumb texture and a unique flavor. The unique flavor is achieved by the release of different aroma compounds and aroma precursors during the sourdough fermentation (Gänzle and Vogel, 2003; De Vuyst and Neysens, 2005). Cereal proteases are liberating amino acids. These amino acids are used by the microbiota and are converted into volatiles, which influence the taste and the flavor of the bread (Brandt *et al.*, 2004; Gänzle *et al.*, 2008). For example, the arginine metabolism of some sourdough lactobacilli leads to the roast flavor of baked goods (De Angelis *et al.*, 2002; De Vuyst and Neysens, 2005). As mentioned above, the decreased pH of the sourdough generates the activation and solubilization of the rye pentosanes. Rye flour has a minor amount of gluten and the activated pentosans fulfill the tasks of gluten instead. Consequently, the pentosans are important for the dough and gas hydration and for the binding capacity of water, which leads to an increased bread volume (Martinez-Anaya and Devesa, 2000; Brandt *et al.*, 2004; De Vuyst and Neysens, 2005; Loponen *et al.*, 2009; Gänzle, 2014). Besides, the proteolytic activity of the cereal enzymes initiated by the low pH increases the nutritional properties and bioavailability of the cereals and the baked product. Furthermore, the hydrolysis of phytate leads to a higher mineral availability of the dough. In conclusion the fermentation of the sourdough by the LAB leads to a more tolerable product (Hammes and Gänzle, 1998; Di Cagno *et al.*, 2002; De Vuyst and Neysens, 2005; Gänzle *et al.*, 2008; Bartkiene *et al.*, 2020). Moreover, the bioavailability of starch is decreased due to the interaction of starch and gluten and the inhibition of amylolytic enzymes (D'Alessandro and De Pergola, 2014). This inhibition leads to a slower abortion of starch by LAB and has several health benefits (D'Alessandro and De Pergola, 2014). As a consequence the starch digestibility is reduced which lowers the glycemic index (GIof the bread (Gobbetti *et al.*, 2018).

Not only the low pH of the sourdough has an influence on the baked goods but also the dough yield (DY), which is calculated as following:

$$DY = (flour + water) * 100/flour$$

The DY describes the hydration of the sourdough and influences the progress and outcome of the sourdough fermentation (De Vuyst and Neysens, 2005). It has also an impact on the acidity of the sourdough and thus on the microbial diversity (Di Cagno *et al.*, 2014).

3.1.4 Different types of sourdough

There are different types of sourdoughs depending on their production type and microbiota. Sourdough type 0 is mainly composed of yeast and wheat flour. The fermentation time differs from 1 to 24 h. The yeast in the dough is important for the leavening of the dough due to its CO_2 production. Although, during long fermentation times up to 24 h lactic acid bacteria arise. So, this dough is a link between type 0 and type 1 sourdoughs.

Type 1 sourdoughs are also traditional sourdoughs with an active microbiota. This microbiota contains mainly LAB like *F. sanfranciscensis*, *Lactiplantibacillus (Lp.*) *plantarum*, *Limosilactobacillus (Li.) pontis*, *Levilactobacillus (Le.) brevis* in cell counts up to 10^9. Two log steps lower yeasts like *Saccharomyces (S.) cerevisiae*, *Kazachstania (K.) humilis* or *K. exigua* also occur in the sourdoughs type 1. This dough is used for leavening of the bread and acidification of the dough. It has a medium acidification down to pH of 4 and medium fermentation times between 4 to 16 h with a medium fermentation temperature. Traditional rye bread is fermented with sourdough in three steps with fresh sour, basic sour, and full sour. The new sourdough is inoculated with the old one, which fosters the continuity of the microbiota. Consecutive re-inoculation of a new batch with the previous batch results in an indirect fermentation with refreshments (back-slopping). Rye and wheat flour are used, or meanwhile other cereals are employed like quinoa for gluten-free sourdoughs. Examples are San Francisco sourdough French bread, Panettone, Briochs, Pugliese and three stage sourdough rye bread (De Vuyst and Neysens, 2005). These sourdoughs are developed from spontaneous fermentation or from defined sourdough starters. Furthermore, they have a stable microbiota and are resistance against contaminants (De Vuyst and Neysens, 2005).

Sourdoughs of the fermentation type 2 are liquid doughs with a long fermentation time up to 5 days with fermentation temperatures up to 43°C. These temperatures accelerate the fermentation process (De Vuyst and Neysens, 2005; Brandt and Gänzle, 2006). As a consequence, they harbor a completely different ecosystem than type 1 sourdoughs. Mostly obligately homofermentative LAB like *Lactobacillus (L.) acidophilus*, *L. delbrueckii*, *L. amylovorus*, *Companilactobacillus (Cl.) farciminis*, and *L. johnsonii*, and obligately heterofermentative *Le. brevis*, *Li. fermentum*, *Li. frumenti*, *Li. pontis*, *Li. panis*, *Li. reuteri*, *L. rossiae* as well as *Weissella (W.)* i.e., *W. confusa* (Vogel *et al.*, 1999; Müller *et al.*, 2001; De Vuyst and Neysens, 2005). These species need to be tolerant to high temperature and high acidity. The sourdough is fermented to the late stationary phase and the microorganisms suffer from a restricted metabolism. These type 2 sourdoughs are semi-fluid silo preparations, suitable

for large scale sourdough production by suppliers and large baking companies. The sourdough is mainly added to the dough for souring activity and because of the long one-step fermentations companies have more flexibility (Brandt and Gänzle, 2006; Brandt, 2019).

Sourdoughs of the type 3 are dried sourdoughs, which can be used as sourdough starters or directly for the bread baking. Theses sourdoughs are mainly interesting for bakers as aroma carriers. They are more standardized end products than active starters. These sourdoughs are not able to leaven the dough although they increase the shelf life of the baked good. Dry resistant microorganisms like *Le. brevis* or facultative heterofermentative *Lp. plantarum* strains are present (Gobbetti, 1998; De Vuyst and Neysens, 2005).

The focus of this work lies on the sourdough type 1 with an active microbiota. In these sourdoughs *F. sanfranciscensis* is the key bacterium, which arises as specialist in these doughs. Changes in the environment or the culture conditions lead to changes in microbiota and so to the quality of the sourdough. Especially the metabolically active sourdough is sensitive to changes in the environment. This product is applied in many products and it is also the starter for type-3-sourdough. A basic understanding is required to enable a knowledge-based product development with a stable microbiota.

3.2 The microbiota of the sourdough

The microbiota of sourdough consists out of LAB and yeasts in the ratio of 1:10 or 1:100 depending on the fermentation type and conditions (Ottogalli *et al.*, 1996; Gobbetti, 1998; De Vuyst and Neysens, 2005; Brandt and Gänzle, 2006; Bartkiene *et al.*, 2020; Comasio *et al.*, 2020). The α-diversity describing the microbial diversity in one single batch of sourdough is relatively low. One sourdough harbors up to six different species or strains in one single fermentation. The γ-diversity describes the diversity of species and strains, which were found in all different sourdough fermentations around the world is high (Gänzle and Ripari, 2016). Up to now 90 different species of LAB and more than 40 species of yeasts were found (De Vuyst and Neysens, 2005), while it remains widely unknown, which of these are autochthonous or major drivers of the fermentation. The composition of LAB and yeasts species depends on a multitude of different factors. On the one hand process parameters like temperature, redox potential, ionic strength, pH and dough yield and on the other hand artisan and region-dependent handling of the sourdoughs influence the composition of the microbial community (De Vuyst and Neysens, 2005). In the sourdough fermentations homofermentative and heterofermentative LAB were

found, whereas heterofermentative LABs are dominating the fermentation process (Kline and Sugihara, 1971; Corsetti *et al.*, 2003). This composition contrasts with many other food fermentations were homofermentative LABs are dominating the process. Mostly the former genus *Lactobacillus* is found in sourdough fermentations followed by species from the genus *Leuconostoc*, *Weissella* and *Pediococcus* (De Vuyst and Neysens, 2005). In spontaneous sourdough fermentations acetic acid bacteria were found but they do not dominate the final fermentation because of their aerobic metabolism (Comasio *et al.*, 2019).

In spontaneous sourdough fermentations homofermentative LAB like *Lacticaseibacillus (Lc.) casei*, *L. delbrueckii, Cl. farciminis*, *Lp. plantarum* and heterofermentative LAB like *Le. brevis*, *Lentilactobacillus (Lt.) buchneri*, and *Li. fermentum* as well es *Pediococci* like *Pediococcus (P.) acidilactici*, *P. pentosaceus* are rapidly dominating over gram-negative enterobacteria (De Vuyst and Neysens, 2005). In these sourdoughs the yeasts *S. turbidans, S. marchalianus, T. albida, K. exigua, S. cerevisiae*, and *Saturnispora saitoi* are isolated. Thereby, there is no difference between rye and wheat sourdough fermentations. In sourdoughs with backslopping events the microbiota differs as the heterofermentative species are dominating the fermentation process. Heterofermentative LAB like *F. sanfranciscensis, Li. pontis*, *Li. panis*, *Cl. paralimentarius*, *Li. frumenti*, *Le. brevis*, *F. fructivorans* are isolated as well as facultative heterofermentative LAB like *Lp. plantarum* and obligate homofermentative like *Cl. mindensis.* The yeasts in propagated sourdoughs belong to the species *Saccharomyces* i.e. *S. cerevisiae* and *Kazachstania* i.e. *K. humilis* (Kline and Sugihara, 1971; Vogel *et al.*, 1994; Vogel *et al.*, 1999; Ehrmann *et al.*, 2003; De Vuyst and Neysens, 2005). Stable in household environments are bacterial species like *Le. brevis* and *Lp. plantarum* together with *S. cerevisiae* (Gänzle and Zheng, 2019; Comasio *et al.*, 2020). Whereas, in Italian households *F. sanfranciscensis* and *Lp. plantarum* were isolated out of a single sourdough fermentation (Gobbetti, 1998). Although, also uncommon LAB species were isolated out of sourdoughs, in Great Britain *Lc. paracasei* was once isolated out of lambic brewery sourdoughs unless it is not common in the lambic beer production (De Vuyst *et al.*, 2017; Comasio *et al.*, 2020). In conclusion, *Lp. plantarum* and *F. sanfranciscensis* were found in more than 50% of the investigated sourdoughs (De Vuyst *et al.*, 2017; Comasio *et al.*, 2020) and appear to be autochthonous.

The dominance of heterofermentative LAB in the sourdough environments is established by their more competitive adaption to this specific niche. Their metabolic equipment fits to the conditions in the sourdough environment based on the carbohydrate usage and growth requirements (De Vuyst and Neysens, 2005). Consequently, they can cope with different energy sources present in the sourdough fermentation like maltose, fructose, glucose and sucrose and they can use

fructose as external electron acceptor in the phosphoketolase pathway or pentose phosphate shunt. Together with the stinting of ATP in the maltose phosphorylase reaction, they can obtain a higher energy yield out of the carbohydrates than the homofermentative LAB with the maltose degradation (Stolz *et al.*, 1995; Hammes *et al.*, 1996; De Vuyst and Neysens, 2005). In the case of *F. sanfranciscensis* its growth conditions match the conditions in most type 1 sourdough fermentations due to temperature, pH, oxygen and backslopping events. Furthermore, heterofermentative LABs isolated out of the sourdough environment have several mechanisms to cope with low pHs, high/low temperatures, high osmolarity, oxygen and starvation (De Angelis *et al.*, 2001; De Vuyst and Neysens, 2005). The production of antimicrobial compounds against other bacterial species as well as the production of organic acids and proteinaceous compounds are further reasons for their dominance in the sourdough fermentation (Gobbetti, 1998; Hammes and Gänzle, 1998; Gänzle and Vogel, 2003).

As aforementioned, in a well-established sourdough fermentation no more than six different species are harbored at relevant numbers. Normally three or less LABs and one or two different yeast are present in on single batch of sourdough (Comasio *et al.*, 2020). This combination can be explained by the complex and competitive ecosystem prevailing in the niche sourdough. In this ecosystem the interactions between the microorganisms can base on mutual relationships, metabolic interactions, and matrix-specific adaptions (De Vuyst and Neysens, 2005; De Vuyst *et al.*, 2017; Van Kerrebroeck *et al.*, 2017). However, the composition of the microbiota also depends on the process technology and the inoculation with a specific starter culture (Gänzle and Ripari, 2016). Under stable fermentation conditions the microbial consortia is constant and stable during several decades although they run under non-sterile conditions (De Vuyst and Neysens, 2005). However, the complexity of a sourdough fermentation and the effect of nutritional requirements and growth parameters on the microbiota need an immense knowledge to gain stable fermentation conditions (Brandt *et al.*, 2004). The microbial community is more affected by non-flour parameters like the pH, DY and the temperature than by the nature of the flour or the geographical region (Vogel *et al.*, 1999; De Vuyst *et al.*, 2014; Di Cagno *et al.*, 2014; Lin and Gänzle, 2014a; Van Kerrebroeck *et al.*, 2017; Comasio *et al.*, 2020). Only the selection for bran, whole flour or white flour alters the buffering capacity of the dough and so the microbial consortia (Meroth *et al.*, 2003). Consequently, the microbial community is mostly selected by the process parameters. At high temperatures with long fermentations times, which is mostly done in sourdough of type 2 fermentations, the microbiota is selected for LAB of the genus *Limosilactobacillus*. As they are also adapted to warm-blooded animals. At ambient temperatures and with short fermentation times the microbiota is selected for

F. sanfranciscensis. As this organism favors ambient temperatures and develops a rapid growth under these conditions (Gänzle *et al.*, 1998; Meroth *et al.*, 2003; Oh *et al.*, 2010; Gänzle and Ripari, 2016). Furthermore, low temperatures between 20°C to 26°C are also more favorable for the development of the sourdough yeasts (Baker's rule) (Gänzle *et al.*, 1998). These facts serve as explanation for the dominance of *F. sanfranciscensis* and the presence of sourdough yeasts in type 1 sourdough fermentations (Böcker *et al.*, 1995; Gänzle *et al.*, 1998; Corsetti *et al.*, 2001; Foschino *et al.*, 2001). *F. sanfranciscensis* is mostly found in artisan sourdoughs in France, Italy, Germany, or the US as it needs stable fermentation conditions (Kline and Sugihara, 1971; Lhomme *et al.*, 2015a; Lhomme *et al.*, 2016; Comasio *et al.*, 2020). In Germany the yeast *K. humilis* is often isolated together with *F. sanfranciscensis* and *Li. pontis* (Gänzle *et al.*, 1998). The same occurrence was found in sourdoughs of the San Francisco Bay Area and in panettone dough where *F. sanfranciscensis* and *K. exigua* are exclusively isolated (Kline and Sugihara, 1971; Foschino *et al.*, 1999; Comasio *et al.*, 2020). Consequently, in combination with the yeasts *K. humilis*, *K. exigua,* and *S. cerevisiae F. sanfranciscensis* belongs to the key species in the type 1 sourdough fermentations (Kline and Sugihara, 1971; Böcker *et al.*, 1995; De Vuyst and Neysens, 2005). However, it is not yet known how these species interact and where the distinct microbiota of the sourdough fermentation originates from.

3.3 *F. sanfranciscensis* a key bacterium in sourdough fermentations

3.3.1 Properties of F. sanfranciscensis and its impact on sourdough

For more than 100 years *F. sanfranciscensis* was propagated in sourdoughs out of the San Francisco Bay Area until it was found by Kline and Sugihara (1971). Although, it took several years prior to its introduction into the approved lists of bacterial names by Weiss and Schillinger (1984) as *L. sanfranciscensis*. 2020 it was reclassified by Zheng *et al.* (2020) into *F. sanfranciscensis* based on its preference to use fructose as external electron acceptor. *F. sanfranciscensis* is the key bacterium of type 1 sourdoughs (Vogel *et al.*, 2011). It is an obligately heterofermentative, gram positive and non-spore forming LAB with lactic and ethanol/acetic acid as well as carbon dioxide as end products. Furthermore, it has one of the smallest genomes within the LAB of approx. 1.23 Mbp together with a low GC content of 36-38% and a high density of ribosomal RNA operons per Mbp genomes (Vogel *et al.*, 2011; Sun *et al.*, 2015). Which is also remarkable as it is the highest ratio among all genomes of known free living bacteria. This incidence explains the rapid growth of the organism in the sourdough habitat which helps to outcompete other sourdough bacteria (Vogel *et al.*, 2011). It is closely related to

the beer spoiling organisms *F. lindneri* and *F. fructivorans* and *F. florum,* which were found on blossoms (Ehrmann and Vogel, 2005; Fraunhofer, 2018; Zheng *et al.*, 2020). Furthermore, *F. sanfranciscensis* has a restricted metabolic potential and its metabolism appears as perfectly adapted to the sourdough environment (Vogel *et al.*, 2011; Gänzle and Zheng, 2019). *F. sanfranciscensis* properties influence the generation of the sourdough. It is important for the souring activity of the dough and due to the phosphoketolase pathway it also produces carbon dioxide for the leavening of the dough (Gobbetti and Corsetti, 1997a; De Vuyst and Neysens, 2005). It is resistant to a low pH down to 4 although it does not convert arginine and glutamate, which is the species-specific acid resistance mechanism of *Li. reuteri* (Gänzle *et al.*, 1998; Zheng *et al.*, 2015). Moreover, due to the souring activity of *F. sanfranciscensis* proteolytic enzymes gain activity, which leads to the liberation of precursors of volatile compounds (Thiele *et al.*, 2002; Vermeulen *et al.*, 2005). Considering its catabolism of amino acids, synthesis of exopolysaccharides, proteolytic activity, and quality of acidification it affects positively the microstructure of bread (De Angelis *et al.*, 2002; Arendt *et al.*, 2007).

However, its dominance in type 1 sourdoughs is not only based on its positive effect on the dough quality but also on its properties to outcompete other organisms in the sourdough (Vogel *et al.*, 2011; Sun *et al.*, 2015). Its small genome and restricted metabolic potential is adapted to the sourdough environment. It prefers maltose as main carbohydrate source, and its metabolism is not affected by the glucose repression present in other sourdough LAB. The glucose gained by the cleavage of maltose to glucose and glucose-1-phosphate is secreted outside of the cell (Gobbetti and Corsetti, 1997b; Korakli *et al.*, 2001; Vogel *et al.*, 2002). Besides, it is capable of formation of amino acids, which are scarcely availably in cereals and in the niche sourdough like aspartate and asparagine (De Angelis *et al.*, 2002; Vogel *et al.*, 2011; Lhomme *et al.*, 2016). However, genes for extracellular proteases are missing as they would not be of any advantage in the sourdough because the hydrolysis is performed by endogenous flour proteases (Lhomme *et al.*, 2016). *F. sanfranciscensis* is able to cope with oxidative stress mediated by thiols through the employment of several transporters like the cysteine/cystine transporters and the glutamate dehydrogenase (Jänsch *et al.*, 2007; Stetina, 2014). In addition, oxygen is exploited by the NADH oxidase Nox2, which enables enhanced growth under aerobic conditions by regeneration of NAD (Vogel *et al.*, 2002). Also, the formation of exopolysaccharides (EPS) from sucrose to fructan or glucan with levan- and glucansucrases is detected in *F. sanfranciscensis* (Tieking *et al.*, 2003; Tieking *et al.*, 2005a; Morita *et al.*, 2008). In 75% of the sourdoughs worldwide *F. sanfranciscensis* is detected with different physicochemical characteristics out of the different sourdough fermentations (Lhomme *et al.*, 2015b; Lhomme *et al.*, 2015a; Gänzle and Ripari,

2016; Lhomme *et al.*, 2016; Yang *et al.*, 2017). The intraspecies diversity of the *F. sanfranciscensis* strains out of the different sourdough is broader than anticipated (Lee *et al.*, 2015; Lhomme *et al.*, 2016; Yang *et al.*, 2017). Furthermore, species diversity appears to be influenced by the sourdough preparation, the baker and the ingredients of the sourdough (Kitahara *et al.*, 2005). Different strains are also present in a single fermentation. Furthermore, there are strain-related functional traits like EPS production (Korakli *et al.*, 2003), or antifungal activities (Schnürer and Magnusson, 2005; Picozzi *et al.*, 2010). Despite the known variety of different *F. sanfranciscensis* strains in sourdough it has remained unclear if these have any effect on bread quality (Yang *et al.*, 2017). Nevertheless, in the business of starter cultures, companies want specific strains as ordered for a stable bread quality. So, *F. sanfranciscensis* strains need to be distinguished from each other (Foschino *et al.*, 2001; Picozzi *et al.*, 2010). Besides, it is important to differentiate the strains from each other and catalogue them (Yang *et al.*, 2017).

Even though various techniques have been used to differentiate LAB especially *F. sanfranciscensis* down to strain level, differentiating strains remains challenging due to their small genome, high similarity, and narrow phylogenetic diversity within this species. In the following the most recent methods that have been applied to differentiate between strains are described. The randomly amplified fragment length polymorphic DNA (RAPD) polymerase chain reaction (PCR) is the mainly used genetic fingerprinting method (De Angelis *et al.*, 2007). However, it has poor reproducibility and the discriminatory power is too low for genetically similar strains (De Angelis *et al.*, 2007). With the addition of primers in a multiplex RAPD the reproducibility is improved but the discriminatory power is still low (Ehrmann and Vogel, 2005; Yang *et al.*, 2017). The PCR amplification of repetitive bacterial DNA elements (rep-PCR) has similar issues as the RAPD due to low reproducibility of the results. Nevertheless, this technique has been reported to have a higher discriminatory power than RAPD and is an easy fingerprinting method, and good for high-throughput strain analyses (De Angelis *et al.*, 2007; Picozzi *et al.*, 2010). Other techniques like the amplified fragment length polymorphism (AFLP), the pulsed field gel electrophoresis (PFGE) and the denaturaturing gradient gel electrophoresis (DGGE) have a high discriminatory power and a good reproducibility (Gobbetti *et al.*, 2016). Nevertheless, these techniques are expensive, time- and energy-consuming, and require special laboratory equipment. As a consequence, this technique is not applicable for high throughput strain differentiation (Foschino *et al.*, 2001; Ehrmann and Vogel, 2005; De Angelis *et al.*, 2007). For multi locus sequence typing (MLST) and 16S sequencing it is necessary to sequence the DNA of each strain. Although, for a high throughout analyze this is time and energy consuming

and expensive as the most institutes are not sequencing their strains by themselves (Picozzi *et al.*, 2010; Yang *et al.*, 2017). For high throughout analyses matrix-assisted laser desorption/ionization (MALDI)-time of flight (ToF) mass spectrometry (MS) is a powerful tool, which is based on the protein spectra of different species. Strain differentiation with MALDI-ToF MS works for several species (Janßen *et al.*, 2018) although for *F. sanfranciscensis* strains the discriminatory power is too low. Consequently, there is a need for a precise strain differentiation system of *F. sanfranciscensis* to monitor its strain-specific behavior in the sourdough system. It is important to analyze the strain-specific properties of *F. sanfranciscensis* to predict its impact on the quality of bread and baked goods. Especially manufacturers can benefit from the strain differentiation and the evaluation of the properties of the *F. sanfranciscensis* strains as this could influence the technologic requirements of doughs and thus patent applications (Yang *et al.*, 2017).

3.3.2 Phylogenetic development of F. sanfranciscensis were does it come from?

F. sanfranciscensis appears to be autochthonous in sourdough environment and its metabolic potential is perfectly adapted to it. Although, it is unlikely that it developed in this specific niche. The existence of the niche sourdough can be traced back until 25,000 years ago and a continuous propagation of sourdoughs has been detected more than 100 years ago (Vogel *et al.*, 2011; Cappelle *et al.*, 2013). Although, 100 backslopping events are roughly 5 x 10^5 bacterial generations, which is not enough regarding the timeline of the molecular clock of bacterial evolution. The adaption of *Li. reuteri* to human took 8-13 million years (Oh *et al.*, 2010; Gänzle and Ripari, 2016). As a result, the time for the adaption of microorganisms to human fermented cereals is still too short to allow species specific adaption (Gänzle and Ripari, 2016). In particular, *F. sanfranciscensis* was still unchanged after 18 years of continuous propagation (Ehrmann *et al.*, 2011). Nonetheless, there are factors, which can have a minor influence on the diversity of one single species. Lytic bacteriophages were isolated out of *F. sanfranciscensis* and other sourdough isolates (Foschino *et al.*, 2005; Ehrmann *et al.*, 2013). The presence of plasmid encoded clustered CRISPR elements carrying phage elements in *F. sanfranciscensis* leads to the assumption that phages alter the sourdough microbiota (Vogel *et al.*, 2011). Although, the viscosity of the sourdough disables the spreading and infection of the bacterial microbiota with phages (Foschino *et al.*, 2005). Consequently, the origin of sourdough bacteria is still highly debated. There are many sources from which the sourdough may be inoculated. Firstly, it can be inoculated from the cereals or from flour contaminants or the bakery environment. Generalists

like *Lp. plantarum* and *Li. fermentum* are often found on cereal flours and the surfaces of plants (Minervini *et al.*, 2010). This inoculation pathway is also the possibility how other heterofermentative generalists like *Le. brevis*, *Li. fermentum*, *P. pentosaceaus, Leuconostoc* and *Weisella* can come from. Secondly, the organisms of the sourdough surroundings might be traced back to an intestinal origin. Mouse and rodent feces as well as feces form the fertilization of the fields or the contamination from the bakery environment (bakers hygiene) and the mills can influence the composition of the microbiota. These include organisms like *L. johnsonii*, *Furfurilactobacillus rossiae* or *L. acidophilus* (Ehrmann and Vogel, 2005; De Angelis *et al.*, 2006; Groenewald *et al.*, 2006; De Vuyst *et al.*, 2014). Furthermore, yeasts like *S. cerevisiae* were not isolated from cereals but recontamination of yeasts or bacterial species or from industrial baker's yeast can explain its occurrence (De Vuyst and Neysens, 2005; Scheirlinck *et al.*, 2009). Thirdly, contamination of flours with insects or flowers with insect as vectors can also be the source of microorganisms from the sourdough environment like *F. sanfranciscensis*. With this inoculation source generalists like *Lp. plantarum* and *Le. brevis* can also occur in the sourdoughs. Finally, sourdoughs can also be inoculated with defined starter cultures like the Böcker-Reinzucht-Sauer starter (Böcker *et al.*, 1990).

F. sanfranciscensis cannot be found in lab-scale sourdoughs, which are fermented only with flour and water (Bessmeltseva *et al.*, 2014; Minervini *et al.*, 2015; Rizzello *et al.*, 2015; Gänzle and Ripari, 2016). The situation changes when the sourdoughs were prepared with flowers and insects. Lin and Gänzle (2014a) isolated *F. sanfranciscensis* in 4 of 7 sourdoughs prepared with flowers, berries, or mother of vinegar. Furthermore, with these additives the sourdough can be fed with competitive strains like *F. sanfranciscensis* (Lin and Gänzle, 2014a; Lin and Gänzle, 2014b; Gänzle and Ripari, 2016). In sequence analyses the DNA of *F. sanfranciscensis* was also successfully detected within fruit flies with ribosomal (r)DNA sequence homologies of 97% (Groenewald *et al.*, 2006; Zheng *et al.*, 2015). Moreover, *F. sanfranciscensis* phylogenetically belongs to the insect and flower-associated organisms of the *Fructilactobacillus* group. This group members like *F. florum* and *F. fructivorans* were continuously isolated from insect frass and flowers and are insect associated organisms (Zheng *et al.*, 2015; Zheng *et al.*, 2020).

3.4 The yeasts *K. humilis* and *S. cerevisiae*

Together with LAB yeasts play a key role in sourdough fermentations. They were often ignored because of the inability to detect them. Due to their leavening properties they are appreciated and often additionally added as starter cultures (De Vuyst *et al.*, 2016). Up to different 40 species of yeast were found in sourdough fermentations (De Vuyst and Neysens, 2005). Because of their fermentative ability ascomycetes yeasts were most often isolated. These include yeasts like *K. humilis*, *K. exigua*, *Candida (C.) krusei*, *Torulaspora (T.) delbrueckii*, *S. cerevisiae* and *Wickerhamomyces anomalus*. *K. exigua* is autochthonous to the niche sourdough while the other species were also found in other ecosystems (Vrancken *et al.*, 2010; Huys *et al.*, 2013; De Vuyst *et al.*, 2014). Furthermore, the presence of *S. cerevisiae* in the sourdough can be rather explained by the addition of Baker`s yeast than its occurrence as natural habitant (De Vuyst and Neysens, 2005; Brandt and Gänzle, 2006). In one single batch of sourdough mostly only one or two yeast species are present. Most common are the species *S. cerevisiae*, *K. humilis* and *C. krusei* (Vrancken *et al.*, 2010). The intra-species diversity of both species leads to strain diversity of *S. cerevisiae* and *K. humilis* in one sourdough fermentation (Huys *et al.*, 2013; De Vuyst *et al.*, 2016). Especially in Italy, *K. humilis* was isolated in 95% of the sourdoughs with durum wheat bran (Gullo *et al.*, 2003). In *Panettone* and *Lagaccio* sourdoughs a stable combination of *F. sanfranciscensis* with *S. cerevisiae* and *K. humilis* was detected. Although, the presence of yeasts is explained by constant contamination with the Baker's yeast during dough propagation (De Vuyst and Neysens, 2005; Venturi *et al.*, 2012). The metabolism of the yeasts has a great impact on quality of the sourdough. Furthermore, the metabolic activity of an organism is explained by its cell surface and *K. humilis* is 20 times larger than *F. sanfranciscensis* (Brandt *et al.*, 2004). The yeasts are able to cope with the different stress factors present in the niche sourdough. Moreover, they are important for different factors in the sourdough fermentation and are contributing not only with their carbon dioxide production to the quality of the product (De Vuyst *et al.*, 2016). As metabolic advantages of the yeast fermentation the production of vitamins especially vitamins of the B-complex and thus reduction of the thiamine content is considered (Batifoulier *et al.*, 2005). Besides, species-specific nutritional values of the yeasts like an increase in the antioxidant capacity of the dough, or phytate activity of the microbial origin or probiotic properties of the yeast are discussed (Türk *et al.*, 2000; Moore *et al.*, 2007; Moore *et al.*, 2009; Moslehi-Jenabian *et al.*, 2010; Gobbetti *et al.*, 2014; De Vuyst *et al.*, 2016). Although, it is not yet known how the interaction of the yeast *K. humilis* and *S. cerevisiae* with *F. sanfranciscensis* alters the dough fermentation and if there are strain-specific differences of *F. sanfranciscensis* in combination with both yeasts.

3.5 Interaction of LAB and yeasts in the sourdough with focus on *F. sanfranciscensis*, *S. cerevisiae* and *K. humilis*

3.5.1 Interaction between LAB and yeasts

Basically, there are different types of interactions possible between members of the microbiota of a specific niche, which are depicted in Figure 1. These interactions are determined by internal parameters, including pH, a_w, redox potential, nutritional content, antimicrobial compounds or protective biological structures, and external parameters, including temperature, humidity, and atmosphere, which represent the physicochemical conditions of the respective habitat. Within this frame it is the implicit factor of the microbiota members, which determines their growth and persistence and their interactive relationship. In Figure 1 the different summarized effects on growth, persistence, and metabolic activity of two members of a microbiota are delineated for different types of interaction in a simplified way. This scheme can be used to probe and possibly elucidate the type of interaction between yeasts and lactobacilli in sourdough fermentation, where they comprise the vast majority of microbes in terms of numbers and metabolic conversion.

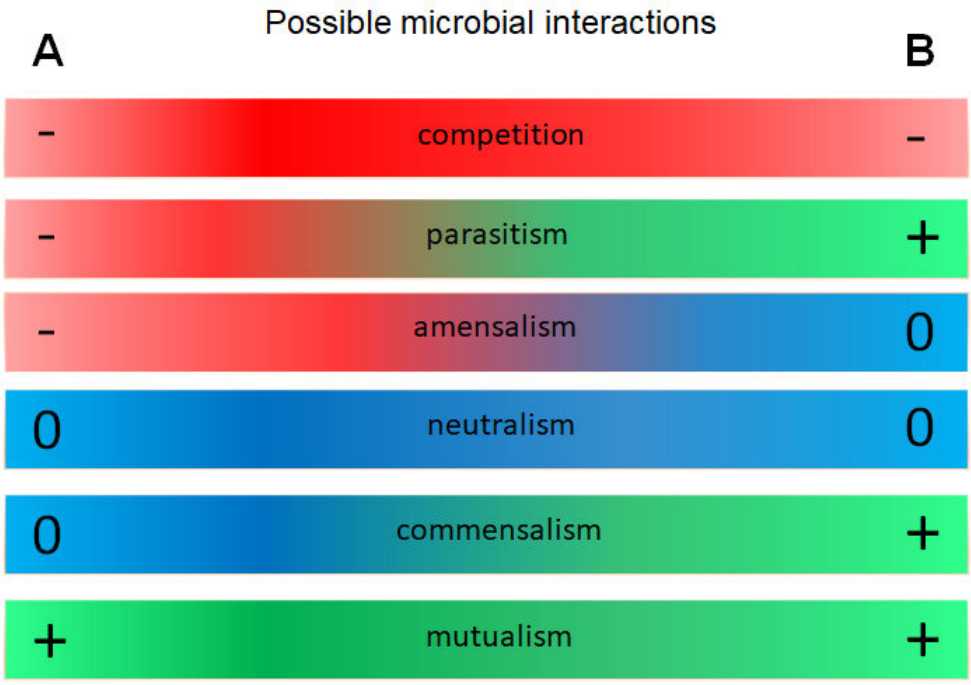

Figure 1: Possible microbial interactions. Minus: negative effects; Plus: positive effects; Zero: no effect.

During a stable sourdough fermentation, the association between yeasts and LAB can endure for years (De Vuyst and Neysens, 2005). The nutrition available in the sourdough needs to be shared and leads to synergistic and antagonistic interactions between the microbiota (Gobbetti and Corsetti, 1997a). The range of interactions in the sourdough lays between mutualism, commensalism, and competition (Figure 1). In the sourdough the microbiota can compete for carbohydrates, amino acids, and external electron acceptors. Moreover, a mutualistic interaction can rely on complementary requirements and mutual stress responses and the metabolic products of one another (Stolz *et al.*, 1995; Gänzle *et al.*, 1998; Jacques *et al.*, 2016; De Vuyst *et al.*, 2017; Comasio *et al.*, 2020). Both matches the observation that the equilibrium in the microbiota is sensible and a substrate change leads to a change in the composition of the microbiota (De Vuyst and Neysens, 2005). Furthermore, the interaction is also altered by the addition of starter cultures or Baker's yeast during propagation. Hitherto, consortia with maltose-positive LAB and maltose-negative yeasts were found. A prominent example is the combination of *F. sanfranciscensis* and *Kazachstania* (Comasio *et al.*, 2020). Although, consortia between glucose-repressed LAB i.e., *Lp. plantarum* and maltose positive yeasts i.e., *S. cerevisiae* were found (Guerzoni *et al.*, 2007; De Vuyst *et al.*, 2017; Sieuwerts *et al.*, 2018; Comasio *et al.*, 2020). The open question is whether this is a mutual or commensal relationship.

3.5.2 F. sanfranciscensis and the yeasts S. cerevisiae and K. humilis

The most frequently found combination in industrial sourdoughs is *F. sanfranciscensis* with *K. humilis*. This interaction can be explained by contemporary growth requirements. *F. sanfranciscensis* prefers maltose instead of glucose, which is produced constantly by flour amylases. The glucose produced from the maltose phosphorylase of *F. sanfranciscensis* is secreted in a ratio of 1:1 to the depleted maltose. The glucose feeds on the one hand maltose-negative yeasts like the aforementioned *K. humilis* and on the other hand confers glucose repression in other sourdough inhabitants, which is a clear advantage of this interaction (Stolz *et al.*, 1993; Hammes *et al.*, 1996; De Vuyst *et al.*, 2017). Whereas the growth with maltose-positive yeasts like *S. cerevisiae* leads to stress responses of *F. sanfranciscensis* and *S. cerevisiae* can be ruled out during propagation. In sourdoughs with this combination the bacterial end product is lower than the yeast end product (De Vuyst and Neysens, 2005). This result is in agreement with the observation of Carbonetto *et al.* (2020): *F. sanfranciscensis* produces lower amounts of lactate when inoculated with the yeasts *S. cerevisiae* and *K. humilis*. Also, for the yeast strains the coexistence with LAB causes stress, yeasts living in coculture with

LAB showed a lower cell count than in monoculture (Carbonetto *et al.*, 2020). This observation does not differ with different combinations of yeasts and LAB including cocultures with *F. sanfranciscensis*, *K. humilis* and *S. cerevisiae*. The cell count of both species is limited by different factors of the sourdough fermentation. As *F. sanfranciscensis* is not able to grow under a pH of 3.8, the cell count of the yeasts is limited by the accumulation of the bacterial end products (Gänzle *et al.*, 1998; Siragusa *et al.*, 2009). Apart from that there are arguments for a mutualistic interaction between *F. sanfranciscensis* and *K. humilis*. *F. sanfranciscensis* is able to regenerate adenosine triphosphate (ATP) by the use of the acetate kinase by the formation of acetate instead of ethanol. Therefore, it needs external electron acceptors to recycle nicotinamide adenine dinucleotide (NAD), which is otherwise recycled by the formation of ethanol. As external electron acceptors *F. sanfranciscensis* can utilize different metabolites i.e., carbon dioxide or fructose (Stolz *et al.*, 1995; De Vuyst and Neysens, 2005). Mostly the oxygen is directly depleted by the aerobic yeasts, and the amount of free fructose in the dough is rather low. Although, the enzymatic activity of *K. humilis* cleaves glucofructans. In this reaction glucose (for the yeast) and fructose (for *F. sanfranciscensis*) originates. Moreover, the fructose can be used by *F. sanfranciscensis* with the mannitol dehydrogenase to form mannitol and recycle NAD. This reaction leads to a higher level of acetate in the sourdough. *F. sanfranciscensis* countenances acetate and lactate up to a level of 250 mM per liter in rye and wheat doughs and its growth is not inhibited by undissociated acetic acid (Gänzle *et al.*, 1998). Albeit the growth of *K. humilis* is inhibited by acetate and to a lesser extent by lactate. Due to the inhibition of *K. humilis* with the formation of acetate, the yeast invertase activity and so the formation of free fructose is also decreased (Brandt *et al.*, 2004). This inhibition alters the FQ, which is at the beginning of the fermentation with lots amount of fructose at 1:1, with the inhibition of the yeast invertase and the depletion of fructose the FQ is changed due to the formation of ethanol instead of acetate (Stolz *et al.*, 1995; Brandt *et al.*, 2004). In conclusion, there are a lot of arguments for a mutual relationship or a competition between LABs especially *F. sanfranciscensis* and yeasts like *S. cerevisiae* and *K. humilis*.

3.6 Hypotheses

The microbiota in sourdoughs underlies a complex and restricted environment where stable conditions lead to stable microbiota composition and finally to reproducible baked goods. *F. sanfranciscensis* is a key bacterium in the type 1 fermentation in industrial and artisan sourdoughs. Together with the yeasts *K. humilis* and *S. cerevisiae* it influences the sourdough microbiota and thus the sensory quality and texture of the baked products (Gänzle and Zheng, 2019). In this study the intraspecies diversity should be investigated of *F. sanfranciscensis* to determine strain-specific differences in the lifestyle of these bacteria. Moreover, the influence of *K. humilis* and *S. cerevisiae* on the strain-specific competitiveness of *F. sanfranciscensis* should be analyzed to obtain mechanistic insight into the interaction of LAB and yeasts in sourdough.

The following working hypotheses will be discussed in this thesis:

a) The core genome of the different strains of *F. sanfranciscensis* is generally related to a plant-based origin.

b) There are strain-specific differences of *F. sanfranciscensis* regarding the competitiveness and persistence in the sourdough system.

c) The strain-specific competitiveness of *F. sanfranciscensis* is influenced by the presence/absence of sourdough yeasts.

d) The strain-specific type of interaction between *F. sanfranciscensis* and the yeasts *K. humilis* and *S. cerevisiae* differs with respect to commensalism, competitiveness, and mutualism.

Different working packages are formed to probe the working hypothesizes and to learn more about the interactions between *F. sanfranciscensis* and the specific yeasts. Firstly, a strain-specific detection system of *F. sanfranciscensis* should be investigated. Because of its small genome the differences between the strains of this species are narrow. Furthermore, the strain-specific behavior of this species should be explored in the sourdough system. Common differentiation systems are either not sufficient to monitor the behavior of *F. sanfranciscensis* in the sourdough or the discriminatory power is too low to differentiate between the *F. sanfranciscensis* strains.

The developed strain-specific differentiation system should then be applied on lab-scale sourdough systems. These systems should differ in their strain-specific combination of *F. sanfranciscensis* as well as on their presence or absence of sourdough yeasts *K. humilis* and *S. cerevisiae*. Furthermore, strain-specific differences should be revealed in the competitiveness of *F. sanfranciscensis* in combination with yeasts and probed for a mechanistic background as depicted by comparative genomics. Metabolic studies should reveal the basics of the competitiveness and interaction principles of *F. sanfranciscensis* in the sourdough. In further investigations the characteristics from genomic predictions should be probed for their coincidence with the phenotypes of *F. sanfranciscensis* strains in order to elaborate the working hypotheses.

4 Material and Methods

4.1 Microorganisms

The microorganisms used in this thesis were formerly isolated out of different sourdoughs. The *F. sanfranciscensis* strains are listed in table 1, and the yeast strains are listed in table 2. For storage, pure overnight cultures of the strains were centrifuged at 7000 x g for 7 min. The cell pellet was diluted with 1500 µl mMRS or YPD media and 800 µl 80% glycerol (Carl Roth, Karlsruhe, Germany). Subsequently the strains were stored at -80°C.

Table 1: Strains of *F. sanfranciscensis* utilized in this thesis.

Organism	Strain	Accession No.	Isolation source	Reference
F. sanfranciscensis	DSM 20451^T	MIYJ00000000	Sourdough, USA	Kline and Sugihara (1971)
F. sanfranciscensis	TMW 1.54 (LTH 1729)	NZ_MIYE01000000	Rye sourdough, Germany	Stolz *et al.* (1995)
F. sanfranciscensis	TMW 1.392 (LTH 2590)	NZ_MIYH01000000	Sourdough, Belgium	Böcker *et al.* (1995)
F. sanfranciscensis	TMW 1.640	SCEZ00000000	Wheat sourdough, Switzerland	Ehrmann and Vogel (2001)
F. sanfranciscensis	TMW 1.726	NZ_MIYD01000000	Sourdough, Italy	Liske *et al.* (2000)
F. sanfranciscensis	TMW 1.897	SCEP00000000	Sourdough, Greece	Rogalski *et al.* (2020c)
F. sanfranciscensis	TMW 1.907	SCEY00000000	Sourdough, Greece	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.936	SCEX00000000	Sourdough, Greece	Rogalski *et al.* (2020c)
F. sanfranciscensis	TMW 1.1150	NZ_MIYG01000000	Sourdough, USA	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.1152	SCEV00000000	Sourdough, USA	Rogalski *et al.* (2020b)

F. sanfranciscensis	TMW 1.1154	SCEU00000000	Sourdough, USA	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.1221	SCET00000000	Sourdough, France	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.1304	SCES00000000	Rye sourdough, Germany	Vogel *et al.* (2011)
F. sanfranciscensis	TMW 1.1470	SCER00000000	Sourdough, Russia	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.1597	NZ_MIYF01000000	Rye sourdough, Germany	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.1730	SCEQ00000000	Rye sourdough, Germany	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.2137 (LS3)	NZ_MIXX01000000	*Wheat sourdough, Italy	De Angelis *et al.* (2007)
F. sanfranciscensis	TMW 1.2138 (LS12)	NZ_MIXY01000000	*Wheat sourdough, Italy	De Angelis *et al.* (2007)
F. sanfranciscensis	TMW 1.2139 (LS27)	NZ_MIXZ01000000	*Wheat sourdough, Italy	De Angelis *et al.* (2007)
F. sanfranciscensis	TMW 1.2140 (LS19)	NZ_MIYA01000000	*Wheat sourdough, Italy	De Angelis *et al.* (2007)
F. sanfranciscensis	TMW 1.2141 (LS48)	NZ_MIYB01000000	*Wheat sourdough, Italy	De Angelis *et al.* (2007)
F. sanfranciscensis	TMW 1.2142 (LS13)	NZ_MIYC01000000	*Wheat sourdough, Italy	De Angelis *et al.* (2007)
F. sanfranciscensis	TMW 1.2314	SCEW00000000	Rye sourdough, Germany	Rogalski *et al.* (2020b)
F. sanfranciscensis	TMW 1.2323	VCSH00000000	Rye sourdough, Germany	Rogalski *et al.* (2020c)

*Highly likely because rye is nearly unused in Italy.

Table 2: Yeast strains used in this thesis.

Organism	Strain	Isolation source	References
K. humilis	TMW 3.1034	Rye sourdough, Germany	Rogalski *et al.* (2020c)
S. cerevisiae	TMW 3.1064	Rye sourdough, Germany	Rogalski *et al.* (2020c)
S. cerevisiae	TMW 3.970	Rye sourdough, Germany	Rogalski *et al.* (2020c)
S. cerevisiae	TMW 3.972	Rye sourdough, Germany	Rogalski *et al.* (2020c)
S. cerevisiae	TMW 3.971	Rye sourdough, Germany	Rogalski *et al.* (2020c)

4.2 Media and cultivation of *F. sanfranciscensis* and yeasts

4.2.1 General information for media preparation

All media for the cultivation of microorganisms were autoclaved or sterile filtered with 0.2 µm sterile filter (Sarstedt, Darmstadt, Germany). The sugars were autoclaved separately and added to the media. The pH was adjusted accordingly before sterilization with NaOH (Carl Roth) or HCl (Carl Roth).

4.2.2 Standard media for cultivation of F. sanfranciscensis

For the cultivation of *F. sanfranciscensis* modified DeMan, Rogosa and Sharpe (mMRS) media were used (Rogalski *et al.*, 2020b). For clarification of the sugar components used in mMRS the abbreviation of these components was set accordingly: maltose, fructose, glucose (mfgMRS. The media were prepared according to table 3. The metal compounds were prepared as 10-fold stock solutions and 2 ml/L were given before autoclaving to the media and the pH was adjusted to 5.4. For agar plates 15 g AgarAgar (Carl Roth) was added before autoclaving. For overnight cultures as well as on agar plates, *F. sanfranciscensis* was cultivated in mfg-MRS in static cultures for up to 48 h at 30°C. Different versions of mfg-MRS were prepared for different experiments. For the acetate test media was prepared without acetate mfg-MRS-(a). For the analysis of the sugar spectrum mfg-MRS without sugars was prepared. Furthermore, sugar was added to a concentration of 2%: maltose (m)-MRS, glucose (g)-MRS, fructose (f)-MRS, sucrose (s)-MRS, ribose (r)-MRS, arabinose (a)-MRS and xylose (x)-MRS.

Table 3: Components for mfg-MRS media.

Compounds	Amount [g/L]	Company
Casein peptone	10	Carl Roth
Meat extract	2	Merck, Darmstadt, Germany
Yeast extract	2	Carl Roth
Sodium gluconate	2	Carl Roth
Sodium acetate x 3 H_2O	5	Carl Roth
Citric acid diammonium salt	5	Carl Roth
Potassium di-hydrogen phosphate	2.5	Merck
L-cysteine hydrochloride x H_2O	0.5	Carl Roth
Polysorbate 80	1	GEBRU Biotechnik GmbH, Heidelberg, Germany
Magnesium sulfate x 7 H_2O	0.2	Merck
Manganese sulfate x H_2O	0.10	Carl Roth
Iron (II) sulfate x 7 H_2O	0.05	Carl Roth
glucose x H_2O	7	Merck
maltose	7	GEBRU Biotechnik
fructose	7	Omni Life Science GmbH & Co. KG, Bremen, Germany

4.2.3 Chemically defined media

Chemically defined media (CDM) was prepared according to table 4 in autoclaved water. The nucleic acids were diluted in 2 M NaOH (Carl Roth). Vitamins were prepared in a 50 x stock and 8 ml were added to the media. After the addition of the compounds the pH was adjusted to 5.4 and the media was sterile filtered with 0.2 µm sterile filter (Sarstedt). The CDM was adjusted according to experimental requirements. In CDM without purines (-ponly pyrimidines were diluted in 2 M NaOH and added to the media CDM-p. For the analysis of the electron acceptors CDM-cwithout citrate was produced and 20 mM either citrate (CDM), fructose (f-CDM-c), Na-gluconate (gc-CDM-c) or malate (mt-CDM-c) was added to the media.

Table 4: Components for CDM media.

Buffer solution	**Amount [g/L]**	**Company**
Sodium acetate	5	Carl Roth
Potassium di-hydrogen phosphate	3	Merck
Di-Potassium hydrogen phosphate	3	Merck
Magnesium sulfate x 7 H_2O	0.5	Carl Roth
Mangan sulfate x H_2O	0.05	Carl Roth
Iron sulfate x 7 H_2O	0.05	Carl Roth
Calcium chloride	2	Carl Roth
Polysorbate 80	1	GEBRU Biotechnik
Vitamins		
p-aminobenzoic acid	0.0005	Sigma-Aldrich
Folic acid	0.0005	Carl Roth
Nicotinic acid	0.002	Sigma-Aldrich
Ca-pantothenate	0.002	Sigma-Aldrich
Biotin	0.001	Carl Roth
Pyridoxal	0.002	Sigma-Aldrich
Riboflavin	0.002	Sigma-Aldrich
Vitamin B_{12}	0.001	AppliChem GmbH, Darmstadt, Germany
Thiamine	0.004	Sigma-Aldrich
Amino Acids		
Cysteine	0.4	Carl Roth
Aspartic acid	0.3	Sigma-Aldrich
Glutamic acid	0.3	Sigma-Aldrich
Alanine	0.2	Sigma-Aldrich
Arginine	0.2	Sigma-Aldrich
Glycine	0.2	GEBRU Biotechnik
Histidine	0.2	Carl Roth
Isoleucine	0.2	Merck
Leucine	0.2	Merck
Lysine	0.2	Carl Roth

Methionine	0.2	Carl Roth
Phenylalanine	0.2	Carl Roth
Proline	0.2	Merck
Serine	0.2	Merck
Threonine	0.2	Sigma-Aldrich
Tryptophane	0.2	Sigma-Aldrich
Tyrosine	0.2	Carl Roth
Valine	0.2	Sigma-Aldrich
Nucleic acid		
Xanthine	0.04	Sigma-Aldrich
Orotic acid	0.5	Sigma-Aldrich
Adenine	0.02	SERVA Electrophoresis GmbH, Heidelberg, Germany
Guanine	0.05	Sigma-Aldrich
Sugar		
Maltose	20	GEBRU Biotechnik

4.2.4 Cultivation of yeasts in YPG media

S. cerevisiae and *K. humilis* were cultivated in yeast peptone glucose (YPG) media. The components were diluted according to table 5. For agar plates 15 g AgarAgar (Carl Roth) was added to the media before sterilization and the pH was adjusted to 6.5. The yeasts were grown for overnight cultures under oxic conditions with 120 rpm for 16 h at 30°C. On agar plates the culture conditions were similar.

Table 5: The compounds for YPG media.

Compound	**Amount [g/L]**	**Company**
Casein peptone	10	Carl Roth
Yeast extract	5	Carl Roth
Glucose x H_2O	20	Merck

4.3 Genome analysis

4.3.1 Generation of bacterial genomes

For whole genome shut gun sequencing of *F. sanfranciscensis* the strains were grown in overnight cultures and the DNA was isolated according to the EZNA ® DNA Kit (OMEGA Bio-teck, Norcross, USA). For sequencing a PCR-free library preparation on the MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA) was utilized and the genomes were processed and assembled with SPAdes V3.9.0 (Bankevich *et al.*, 2012) through the method of Huptas *et al.* (2016). The sequences were annotated with the NCBI Prokaryotic Genome Annotation Pipeline (Haft *et al.*, 2005; Tatusova *et al.*, 2013). Furthermore, the genomes were also processed with the Rapid Annotation Subsystem Technology (RAST) to get the enzyme commissions (EC) numbers of the proteins and the subcellular localization (Aziz *et al.*, 2008). The blast hits and the Gene Ontology (GO) terms were determined by the TIGR Annotation Engine (Rogalski *et al.*, 2020a).

4.3.2 In silico genome analysis

The average nucleotide identity (ANI) values were utilized to reveal the relationship between the *F. sanfranciscensis* strains (Goris *et al.*, 2007). The ANI values are a robust measurement for the genetic and evolutionary distance between a given pair of genomes (Goris *et al.*, 2007; Richter and Rosselló-Móra, 2009). For the analysis, the genomes were separated artificially in 1020 nucleotide (nt) sequences and were compared to a reference genome. This calculation was done by the software tool Jspecies vers. 1.2.1 were the calculation of the ANIb values is based on the BLAST algorithm (Richter and Rosselló-Móra, 2009; Hilgarth *et al.*, 2018). In addition, a phylogenetic tree based on the ANIb values was calculated with the Molecular Evolutionary Genetics Analysis (MEGA) 7.2 tool. The calculation was performed with neighbor-joining and the unweighted pair group method (UPGMA) (Rogalski *et al.*, 2020c).

For the differentiation of the diagnostic marker genes (DMGs) of the 24 *F. sanfranciscensis* strains the BlAst Diagnostic Gene findEr (BADGE) was applied (Behr *et al.*, 2016). The results of the BADGE analysis were visualized *via* Blast Ring Image Generator (BRIG) (Alikhan *et al.*, 2011). Furthermore, the pan- core and accessory genome of the *F. sanfranciscensis* group was calculated with the BADGE results (Eisenbach *et al.*, 2018). The genes coding for important metabolic pathways enzymes were controlled *via* the Basic Local Alignment Search Tools (BLAST) SmartBlast, nBLAST and pBLAST at National center for Biotechnology Information

(NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Basic local alignment search tool; Marchler-Bauer *et al.*, 2017). The nucleotide structure and the place in the genome was analyzed with the CLC main workbench 8.0 (https://digitalinsights.qiagen.com/). The alignment of the genes was performed with Clustal Omega (Larkin *et al.*, 2007) and the results viewer Jalview (Waterhouse *et al.*, 2009). The membrane binding capacity of predicted membrane proteins was analyzed with the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Furthermore, the occurrence of bacteriocin islands were analysis with BAGEL (de Jong *et al.*, 2006) and the prediction of phages was conducted with Phaster (Arndt *et al.*, 2016; Arndt *et al.*, 2017).

The results of the genetic analysis and genes predicted for important metabolic pathways involved in the utilization of different sugars and external electron acceptors and the resulting differences between the *F. sanfranciscensis* strains were visualized with tree diagrams. These tree diagrams were calculated with BIONUMERICS V7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium) were the UPGMA and the neighbor joining or the similarity coefficient with categorical differences were applied.

4.3.3 Analysis of the CRISPR-Cas system

The bacterial cells apply the CRISPR-CRISPR associated genes (Cas) system as adaptive immune system to combat attacks from known phages, plasmids, and DNA elements (Barrangou and Marraffini, 2014). After the survival of an attack characteristic genetic elements like the protospacer adjacent motif (PAM) of the bacteriophages are implemented as spacer sequence into the CRISPR-Cas locus. Small CRISPR-(cr)RNA sequences were performed with the specific spacer region of the phage. When the same phage attacks again, the crRNA sequences with the Cas-proteins binds the PAM sequences complementary and the nucleotide cleavage is performed (Barrangou *et al.*, 2007; Barrangou and Marraffini, 2014). Thereby, every bacterial strain needs to combat various bacterial phages attacks, the CRISPR locus length is strain-specific (Horvath *et al.*, 2009; Crawley *et al.*, 2018).

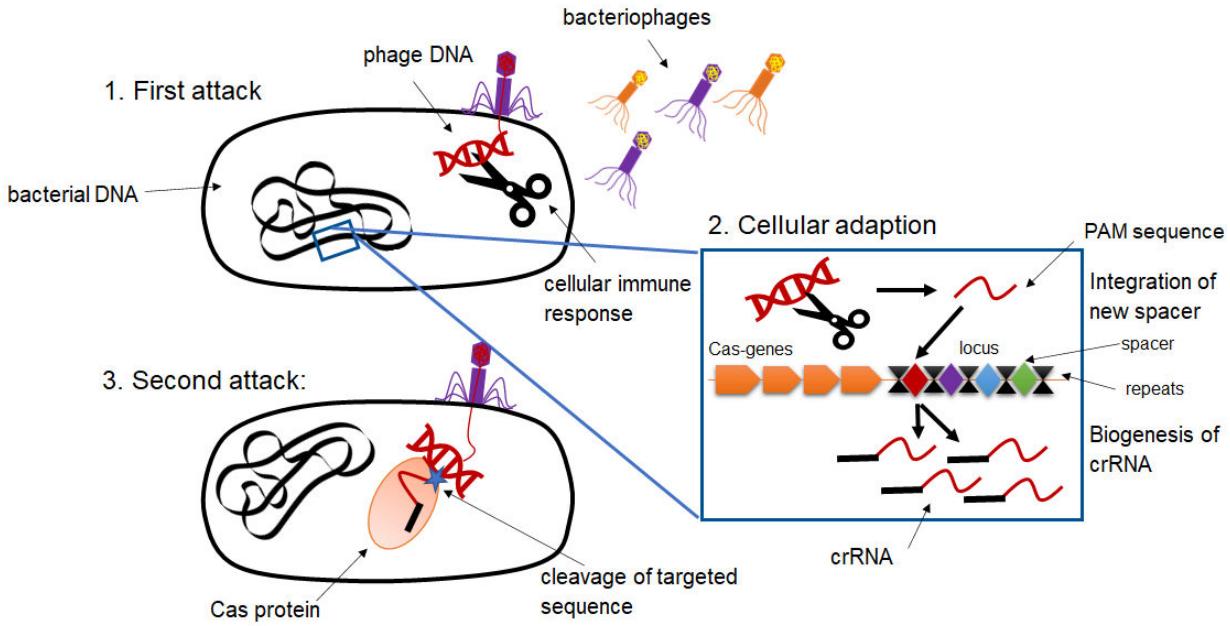

Figure 2: Scheme of the CRISPR-Cas system. 1. First attack, the bacteriophages invade the bacterial cell. The cellular immune system combats the phage attack. 2. Cellular adaption with the CRISPR-system. The PAM sequence of the phage DNA is integrated as new spacer into the CRISPR locus. Small crRNAs are transcribed. 3. Second attack of the previous bacteriophage. Cas protein complex with crRNA binds complementary to the phage DNA and cleaves the target sequence.

The CRISPR-Cas system was analyzed in the 24 *F. sanfranciscensis* strains. The CRISPR loci and the Cas-Genes were detected in the genome *via* the CRISPR finder (Couvin *et al.*, 2018). Sequence homologies of the CRISPR-Cas Type II-A locus especially the CRISPR associated gene *csn2* as well as the spacer and repeat sequences between the *F. sanfranciscensis* strains were identified with Clustal Omega. To visualize the CRISPR loci with its different spacer sequences CRISPR studio (Dion *et al.*, 2018) was applied. For the analysis with CRISPR studio the output of the CRISPR analysis software CRISPR detect (Biswas *et al.*, 2016) was used. Furthermore, the Protospacer adjacent Motif (PAM) of the CRISPR Type II-A loci was identified querying all spacer sequences with BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The spacer sequence matching a virus sequence with less than two mismatches was applied. In WebLogo 10 nt of the 5' and 3' end was implemented for the visualization of the PAM sequence (Crooks *et al.*, 2004). The trans activating CRISPR RNA (tracrRNA) was predicted according to Hupfeld *et al.* (2018).

4.4 Strain differentiation and fingerprint methods

4.4.1 Strain differentiation of F. sanfranciscensis with the CLLP-PCR

The 24 *F. sanfranciscensis* strains were differentiated with the help of the CRISPR locus length polymorphism (CLLP)-PCR (Rogalski *et al.*, 2020b). The detected sequence homologies up- and downstream of the CRISPR Type II-A locus were applied to constitute the strain-specific CRISPR locus length *via* PCR. Therefore, 500 nts up- and downstream of the CRISPR locus were aligned with Clustal Omega to determine sequence homologies. In these homolog regions primers were set. Upstream of the CRISPR locus the sequence of the *csn2* gene is conserved in the strains and the forward primer was set in this region (CR_fow3). Downstream of the CRISPR locus two different primers were set as the 24 *F. sanfranciscensis* strains differ in their downstream sequence of the CRISPR locus. For the strains of the CRISPR Type II-Aa the reverse primer was set in the predicted nicotinamide mononucleotide transporter (CR_rev2a) and for the strains of the CRISPR Type II-Ab the reverse primer was set in the predicted citrate sodium symporter (CR_rev4b). The designed primers are listed in table 6 (Rogalski *et al.*, 2020b). For *F. sanfranciscensis* TMW 1.1597 strain-specific primers were designed for identification as it lacks a CRISPR-Cas locus in its genome (Table 6) (Rogalski *et al.*, 2020c). For typing of the *F. sanfranciscensis* strains DNA isolated with the EZNA ® DNA Kit (OMEGA Bio-teck) was utilized for the CLLP-PCR. For tracing of the strains in the sourdough DNA was isolated directly out of the sourdough. Therefore, 1 g of sourdough was collected and washed with ¼ Ringer's solution (Merck, Darmstadt, Germany) and the DNA was subsequently isolated with the FastDNA™ Spin Kit for Soil (MP, Santa Ana USA). With the isolated DNA and the designed CRISPR primers a multistep PCR was performed (Rogalski *et al.*, 2020b). The detailed experimental setup can be found in the results section (5.1). The PCR reaction for the identification of TMW 1.1597 was conducted similar with a different set of primers. For a clearer visualization, the PCR results of the CLLP-PCR and the PCR results for TMW 1.1597 identification were pooled together bevor agarose gel electrophoresis (Rogalski *et al.*, 2020c).

Table 6: The PCR-Primers for the *F. sanfranciscensis* strains.

Primer	Sequence 5'-3'	Length [nt]	Tm [°C]	Binding side
CR_fow3	GCTGATAGGTGAATATTAC	19	50.2	*csn2*
CR_rev4b	GATAATTCCAATAATAGCGTAG	22	52.8	Citrate sodium symporter
CR_rev2a	CTCTCTTATAACTCTCAAAC	20	51.1	Nicotinamide mononucleotide transporter
1.1597_Mtf_for	GCAAACAAGCCAAGGGAAG	19	56.7	Strain-specific methyl transferase
1.1597_Mtf_rev	CCATCCTCGCCCATGTTATC	20	59.4	Strain-specific methyl transferase
Lev_1_f	ATGACTAAAGAACATAAGAAAATG	24	52.5	Levansucrase
Lev_2_r	CAAGAAACGTCGTAATGATTAA	22	52.8	Levansucrase

4.4.2 Microsatellite typing of S. cerevisiae

The *S. cerevisiae* isolated out of the sourdough were differentiated with the microsatellite typing technique invented by Legras *et al.* (2005). Therefore, the DNA of the *S. cerevisiae* strains was isolated with modifications according to Cenis (1992). Overnight cultures in YPD media of the *S. cerevisiae* were grown and afterwards washed in TE buffer (10 mM Tris-HCl (Carl Roth), 1 mM EDTA pH 8 (Carl Roth)). After centrifugation at 13,000 rpm for 5 min the supernatant was discarded. The cells were disrupted with 0.5 mm glass beads (Carl Roth) and 300 µl extraction buffer (200 mM Tris HCl pH 8.5 (Carl Roth), 250 mM NaCl (Carl Roth), 25 mM EDTA (Carl Roth), 0.5% SDS (Carl Roth) with the FastPrep®-24 (MP, Biomedicals) for 5 m/s for 45 s. Afterwards, 150 µl of 3 M Na-acetate (Carl Roth) pH 5.2 was added and briefly vortexed. The tubes were placed for 10 min at 20°C and then centrifuged at 13,000 rpm for 5 min. The supernatant was transferred into a new tube and an equal volume of cold isopropanol (Carl Roth) was added and the tube was mixed by inversion. Incubation for 5 min to 24 h at -20°C was performed and afterwards centrifuged at 13,000 rpm for 5 min. Furthermore, the supernatant was discarded, and the pellet was washed with 500 µl cold ethanol (Carl Roth) and mixed by inversion. After centrifugation at 13,000 rpm for 5 min the supernatant was discarded, and the

pellet was centrifuged again. The last drops of ethanol were removed, and the pellet was air dried. Finally, the isolated DNA is resuspended in 50 µl water for 10 mins at 50°C.

Table 7: Primer for microsatellite typing of *S. cerevisiae.*

Primer	Sequence 5'-3'	Length [nt]	Tm [°C]
SCYOR267C_fow	TACTAACGTCAACACTGCTGCCAA	24	61
SCYOR267C_rev	GGATCTACTTGCAGTATACGGG	22	60.3
YPL009c_fow	AACCCATTGACCTCGTTACTATCGT	25	61.3
YPL009c_rev	TTCGATGGCTCTGATAACTCCATTCATCCTG	31	66.8
C11_fow	TTCCATCATAACCGTCTGGGATT	23	58.9
C11_rev	TGCCTTTTTCTTAGATGGGCTTTC	24	59.3

For the hypervariable microsatellite based typing technique the microsatellites loci SCYOR257C, YPL009c and C11 were chosen as they were the most promising for strain differentiation (Legras *et al.*, 2005). The primers for the microsatellite loci PCR are listed in table 7. The PCR was performed for each primer pair in 50 µL reaction volume containing 100 ng *S. cerevisiae* DNA. The detailed experimental setup can be seen in the results part (see 5.2).

4.5 Detection of physiological behavior

4.5.1 The utilization of carbon sources during growth

The disaccharides maltose and sucrose, the monosaccharides glucose and fructose and the pentoses ribose, xylose and arabinose belong to the spectrum of sugars, which are related to the growth of lactic acid bacteria. On this account the usage of these carbohydrate as sole carbon sources by *F. sanfranciscensis* was investigated in growth experiments. MRS media with aforementioned sugars as sole carbon sources were prepared. Moreover, overnight cultures of *F. sanfranciscensis* strains were grown and after centrifugation at 7,000 x g for 7 min washed in ¼ Ringer's solution. The strains were set to an OD_{600} of 0.05 in the applied media (Rogalski *et al.*, 2020a).

Furthermore, the utilization of different electron acceptors like fructose, citrate and oxygen and the usage of Na-gluconate and malate for growth benefit was analyzed. As the mfg-MRS contains ingredients like yeast and meat extract where the chemical composition is not clear a

CDM was produced without external electron acceptors (Table 3, Table 4). In these experiments' maltose was applied as sole carbon source and 20 mM of one of each electron acceptor was added. The chosen strains were handled as above and the OD_{600} was set to 1. Afterwards, 200 µl were added into 96-well plates (Sarstedt). As oxygen is also an external electron acceptor for *F. sanfranciscensis* the experiments were performed under anoxic conditions (Gänzle *et al.*, 2007; Rogalski *et al.*, 2020a). The utilization of malate and Na-gluconate for growth enhancement was performed under oxic conditions with oxygen as external electron acceptor. Furthermore, the requirement for purines was determined in growth experiments under oxic conditions in CDM however in CDM with and without (CDM-p) purines. All growth experiments were performed in a SPECTROstar^{nano} plate reader (BMG, Labtech, Ortenberg, Germany) at 30°C in 96-well plates for 48 h and in 200 µL reaction volume. For anoxic conditions, the wells were covered with 100 µL paraffin oil (Carl Roth).
In addition, the different fermentation patterns of all 24 *F. sanfranciscensis* strains were analyzed with the standard system API 50 CHL (BioMérieux, Marcy l'Etoile, France), The test was performed according to manual instructions (API systems, BioMérieux).

4.5.2 The production of exopolysaccharides in F. sanfranciscensis

The production of EPS in the 24 *F. sanfranciscensis* strains was investigated. Therefore, the strains were grown on m-MRS plates with additional 50 g of sucrose for 48 h at 30°C. The levansucrase gene *levS* consists of several repeat sequences at the 5' and 3' end (Tieking *et al.*, 2005b). Traditionally, the repeat sequences are difficult to assemble in whole genome shotgun reactions (Alkan *et al.*, 2010). To prove the correct length of *levS* a PCR was performed with *levS* primers (Table 6) in a 50 µL reaction volume with 50 ng DNA. The PCR ingredients and the Mastercycler gradient were similar to the CLLP-PCR although with different primers. A standard PCR protocol for 16S RNA with the following settings was conducted: The initial denaturation starts with 2 min at 94°C, afterwards 32 cycles with 45 sec denaturation at 94°C, 1:30 min annealing at 52°C, followed by 2 min elongation at 72°C and one step final elongation for 5 min at 72°C.

4.6 Lab scale sourdough systems

4.6.1 Strain preparation

The characterization of competitiveness and persistence upon backslopping was conducted in a lab scale sourdough system. Eight strains of *F. sanfranciscensis* were chosen according to their CRISPR-Cas locus length polymorphism and their properties. These strains were sorted into two different groups, group 1 with *F. sanfranciscensis* TMW 1.1150, TMW 1.392, TMW 1.2138, TMW 1.2137 and group 2 with *F. sanfranciscensis* TMW 1.1597, TMW 1.1221, TMW 1.907 and TMW 1.726. These two groups were inoculated in the sourdough with either *K. humilis*, *S. cerevisiae* or without the addition of a specific yeast. In addition, sourdough trials were also performed with either *F. sanfranciscensis* TMW 1.907 or *F. sanfranciscensis* TMW 1.392. These two strains were also inoculated either with *K. humilis* or *S. cerevisiae* or without any specific yeast. Furthermore, the *F. sanfranciscensis* strains and the yeasts were grown in overnight cultures, set to an OD_{600} of 1, and 1 ml of each strain and the yeasts/no yeast were added to a sourdough bevor incubation.

4.6.2 Preparation of the lab-scale sourdough model

The propagation of the sourdough is described in Figure 3. The sourdough was prepared with 100 g whole rye flour (dm, Karlsruhe, Germany) and 100 g tap water (Freising, Germany) to a DY of 200. Before incubation, the mixed cultures were added to the pre-fermented sourdough. The sourdough was fermented for 24 h at 28°C and propagated from four up to ten days with 5% to the flour mass. During sourdough fermentation several measurements were taken. The pH, the colony forming units (cfu) per ml and the development of a distinct microbiota was measured (4.6.3). For the characterization of competitiveness between the *F. sanfranciscensis* strains or the distinct microbiota 1 g of sourdough was taken and processed for the CLLP-PCR (see 4.4.1).

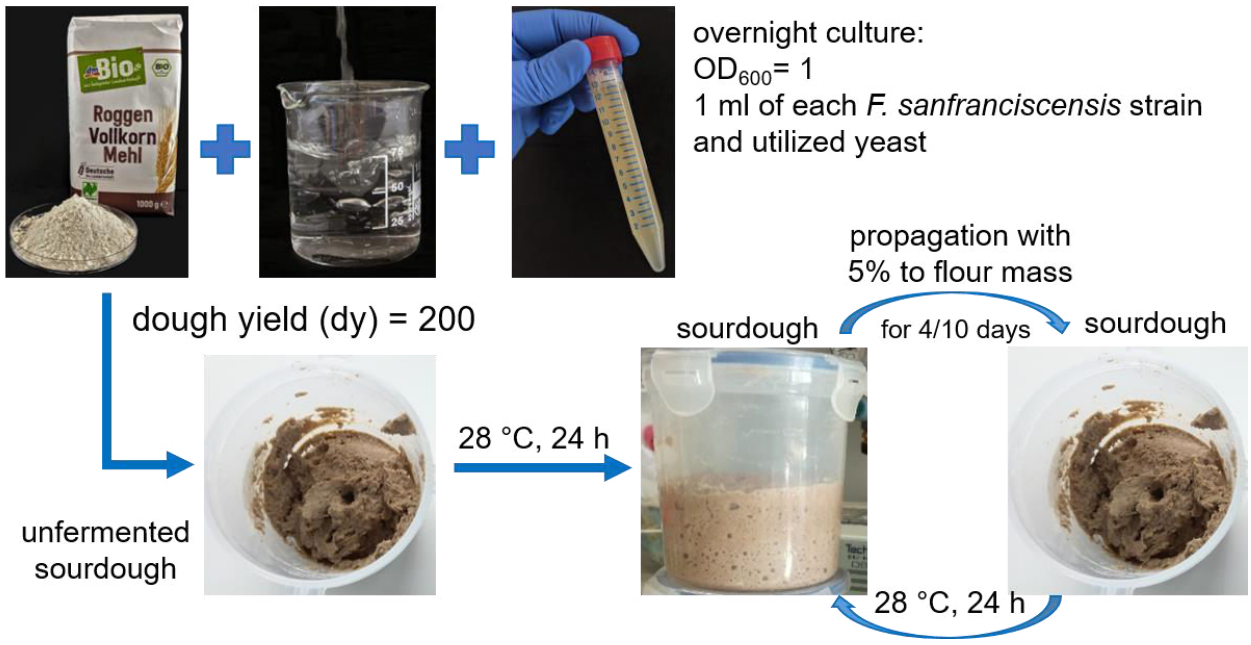

Figure 3: Scheme of the sourdough propagation.

4.6.3 Monitoring of the fermentation process of the sourdough

The pH of the sourdough was measured before and after propagation with a pH electrode from Mettler Toledo InLab® Routine (Columbus, USA). The electrode was rinsed with 70% ethanol and deionized water. The cfu per ml was measured at day 0, 1, 5 and 10 and in fermentation with only one *F. sanfranciscensis* strain also at day 3 and 7. At the same time points the fermentation process and the development of a distinct microbiota was monitored with MALDI ToF MS. With this method bacterial and yeast colonies were identified according to their protein spectrum. This protein spectrum is squared to a deposited database and the bacteria and yeast species can be determined. Sterile toothpicks were used to pick up colonies of bacteria and yeast from agar plates and were swiped on a 96-spot target. The spots were overlaid with 70% formic acid, air dried and overlaid with a α-cyano-4-hydroxycinnamidacid (HCCA) matrix and again air dried. Furthermore, the prepared target was channeled in the MALDI-ToF MS. For the MALDI-ToF MS and cfu/ml measurements 1 g or 10 g of the sourdough was taken for a ten-fold serial dilution up to 10^{-7} in ¼ Ringer's solution. The dilutions were plated out on mfg-MRS for *F. sanfranciscensis* and on YPG agar plates for yeasts and other generalists and incubated for 48 h at 30°C. The so generated colonies were taken for the abovementioned analysis.

4.7 Analytical analysis *in vitro* and *in vivo*

For the measurements of acetic acid, lactic acid, citric acid, ethanol and the sugars and sugar alcohols maltose, fructose, glucose, sucrose, mannitol and erythritol in the sourdough or in buffer solution the high-performance liquid chromatography (HPLC) (Dionex Ultimate 3000, Thermo Fisher Scientific, USA) was utilized.

4.7.1 Preparation of sourdough samples

The sourdough was prepared as described (see 4.6.2) for *F. sanfranciscensis* TMW 1.392, TMW 1.1150, TMW 1.2138 and TMW 1.907 with *K. humilis*, *S. cerevisiae* or without yeast. For each fermentation, a high OD_{600} of 5 in a volume of 14 ml of each bacterial and 140 µl of each yeast culture was applied to the pre-fermented dough. Furthermore, the sourdough was fermented up to three days, backslopped and afterwards samples were taken at 0 h and 24 h. The sourdough samples were diluted 1:2 in deionized water and centrifuged for 30 min, at 8,000 x g, 10°C. For the analysis of the organic acids and alcohol 1.5 ml supernatant was treated over night with 75 µl of 70% perchloric acid (Carl Roth). For the analysis of the sugar and sugar alcohols 1.5 ml supernatant was incubated with 12.52 mM $ZnSO_4*7H_2O$ (Carrez solution 2), 10 mM NaOH and 4.26 mM $K_4[Fe(CN_6)]*3H_2O$ (Carrez solution 1) and inverted. Both preparations were centrifuged for 10 min, at 13,000 x g, RT and then two times filtered through a 2 µM membrane filter (Phenomenex, Torrance, USA). After this treatment, the samples were utilized for HPLC analysis (Cabálková *et al.*, 2004; Rühmkorf *et al.*, 2012).

4.7.2 Preparation of samples in buffer solution

The turnover of predicted components of the sourdough environment due to *F. sanfranciscensis* was determined with HPLC. The same *F. sanfranciscensis* strains as for the HPLC sourdough analysis were chosen. These strains were grown in overnight cultures and were set to an OD_{600} of 5 in ¼ Ringer's solution. 20 mM of maltose, fructose, glucose, or sucrose were added. The usage of citrate, fructose as electron acceptor, oxygen, malate, and gluconate in combination with maltose was also determined. On that account 20 mM of the mentioned compounds were added to a bacterial culture with 20 mM of maltose. The cultures were grown in static conditions at 30°C for 6 h. For the determination of the effect of oxygen the culture with 20 mM of maltose was grown in oxic conditions with 150 rpm. After 0 h and 6 h samples were taken and centrifuged at 13,000 x g for 2 min. The supernatant was sterile filtered and added into HPLC vials for analysis.

4.7.3 High-performance liquid chromatography (HPLC)

HPLC analysis is an analytical method in Chemistry and Biochemistry. It is a useful method for the analysis and quantification of liquid compounds *via* standards. In Biochemistry the HPLC can be applied to determine sugars, organic acids and sugar alcohols as well as amino acids and proteins from various sources and it is a useful tool in Food Chemistry (Andersson and Hedlund, 1983; Lefebvre *et al.*, 2002; Zaky *et al.*, 2017; Coelho *et al.*, 2018; Eisenbach *et al.*, 2018; Schmid *et al.*, 2019). A liquid mixture is solved in a mobile phase which flows with high pressure in a column filled with a separation medium called stationary phase. The mobile and stationary phase is dependent from the analyte. The stationary phase in the column interacts physically with the analyte and separates the liquid mixture according to the interactions with the stationary phase (Figure 4). The time required by the analyte to solve from the physical interaction with the mobile phase is called the retention time. The retention time can be measured *via* different detectors and is characteristic for a specific analyte in defined conditions (Swartz, 2010). The analyte is depicted as a peak with respect to a standard in a chromatogram (Figure 4). The standards are also applied for the quantification of the analyte (SHIMADZU; Gerber *et al.*, 2004). The refraction index (RI) detector is applied for the sugars and amino acids measured in this thesis. The depletion and production of sugars, sugar alcohols, organic acids, and alcohols of *F. sanfranciscensis* strains and yeasts important in the sourdough fermentation were measured during this thesis. The detailed experimental setup is described in 5.4.

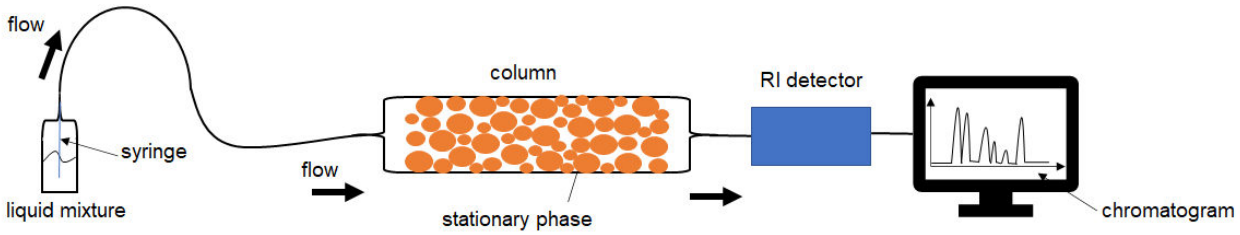

Figure 4: Scheme of the HPLC system. With a syringe small amounts of the liquid mixture are introduced into the HPLC system. The mobile phase transports the solvent with high pressure into the column. The stationary phase binds to the solvent. The specific characteristic of the solvent leads to the separation from the stationary phase. The RI detector measures the retention time of the solvent and displays it as a specific peak in the chromatogram.

4.7.4 Determination of the cellular dry weight and cell size

The cellular dry weight is a useful tool to compare the metabolic turnover of different bacterial strains which each other. This tool is independent of the cell size and the optical density of the bacterial culture. The optical density is sometimes misleading as it is dependent from the cell size of bacterial strains. Strains with broader cells reach a higher optical density compared to smaller strains with the same number of cells. Details for the experimental setup are described in the results section (see 5.4). The cell size is among others important for the metabolic turnover. Furthermore, the differences between the cell size and the volume of the cells can influence the import of extra cellular substances (Bakken and Olsen, 1987; Bååth, 1994). Therefore, the cell sizes of four *F. sanfranciscensis* strains were determined. Experimental details can be found in the results section (see 5.4).

4.8 Statistical analysis

Statistical analysis was performed to prevent artefacts and outliers. Details can be found in the results section (see 5.1; 5.2; 5.3; 5.4).

5 Results (Thesis publications)

5.1 Monitoring of *Lactobacillus sanfranciscensis* strains during wheat and rye sourdough fermentations by CRISPR locus length polymorphism PCR

Preface: At the time of publication *Lactobacillus sanfranciscensis* was the valid taxon, which was later on re-named to *Fructilactobacillus sanfranciscensis*. For any coherence of this thesis´ text, the new designation *Fructilactobacillus sanfranciscensis* is used throughout despite that the reprinted pdf uses the old taxon designation.

In sourdough fermentations with an active microbiota and at moderate temperatures *Fructilactobacillus* (*F.) sanfranciscensis* is a competitive key species together with yeasts like *Saccharomyces* (*S.) cerevisiae* and *Kazachstania* (*K.) humilis*. Different strains of *F. sanfranciscensis* were isolated out of different artisanal and industrial sourdoughs in countries like Germany, Italy, the Netherlands, and China. It is well known that different strains of *F. sanfranciscensis* have different properties and these properties can influence the quality of the sourdough and in the end the bread quality. However, a fast and reliable identification of *F. sanfranciscensis* strains especially in the niche sourdough was not available. Therefore, intra-species interactions and competitions of *F. sanfranciscensis* in the sourdough were still unexplored.

On this account, a species-specific PCR-based method for strain identification and strain monitoring in the sourdough is presented in this communication. This typing method is based on the CRISPR locus length polymorphism of the *F. sanfranciscensis* strains. The length of the CRISPR locus is highly variable and strain specific. For the development of this method the genomes of 21 *F. sanfranciscensis* strains were sequenced and annotated, and the CRISPR-Cas system was analyzed. Nineteen out of 21 strains contain the CRISPR-Cas Type II-A system and three strains contain the CRISPR-Cas Type I-E in addition. Two of the strains do not contain any CRISPR-Cas loci. The CRISPR-Cas Type II-A system was applied for strain differentiation and 14 different CRISPR-Cas genotypes were observed *in silico* in the analysis of the loci. In the region of the conserved 5`-end of the CRISPR-Cas locus the forward primer was created. Furthermore, there were two groups of CRISPR-Cas Type II-A systems and the reverse primer was placed in the conserved 3`-end of these two groups respectively. The three primers were applied in a multiplex PCR for *in-situ* analysis of the CLLP and therefore for strain differentiation.

For monitoring of the *F. sanfranciscensis* strains in the sourdough four different strains were chosen according to their CLLP (*F. sanfranciscensis* TMW 1.392, TMW 1.1150, TMW 1.2138 and TMW 1.2142) and sourdoughs were prepared. The DNA of the *F. sanfranciscensis* strains in the sourdough was isolated and the CLLP-PCR was processed. In the rye and wheat sourdough fermentation *F. sanfranciscensis* TMW 1.392 was after two days competitive against the others until the end of the experiment time. Consequently, it was possible to demonstrate that the CLLP-PCR is suitable for strain differentiation and species monitoring in complex environments like sourdoughs.

Author contributions: Esther Rogalski conducted all experiments and was responsible for the experimental design. Furthermore, she visualized and evaluated the data, and wrote the original draft of the manuscript.

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Monitoring of *Lactobacillus sanfranciscensis* strains during wheat and rye sourdough fermentations by CRISPR locus length polymorphism PCR

Esther Rogalski, Rudi F. Vogel, Matthias A. Ehrmann*

Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany

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ABSTRACT

Lactobacillus (*L.*) *sanfranciscensis* is a competitive key species in sourdough fermentations. However, the principles involved in establishing the commonly observed phenomenon of strain dominance are unresolved. This has been studied little because the methods for fast and reliable differentiation of strains and their monitoring during fermentation are tedious and cannot be done with large numbers of isolates. In this contribution, we present a strain-specific, PCR-based typing method that uses length heterogeneities of the clustered regularly interspaced short palindromic repeats (CRISPR) loci as they occur in the genomes of different strains. *In silico* analysis of 21 genomes revealed 14 different CRISPR genotypes. We then designed a primer set to simultaneously detect different strains in a multiplex PCR assay designated CRISPR locus length polymorphism PCR (CLLP-PCR). The usefulness of this method was evaluated in lab-scale sourdough fermentations conducted with rye and wheat flours. First, the flour was mixed with water to a dough yield of 200. Then each dough was inoculated with four different *L. sanfranciscensis* strains (TMW 1.1150, TMW 1.392, TMW 1.2142, and TMW 1.2138) at levels of 10^9 cfu/g each. Sourdoughs were propagated at 28 °C for 5 days by back slopping 5% to the flour mass every 24 h. Samples were collected each day; DNA was isolated, and the presence of strains was detected qualitatively in the sourdoughs with PCR.

L. sanfranciscensis TMW 1.392 became dominant as early as 2 days into the fermentation and remained the only detectable strain for the rest of the sampling period. CLLP-PCR proved to be useful in investigating the assertiveness of different strains of *L. sanfranciscensis* in sourdoughs. Therefore, CLLP-PCR may be used as a tool to investigate assertiveness of microorganisms in food fermentations at the strain level.

1. Introduction

Sourdough has been used since ancient times for baking bread and preserving baked goods (De Vuyst and Neysens, 2005; Lönner and Ahrné, 1995). Because of its desirable effects on the resulting bread, such as extended shelf life, unique flavor, texture, nutritional properties, and increased bread volume due to gas production, sourdough has remained popular to this day (De Vuyst and Neysens, 2005; Gänzle et al., 2007; Hammes and Gänzle, 1998; Korakli et al., 2000). Lactic acid bacteria (LAB) and yeasts are the drivers for the fermentation of the flour and water mixture into sourdough (De Vuyst and Neysens, 2005; Di Cagno et al., 2002; Gänzle et al., 2007). The most commonly isolated bacterial species in sourdough are *Lactobacillus* (*L.*) *pontis*, *L. brevis*, *L. plantarum*, *L. rossiae*, and *L. sanfranciscensis* (Brandt and Gänzle, 2006; Gänzle, 2005; Gänzle et al., 2008), and the most common yeasts are *Saccharomyces cerevisiae* and *Kasachstania humilis* (syn. *Candida milleri*) (Gobbetti and Corsetti, 1997; Sieuwerts et al., 2018).

L. sanfranciscensis is seen as the key species in sourdough fermentations; it has not been isolated from any other ecological niche thus far. First isolated from San Francisco sourdough (De Vuyst and Neysens, 2005; Kline and Sugihara, 1971), it is the predominant LAB in Type 1 fermentations (De Vuyst and Neysens, 2005; Yang et al., 2017), which is maintained through a daily and continuous propagation every 4–16 h at 25 °C–35 °C and a pH value of 3.5 to 4.0 (Brandt and Gänzle, 2006). Moreover, it is common in Chinese traditional sourdough (Yang et al., 2017) and in sourdoughs used for Italian pastries like panettone, in brioches, pizza, rye, and wheat bread (Gobbetti and Corsetti, 1997; Picozzi et al., 2010) and in Belgian sourdoughs (Scheirlinck et al., 2007).

Although the identification of individual species in a given fermentation process and their dynamic growth behavior can be investigated relatively easily using established methods such as matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS) (Janßen et al., 2018) and multiple DNA-based

* Corresponding author at: Lehrstuhl für Technische Mikrobiologie, TU München, Gregor-Mendel-Straße 4, 85354 Freising, Germany.
E-mail address: matthias.ehrmann@tum.de (M.A. Ehrmann).

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Table 1
Strains of *L. sanfranciscensis* used in this study.

Organism	Strain	Accession no.	Isolation source	References
L. sanfranciscensis	DSM 20451^T	MIYJ00000000	Sourdough, USA	Kline and Sugihara (1971)
L. sanfranciscensis	TMW 1.54 (LTH 1729)	NZ_MIYE01000000	Rye sourdough, Germany	Stolz et al. (1995)
L. sanfranciscensis	TMW 1.392 (LTH 2590)	NZ_MIYH01000000	Sourdough, Germany	(Böcker et al., 1995)
L. sanfranciscensis	TMW 1.640	SCEZ00000000	Wheat sourdough, Switzerland	Ehrmann and Vogel (2001)
L. sanfranciscensis	TMW 1.726	NZ_MIYD01000000	Sourdough, Italy	Liske et al. (2000)
L. sanfranciscensis	TMW 1.907	SCEY00000000	Sourdough, Greece	This study (1998)
L. sanfranciscensis	TMW 1.1150	NZ_MIYG01000000	Sourdough, USA	This study (2000)
L. sanfranciscensis	TMW 1.1152	SCEV00000000	Sourdough, USA	This study (2000)
L. sanfranciscensis	TMW 1.1154	SCEU00000000	Sourdough, USA	This study (2000)
L. sanfranciscensis	TMW 1.1221	SCET00000000	Sourdough, France	This study (2000)
L. sanfranciscensis	TMW 1.1304	SCES00000000	Rye sourdough, Germany	Vogel et al. (2011)
L. sanfranciscensis	TMW 1.1470	SCER00000000	Sourdough, Russia	This study (2008)
L. sanfranciscensis	TMW 1.1597	NZ_MIYF01000000	Rye sourdough, Germany	This study (2009)
L. sanfranciscensis	TMW 1.1730	SCEQ00000000	Sourdough, Germany	This study (2010)
L. sanfranciscensis	TMW 1.2137[1] (LS3)	NZ_MIXX01000000	Sourdough, Italy	De Angelis et al. (2007)
L. sanfranciscensis	TMW 1.2138[1] (LS12)	NZ_MIXY01000000	Sourdough, Italy	De Angelis et al. (2007)
L. sanfranciscensis	TMW 1.2139[1] (LS27)	NZ_MIXZ01000000	Sourdough, Italy	De Angelis et al. (2007)
L. sanfranciscensis	TMW 1.2140[1] (LS19)	NZ_MIYA01000000	Sourdough; Italy	De Angelis et al. (2007)
L. sanfranciscensis	TMW 1.2141[1] (LS48)	NZ_MIYB01000000	Sourdough, Italy	De Angelis et al. (2007)
L. sanfranciscensis	TMW 1.2142[1] (LS13)	NZ_MIYC01000000	Sourdough, Italy	De Angelis et al. (2007)
L. sanfranciscensis	TMW 1.2314	SCEW00000000	Rye sourdough, Germany	This study (2018)

Strains kindly provided by [1]Fabio Minervini.

techniques (De Angelis et al., 2007; Dellaglio et al., 1998), the tracking of single strains that are part of a complex microbial community is still a major challenge. Sourdough microbiota may consist not only of different microbial species but also of different strains of a given species. Therefore, inter-strain relationships may also contribute to the stability of a microbial consortium (Kitahara et al., 2005). The very small genome of *L. sanfranciscensis* TMW 1.1304 (approximately 1.23 Mbp) and this bacterium's very narrow niche suggest a strong adaptation to the sourdough environment (Vogel et al., 2011). Nevertheless, previous studies using molecular methods have demonstrated intraspecies diversity of *L. sanfranciscensis* (Corsetti et al., 2003; Dellaglio et al., 1998). Moreover, genomic comparison of *L. sanfranciscensis* strains from different sourdough types reveal a level of genomic diversity that is larger than anticipated, suggesting that strains occur in different environments (De Angelis et al., 2007; Geißler et al., 2017). However, drivers for the establishment of strain dominance in a specific sourdough process are unknown. Characterization of assertiveness at the strain level requires methods capable of differentiating and monitoring single strains in sourdough fermentations. Techniques used so far include randomly amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) analysis (De Angelis et al., 2007), ribotyping (Kitahara et al., 2005), pulsed field gel electrophoresis (PFGE), restriction fragment polymorphism (Ehrmann and Vogel, 2001), and ribosomal intergenic spacer analysis (De Angelis et al., 2007). In addition, these methods require the isolation of individual or single colonies as a prerequisite. Denaturation gradient gel electrophoresis (DGGE) allow the detection of non-cultivatable organisms, but as in previous cases, these techniques do not discriminate at the strain level.

CRISPR and CRISPR-associated (Cas) genes constitute the adaptive immune system of bacteria, protecting against mobile genetic elements (MGE) or bacteriophages (Barrangou et al., 2007; Barrangou and Marraffini, 2014; Crawley et al., 2018). After surviving a viral or MGE attack, immune markers of these aggressors (spacer) get integrated into the CRISPR locus that is flanked by highly conserved repeats (Barrangou et al., 2007; Barrangou and Marraffini, 2014; Garneau et al., 2010). The different number of such events results in a strain-specific length heterogeneity. Therefore, the CRISPR locus reflects changes in environment and changes over time (Barrangou et al., 2007). A major point is the transcription and processing of CRISPR loci into small interfering RNAs (crRNA). These RNAs guide nucleases (cas proteins) to complementary DNA which lead to digestion of foreign elements. A specific protospacer adjacent motif (PAM) sequence on the invading DNA allows to distinguish bacterial self from non-self-DNA (Barrangou and Marraffini, 2014; Louwen et al., 2014; Wright et al., 2016). The mechanism in detail is described elsewhere (Barrangou and Horvath, 2012; Barrangou and Marraffini, 2014; Horvath and Barrangou, 2010 ; Horvath et al., 2008; Makarova et al., 2011; Makarova et al., 2015). Targeted modification of DNA fragments with endonucleases makes the system interesting for programmable genome editing (Briner et al., 2014; Sander and Joung, 2014;Wright et al., 2016). In clinical microbiology CRISPR loci are used as epidemiological markers to detect *Mycobacterium tuberculosis* strains since 1993 (Groenen et al., 1993; Sorek et al., 2008;Zhang et al., 2010). But there are several applications possible in food production related to fermentation processes were lactobacilli are omnipresent (Selle and Barrangou, 2015). Especially in Lactobacillus spp. the CRISPR system is disproportional abundant and could be used for typing or genetic modifications in starter cultures or probiotics (Selle and Barrangou, 2015). The most common CRISPR-Cas system in lactobacilli is the Type II system with the Type II-A subtype (Crawley et al., 2018). Briner and Barrangou (2013) successfully used 10 unique locus genotypes that contained between 9 and 29 variable spacers within the CRISPR locus to differentiate between *L. buchneri* pickle fermentation isolates. In this contribution, we analyzed the CRISPR loci of 21 isolates of *L. sanfranciscensis* and demonstrate its potential as a tool to track single strains during sourdough fermentations.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

The *L. sanfranciscensis* strains used in this work are listed in Table 1. The strains were routinely grown on modified De Man, Rogosa, and Sharpe (mMRS) agar at pH 5.4 at 30 °C for 48 h. The mMRS medium contains 1% w/v casein peptone (Roth, Karlsruhe, Germany), 0.2% w/v meat extract (Merck, Darmstadt, Germany), 0.2% w/v yeast extract (Roth), 0.2% w/v sodium gluconate (Roth), 0.5% w/v sodium acetate x 3 H_2O (Roth), 0.5% w/v citric acid diammonium salt (Roth), 0.25% w/v potassium di-hydrogen phosphate (Merck), 50‰ w/v L-cysteine hydrochloride monohydrate (Roth), 0.1% w/v polysorbate 80 (GEBRU Biotechnik GmbH, Heidelberg, Germany), 20‰ w/v magnesium sulfate x 7 H_2O (Merck), 10‰ w/v manganese sulfate x H_2O (Roth), 5‰ w/v iron (II) sulfate x 7 H_2O (Roth) and 0.7% w/v glucose monohydrate

(Merck), 0.7% w/v maltose (Merck), and 0.7% w/v fructose (Omni Life Science GmbH & Co. KG, Bremen, Germany). For mMRS agar, 1.5% w/v AgarAgar (Roth) was added. For liquid culture, the strains were grown in mMRS medium at 30 °C for up to 48 h. The strains were isolated and identified to species level with MALDI-TOF MS (biotyper, Brucker Billerica, USA) and 16S rRNA gene sequencing (Table 1). All isolates were stored at −80 °C in frozen glycerol stocks. The growth of cultures was monitored using a SPECTROstar^{nano} plate reader (BMG Labtech, Ortenberg, Germany) with 96-well plates (Sarstedt, Nümbrecht, Germany). To grow cultures, mMRS medium was first inoculated with an OD_{600} of 0.05 of an overnight culture incubated at 30 °C and then incubated for 48 h at 30 °C. Three biological and technical replicated were conducted. The growth curves were analyzed with the grofit package (Kahm et al., 2010).

2.2. Generation of draft genomes and identification of CRISPR-Cas loci

DNA isolation for whole genome shotgun sequencing was performed with E.Z.N.A. ® DNA Kit from OMEGA Bio-tek (Norcross, USA). For the isolation the strains were grown over night in mMRS for 30 °C. Genome sequencing of strains was carried out using a MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA) with a PCR-free library preparation. Processing and assembly with SPAdes V3.9.0 (Bankevich et al., 2012) were conducted using the method described by Huptas et al. (2016). The NCBI Prokaryotic Genome Annotation Pipeline (Haft et al., 2018; Tatusova et al., 2016) was used to annotate the sequences, and the NCBI accession numbers are shown in Table 1.

Twenty-one genome sequences were uploaded to the CRISPR-Cas finder (Couvin et al., 2018), a tool that locates CRISPR loci and the Cas genes. Homologies in the spacer sequences were evaluated by aligning and comparing the spacer of the Type II-A loci together with the repeat sequences using Clustal Omega (Larkin et al., 2007). For the visualization of the CRISPR spacers sequences CRISPRStudio was used together with CRISPRDetect for a correct output (Biswas et al., 2016; Dion et al., 2018).

2.3. In silico analyses of the CRISPR-Cas system Type II-A

To identify the PAM sequences first the spacer sequences of all CRISPR Type II-A loci were queried with BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). For protospacer sequences all results matching a virus sequence with less than two mismatches were used. Together with 10 nt at the 5′-end and the 3′-end of the protospacer the PAM sequence was analyzed with WebLogo (Crooks et al., 2004). The detection of the predicted tracrRNA was performed according to Hupfeld et al. (2018).

2.4. CRISPR genotyping of L. sanfranciscensis isolates

Five hundred nucleotides upstream and downstream of the Type II-A CRISPR locus (the repeat spacer sequence) of all selected strains were aligned with Clustal Omega to find regions for primer binding. For the forward primer, it was possible to choose a region in the *csn2* genome to design the forward primer CR_fow3 (5′-GCTGATAGGTGAATATTAC-3′). Because of the two versions of the CRISPR locus II-A, it was necessary to create a reverse primer for each group separately. One primer was designed for type a: CR_rev2a (5′-CTCTCTTATAACTCTCAAAC-3′), and another was designed for type b: CR_rev4b (5′-GATAATTCCAATAATA GCGTAG-3′). With these primers, a multiplex PCR was performed in 50 µL containing 5 µL 10× PCR-Mix (MP Biomedicals, Santa Ana, USA), 200 µM dNTPs (MP Biomedicals), 0.25 µM of each primer (Eurofins Genomics, Ebersberg, Germany), 1.5 U TAQ Polymerase (MP Biomedicals) and 10–50 ng of pure DNA from isolation. PCR conditions were optimized to a standard protocol with 2 min of initial denaturation at 95 °C. The cycle starts with 45 s denaturation at 95 °C, 1.30 min annealing at 53 °C, and 2 min elongation at 72 °C. Thirty-two cycles were performed, and the PCR was finished with 5 min final elongation. The same approach was also used for the PCR of the DNA isolated from laboratory-scale sourdough samples. PCR fragments were visualized by agarose gel electrophoresis. For strains DSM 20451^T and TMW 1.2140, the amplicons were purified using the E.Z.N.A® Cycle Pure Kit (Omega Bio-Tek) and their sequences were analyzed (Eurofins Genomics, Germany).

2.5. Sourdough preparation, cfu measurement, and DNA isolation

To investigate strain assertiveness, four *L. sanfranciscensis* strains (TMW 1.392, TMW 1.2138, TMW 1.1150, and TMW 1.2142) were chosen for setting up a laboratory-scale sourdough model. After growing for 48 h in mMRS medium, the strains were adjusted to an OD_{600} of 1 in Ringer's solution (Merck, Darmstadt Germany), and then equal volumes (1 mL) of all strains were mixed together. Afterwards, rye or wheat flour was mixed with water to a dough yield of 200, and the strain solution was added. Every 24 h, the sourdough was propagated by back slopping 5% of the flour volume and incubating at 28 °C. The experiment was performed over 5 days. The pH was measured before and after back slopping and at the beginning and the end of the experiment. Sourdough samples were collected every day, 500 mg of which was used for DNA isolation with the FastDNA™ Spin Kit for Soil (MP, Santa Ana USA). For cfu/mL measurements, 1 g of the sourdough was mixed with 9 mL of Ringer's solution and a ten-fold serial dilution up to 10^{-7} in Ringer's solution was performed. Each dilution step was plated out on mMRS agar plates and incubated for 48 h at 30 °C. For determination of colony forming units (cfu) samples were collected at 0, 1, 3, and 5 days of fermentation. Also the cfu of all four strains at OD_{600} at 1 was determined bevor inoculation of the sourdough, except for TMW 1.2138 (10^8 cfu/mL) the cfu was at 10^9 cfu/mL. DNA isolated from each sampling date was used to perform PCR and agarose gel electrophoresis. For the PCR 100 ng/µL of isolated DNA was used.

3. Results

3.1. Identification of CRISPR-Cas systems in L. sanfranciscensis genomes

CRISPR loci were found in 19 of 21 tested strains of *L. sanfranciscensis*. The major CRISPR-Cas Type was II-A, and a second Type I-E system was found in TMW 1.54, TMW 1.640, and TMW 1.1730. The repeat sequence of the Type II-A system (5′-GTTTTAGAAGTACGTCAT TCTAATGAGATTAAGAGC-3′) was highly conserved in all the strains, although in a few strains, the C at the 3′-end was missing. TMW 1.640 had a single mutation at position 13 were C is changed to T. In 11 of the 19 strains tested, the number of spacers differs and was between 2 and 31 (Fig. 1). The repeat sequence of the Type I-E system was 5′-GTAT TCCCCACGCATGTGGGGGTGATCCT-3′ (29 nucleotides). TMW 1.640 also had a second Type I-E system with only one spacer sequence and a repeat sequence of 5′-CTGGAGCAGCTTGTTGAACTGGTGCT-3′ (26 nucleotides). The strains TMW 1.2141 and TMW 1.1597 had no CRISPR-Cas system. In this work, we focused on the CRISPR-Cas Type II-A locus because most of the strains had this system and the Type I-E was too rare to use as a typing system. The CRISPR-Cas Type II-A system of *L. sanfranciscensis* was analyzed more in detail (S1). The Type II-A system was composed of the endonuclease Cas9, Cas1 and Cas2, which were upstream of the CRISPR-associated protein Csn2 which was directly upstream of the CRISPR locus just like described in the literature (Barrangou and Horvath, 2012; Bhaya et al., 2011; Crawley et al., 2018). The most closely related Cas nucleases were found in *L. lindneri* (*79%* Cas9 identity) followed by *L. buchneri* (66% Cas9 identity) (S2). As described in the literature for CRISPR-Cas Type II-A systems the tracrRNA is between *cas9* and *cas1* (Fig. 1, S5). For detection of the PAM recognition site 19 protospacers with 10 nt of the 3′- and 5′-flanking region were analyzed and visualized with WebLogo (Crooks et al., 2004). The PAM recognition site is in the 3′- flanking region and

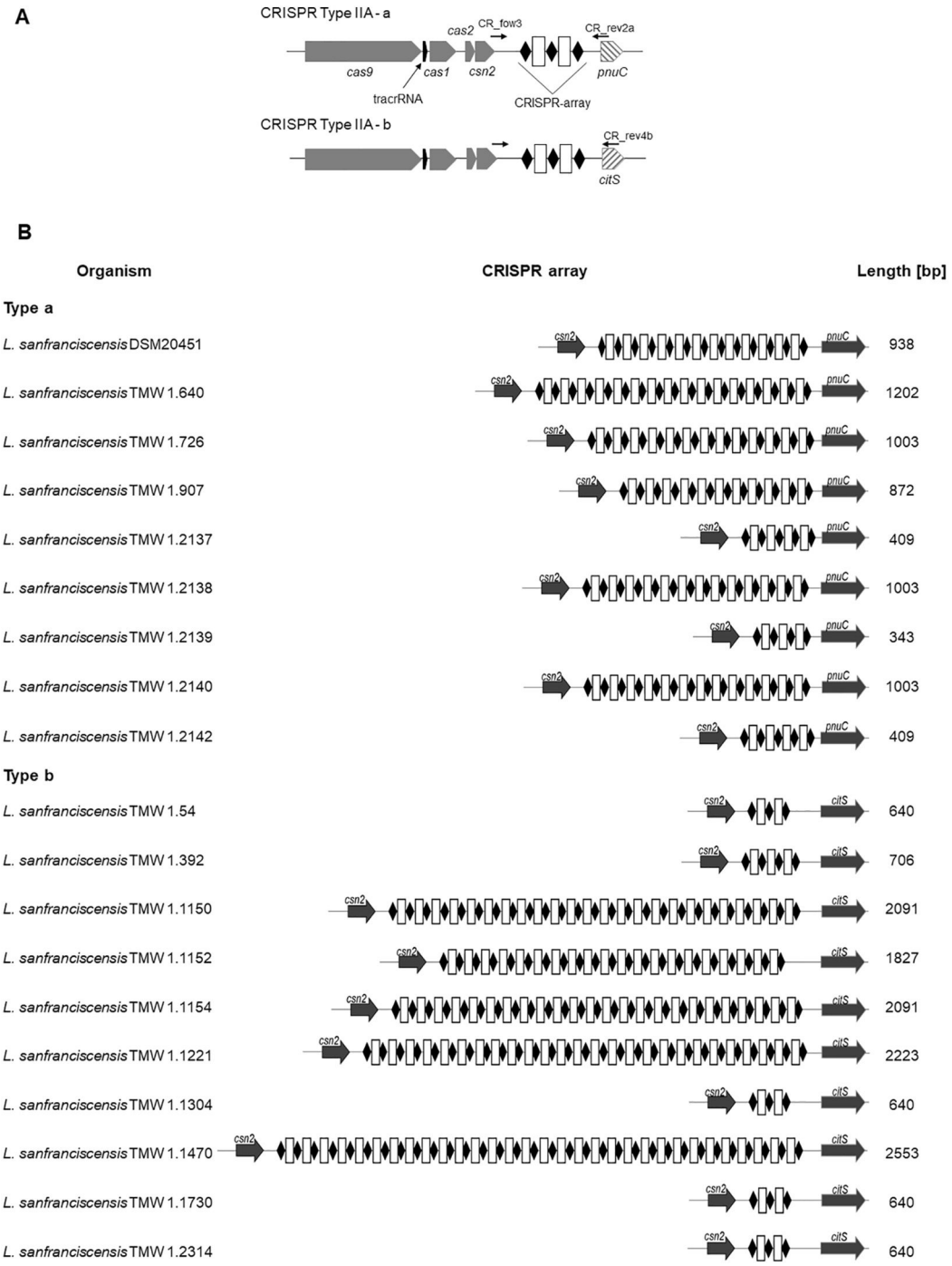

Fig. 1. *In silico* analysis of the CRISPR Type II-A loci in different *L. sanfranciscensis* strains. A: the CRISPR-Cas Type II-A system of *L. sanfranciscensis*. B: The strains are sorted according to their CRISPR Type II-A type (a or b). The gene *csn2* is upstream, and the genes *pnuC* (type a) or *citS* (type b) are downstream of the CRISPR locus. The white diamonds and the black figures symbolize the repeat spacer array. The length in the third column refers to the number of nucleotides between these primers. *pnuC* is the nicotinamide mononucleotide transporter, and *citS* is the citrate sodium symporter.

consists of two conserved A's at position 3 and 5. At position 4 an A or C is possible as well as two C's at position 6 and 7 (S4). The PAM recognition site of *L. sanfranciscensis* shows similarities to this of the previously described sequence of *S. thermophilus* (Briner and Barrangou, 2013; Horvath et al., 2008). As described in the literature the crRNA consists, after expression of the CRISPR spacer array and maturation, of small spacer-repeat RNAs (Selle and Barrangou, 2015; Stout et al., 2017). Downstream of the CRISPR-Cas II-A locus, we found either a nicotinamide mononucleotide transporter (*pnuC*); in nine strains; hereinafter referred to as type a) or a citrate sodium symporter (*citS*); in ten strains; hereinafter referred to as type b). We also analyzed the spacers of the different strains (Fig. 2). The spacer sequences were ordered like they were presumably acquired, with the first spacer at the 5′-end and the newest at the 3′-end (Dion et al., 2018). Overall, we identified 11 different CRISPR genotypes. Strains sharing identical genotypes were most often isolated from the same source (Table 1). Strains with equal numbers of spacers also shared identical spacer sequences. The exceptions were isolates TMW 1.2139 and TMW 1.392, which share the same number of spacers, but their sequences differed. However, these two strains differed in the type of their CRISPR-Cas

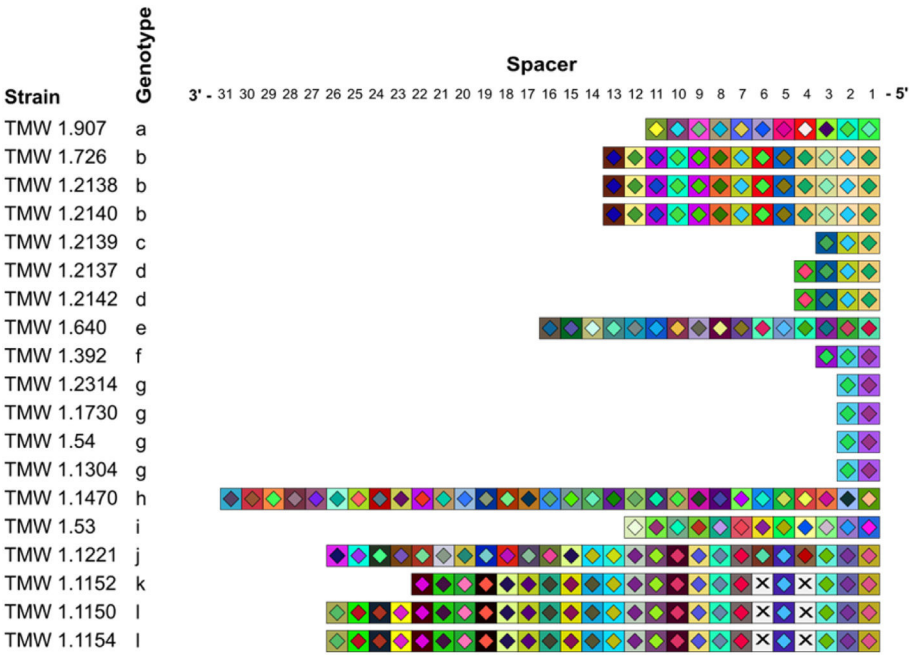

Fig. 2. Visualization of the spacer sequences of the Type II-A CRISPR loci. Each square represents a spacer whose positions are enumerated from 1 to 31 from first acquired to new spacers. Different color combination means different spacer sequences. Each specific spacer combination represents a spacer genotype (a–k). The 'x' means a deleted or missing spacer.

Type II-A loci. This showed how the variability of the CRISPR locus can be used for typing strains. Some spacer sequences matched with the *L. sanfranciscensis* phage EV3 (Ehrmann et al., 2013; Foschino et al., 2005). Strains with the phage sequences, which were inserted in the CRISPR locus, differed on the basis of genotype and source of isolation (S3).

3.2. Design of a CRISPR length polymorphism PCR (CLLP-PCR)

In order to determine the sizes of the CRISPR repeat spacer arrays *in situ*, a semi-multiplex PCR consisting of one forward and two reverse primers was performed with all *L. sanfranciscensis* strains. To detect type a strains, one reverse primer bound directly after the beginning of *pnuC*, and to detect type b strains, the other reverse primer bound within *citS*. Thus, the combination of two reverse primers with one forward primer (binding at *csn2*) enabled the simultaneous detection of type a and b of all CRISPR locus type II-A systems (Fig. 3). The amplicon sizes matched the *in silico* predicted locus length (Fig. 1 and Fig. 3) (except for strains DSM 20451^T and TMW 1.2140). The type b arrangement included more nucleotides between the end of the CRISPR locus and the beginning of *citS*. This is the reason why the length of the CRISPR locus differed also between type a and b, and strain TMW 1.2142 with four spacers had a lower molecular size and thus a lower gel band than TMW 1.392 with three spacers (Fig. 1). These results explain why CRISPR locus length polymorphism was easy to detect with PCR and was hypervariable from strain to strain.

3.3. Tracking of L. sanfranciscensis strains in a lab-scale sourdough model

To evaluate the CRISPR locus length polymorphism detection system, four strains (TMW 1.392, TMW 1.1150, TMW 1.2138 and TMW 1.2142) differing in isolation source and CRISPR pattern were chosen (Table 1). The strains also differed in their growth in mMRS medium (Fig. 4). TMW 1.392 had the shortest lag phase (3.8 h), whereas TMW 1.1150 had the longest lag phase (7.5 h). The strain TMW 1.2138 grew fastest to the maximum OD_{600} of 3.5 with a slope of 0.5 OD_{600}/h; TMW 392 was the slowest grower with a slope of 0.25 OD_{600}/h and a maximum OD_{600} of 3.6. TMW 1.1150 had the lowest OD_{600} maximum of 2.6, whereas TMW 1.2142 had the highest OD_{600} maximum of 3.7 (Fig. 4).

As expected, all four strains were detected in one reaction when their purified genomic DNAs were mixed together (Fig. 5A). In the next

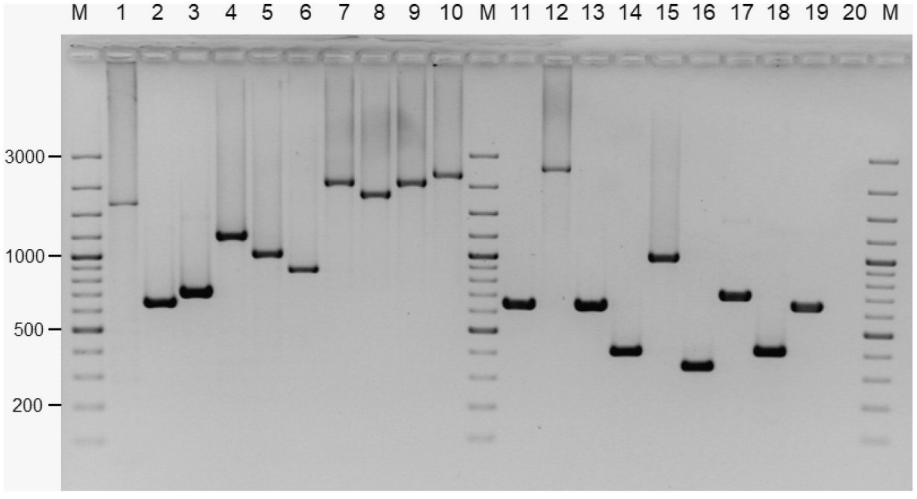

Fig. 3. Amplicons of Type II-A CRISPR loci of analyzed *L. sanfranciscensis* strains. Lanes 1–20, *L. sanfranciscensis* DSM 20451^T, TMW 1.54, TMW 1.392, TMW 1.640, TMW 1.726, TMW 1.907, TMW 1.1150, TMW 1.1152, TMW 1.1154, TMW 1.1221, TMW 1.1304, TMW 1.1470, TMW 1.1730, TMW 1.2137, TMW 1.2138, TMW 1.2139, TMW 1.2140, TMW 1.2141, TMW 1.2142, TMW 1.2314, and TMW 1.1597. Lane M, 100 bp plus DNA Ladder, Thermo Fisher Scientific (Waltham, USA).

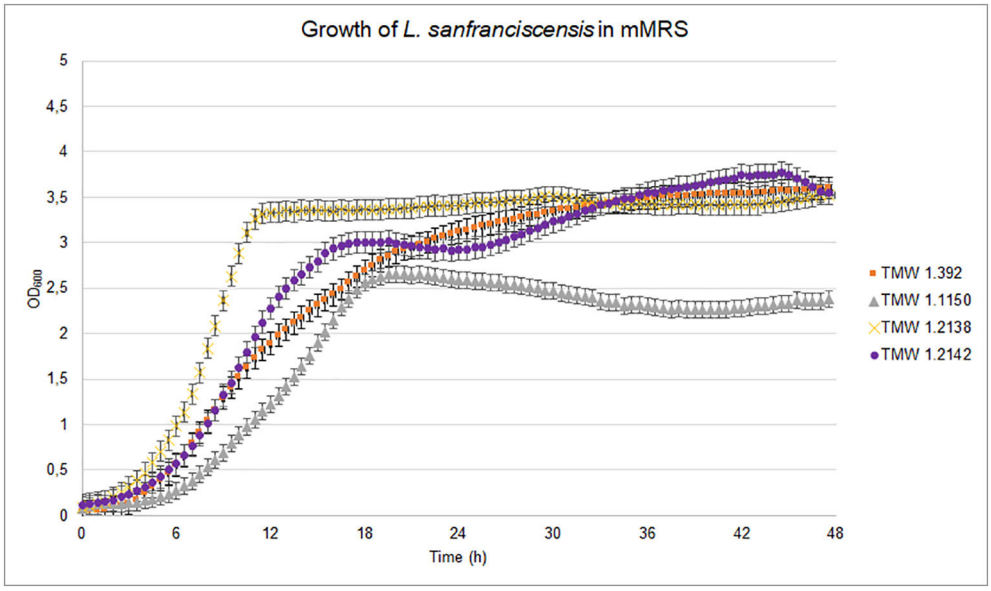

Fig. 4. Different growth behavior (optical density [OD] *versus* time [h]) of *L. sanfranciscensis* strains in mMRS medium for 48 h at 30 °C under aerobic conditions in a 96-well plate. The values are means of three independent experiments.

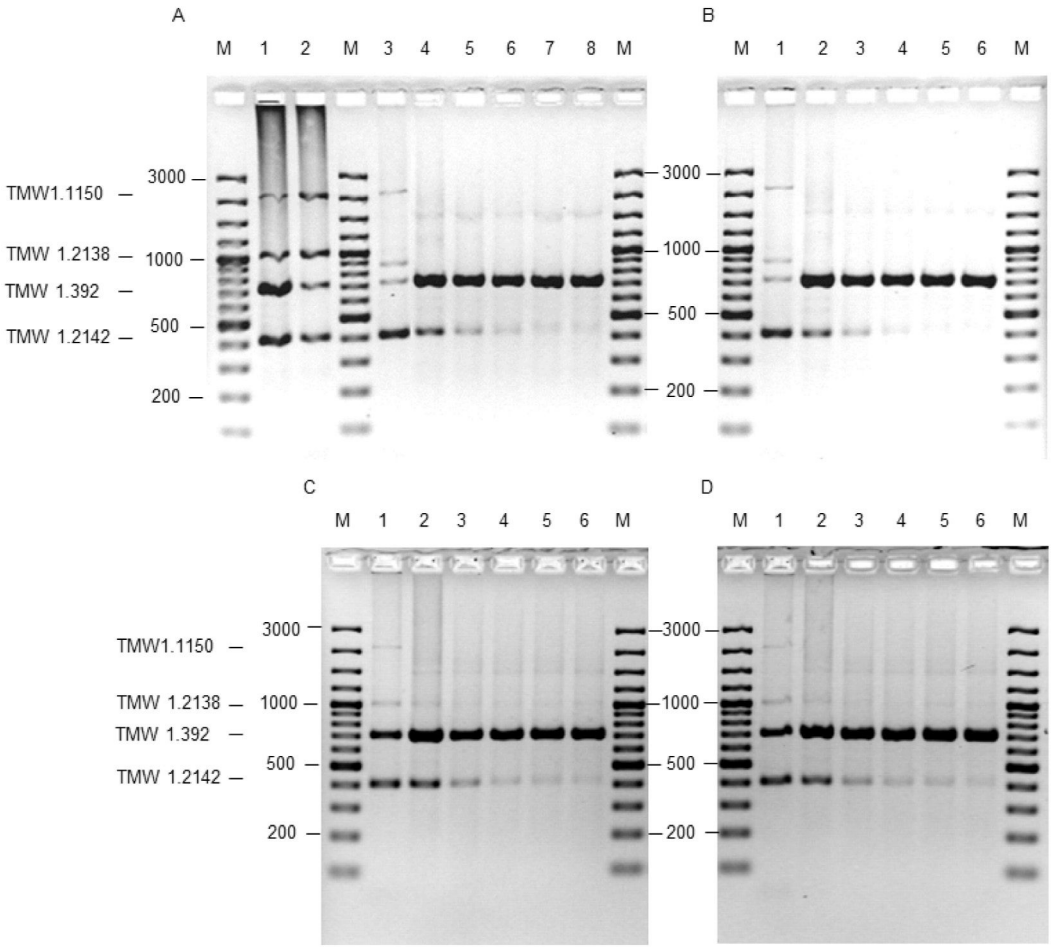

Fig. 5. Characterization of assertiveness of *the L. sanfranciscensis* strains TMW 1.1150 (2091 bp), TMW 1.2138 (1003 bp), TMW 1.392 (706 bp), and TMW 1.2142 (409 bp). TMW 1.392 is assertive over the others in all sourdoughs. A: Rye sourdough, mixture of pure DNA [1], mixture of cultures [2], and day 0 to day 5 [3–8]. B is also a rye sourdough and a biological replicate of A, and C and D are wheat sourdoughs and biological replicates. From B to D: Lanes 1–6 represent day 0 to day 5. Marker (M) Gene ruler 100 bp plus DNA Ladder, Thermo Fisher Scientific.

experiment, equal numbers of growing cells of the chosen strains were mixed together. When the DNA from this mixture was isolated, the multiplex PCR was still able to detect the four strains (Fig. 5A). Finally, the system was tested in lab-scale sourdough models. Both wheat and rye sourdoughs were prepared in a lab-scale model, each containing *L. sanfranciscensis* TMW 1.392, TMW 1.1150, TMW 1.2138, and TMW 1.2142. Sourdoughs were inoculated with the strain mixture at a final cell count of 2.17×10^7 cfu/g. At the end of each fermentation cycle (24 h), the number of cfus remained constant at 1×10^{10} cfu/g throughout the whole experimental period. In both doughs, the initial pH of approximately 6.24 dropped to values between 3.5 and 3.8 after each cycle of fermentation.

At the beginning of the experiment, it was possible to detect amplicons of all four strains. After 3 days of back slopping, the consortium was then stable till the end of the experiment. In the first 2 days, *L. sanfranciscensis* strains TMW 1.392 and TMW 1.2142 were detectable. After 3 days of fermentation, only a single 0.7 kb amplicon was detected, indicating the dominance of *L. sanfranciscensis* TMW 1.392 (Fig. 5). This was observed in the rye as well as in the wheat sourdough and in both biological replicates.

4. Discussion

4.1. The CRISPR-Cas system in L. sanfranciscensis

Widely distributed in LAB, CRISPR-Cas systems provide acquired resistance against viruses and foreign DNA attacks (Barrangou et al., 2007; Deveau et al., 2008; Horvath et al., 2009; Horvath et al., 2008). The CRISPR-Cas system could be used in many different fields for genotyping or genome engineering especially in medicine (Hart et al., 2015; Zhang et al., 2010) or in food production as described detailed elsewhere (Donohoue et al., 2018; Selle and Barrangou, 2015; Stout et al., 2017). Recent reports focusing on lactobacilli describe the presence and intraspecies diversity of CRISPR-Cas systems in *L. buchneri*, *L. sakei*, and *L. helveticus* (Briner and Barrangou, 2013; Scaltriti et al., 2019; Schuster et al., 2019). But also the CRISPR patterns of food pathogens like *Listeria monocytogenes* or *Salmonella enterica* are described intensively (Di et al., 2014; DiMarzio et al., 2013; Liu et al., 2011)

In this contribution, we demonstrate the occurrence and diversity of the CRISPR-Cas immune system in *L. sanfranciscensis*. A CRISPR-Cas II-A system was present in 90.5% of the tested *L. sanfranciscensis* strains ($n = 21$). An additional CRISPR-Cas I-E system was present in 14% of the strains. Length of the system was heterogeneous in 58% of the strains; we found 11 different genotypes. Although sequences of the repeats in the II-A system are highly conserved, spacer composition differs extremely between the different genotypes. Notably, spacer sequence similarity was higher between strains isolated from the same country compared with those from different regions.

It is typical for spacer sequences to match with sequences occurring in other strains, species, or phages (Louwen et al., 2014; Yin et al., 2013). Indeed, we found spacers perfectly matching with sequences of phage EV3, a previously described *L. sanfranciscensis* phage isolated from Italian sourdoughs (Foschino et al., 2005). All phage spacers (perfectly and partly matching) are recruited from different regions within the phage genome. Strains with the same CRISPR genotype share identical phage sequences (Foschino et al., 2005; Bernheim and Rocha, 2016) (S2).

CRISPR loci in organisms sharing the same environment are more similar than those in phylogenetically related species but isolated from different environmental sources (Pearson et al., 2015). This context partly explains why strains isolated from German sourdoughs do not have EV3 phage matching sequences in their CRISPR loci. Some genotypes share the same ancestral spacers, like genotype j, k and l have at least 11 ancestral spacers in common. As well as the genotypes b to d share the first spacer (Fig. 2).

In most cases, amplicon sizes predicted *in silico* correspond well to the experimentally determined amplicon sizes. However, amplicons from DNA of *L. sanfranciscensis* DSM 20451^T and TMW 1.2140 were longer than predicted. It is likely that this is due to incorrect assembly of shotgun reads caused by the highly repetitive nature of CRISPR loci the sanger sequencing confirmed that.

The difficulties associated with sequence assembly increase as the repetitive sequences become larger (Alkan et al., 2010; Nagarajan and Pop, 2009; Treangen and Salzberg, 2011). Nevertheless, the high variability of the CRISPR locus length means that it is a potential parameter for strain differentiation. Strains with the genotype h have been isolated from doughs of one producer at different time points over 20 years, which indicates that CRISPR locus length heterogeneity is a stable trait.

In summary, strain typing using CRISPR locus length heterogeneity is a valuable tool to differentiate between strains of *L. sanfranciscensis*. But a prerequisite to use this method is the presence of the CRISPR-Cas system.

4.2. CRISPR locus as a tracking tool of strains in sourdough

The CRISPR repeat spacer array pattern of several strains were detectable within a mixture of pure DNA from several different strains, as well as in DNA isolated from a mixture of overnight cultures of these strains. Moreover, it was possible to detect all strains in a lab-scale sourdough model different flours such as rye and wheat. All four *L. sanfranciscensis* strains (TMW 1.392, TMW 1.1150, TMW 1.2138, and TMW 1.2142) were detectable at day 0 of the sourdough fermentation, and it was possible to track some strains over the experimental period. In doing so, the shift in abundance of the strains toward the most dominant strain was observed. At the beginning, *L. sanfranciscensis* strains TMW 1.392 and TMW 1.2142 seemed to co-exist. But after day 3, *L. sanfranciscensis* TMW 1.392 was clearly the most assertive of all strains. Despite nearly the same cell count of the strains at day zero (see Section 2.4.) the band intensity differed from strain to strain. This could due to many factors like different cell disruption of the strains and different binding capacity of the primers to the different strains. Also in strains were the CRISPR locus has a higher molecular mass the band intensity is lower than in strains were the molecular mass of the CRISPR locus is lower.

In comparing strain growth, TMW 1.2138 had the highest growth rate whereas TMW 1.2142 reached the highest cell density. However, TMW 1.392 was the most assertive strain in both sourdoughs (Fig. 4). This demonstrates that the behavior of strains in media (here mMRS) cannot predict their behavior or assertiveness in sourdough; assertiveness is likely related to strong adaptation to the sourdough environment.

This experiment proved the suitability of the CRISPR locus length heterogeneity to be the basis of a monitoring method of strain dynamics in complex environments. Both the identification of specific strains of *L. sanfranciscensis* and the monitoring of *L. sanfranciscensis* in the sourdough show the resolving power of CLLP-PCR in strain tracking. This provides a powerful method for characterizing strain assertiveness of *L. sanfranciscensis* in sourdoughs.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108475.

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5.2 Role of *Kazachstania humilis* and *Saccharomyces cerevisiae* in the strain-specific assertiveness of *Fructilactobacillus sanfranciscensis* strains in rye sourdough

Sourdough is a popular ingredient in bread and other baked goods. In traditional sourdough LABs like *Fructilactobacillus* (*F.) sanfranciscensis* and yeasts like *Saccharomyces* (*S.) cerevisiae* and *Kazachstania* (*K.) humilis* are competitive key species. However, their inter- and intra- species interactions in the sourdough are still unknown. In addition, the competition between strains of *F. sanfranciscensis* as well as the influence of different yeasts on this competition are gaining more research interest.

To investigate the intra-species competitiveness of *F. sanfranciscensis* in the niche sourdough the CLLP-PCR was applied. Eight strains of a strain set of 24 strains were chosen regarding their genomic differences. These strains were sorted into two different groups. The different behavior of these strains in the groups were observed in combination with the yeasts *K. humilis*, *S. cerevisiae* or without any yeast in rye sourdoughs. In addition, the behavior of *F. sanfranciscensis* TMW 1.392 and TMW 1.907 in the presence or absence of the different yeast was observed, too. The strains showed different behavior during the sourdough fermentation. With this lab-scale sourdough experiments it was possible to sort the eight strains into three different groups. Group A strains were competitive in the sourdough experiment independently of the yeast inoculation, Group B strains were only competitive with a specific yeast, and Group C strains were not competitive in the sourdough independently of yeast inoculation. In the sourdough fermentation with only one *F. sanfranciscensis* strain the development of the pH and the cfu/ml as well as the competition against the autochthonous flour microbiota was strain-specific. Interestingly, in fermentations without yeast inoculation the occurrence of *S. cerevisiae* or *K. humilis* in the sourdough was also strain-specific and according to the priorly conducted experiments.

Author contributions: Esther Rogalski was responsible for the experimental setup and the implementation of the experiment. She visualized and evaluated the data and wrote the original draft of the manuscript.

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ORIGINAL PAPER

Role of *Kazachstania humilis* and *Saccharomyces cerevisiae* in the strain-specific assertiveness of *Fructilactobacillus sanfranciscensis* strains in rye sourdough

Esther Rogalski[1] · Matthias A. Ehrmann[1] · Rudi F. Vogel[1]

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Abstract
Sourdough is a common ingredient for baked goods as it improves their texture, shelf life and flavor. One of the dominant key species in type I sourdoughs is *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*), which occurs with a multitude of different strains. Noticeably, this species often shares its habitat with the yeasts *Saccharomyces cerevisiae* or *Kazachstania humilis*. It is still unclear, which relationship exists between these organisms and whether it is characterized by coexistence, interaction, or mutualism. In this study, competitiveness of different *F. sanfranciscensis* strains in rye sourdough was examined and its dependence of co-existing yeasts was explored. In particular, it was investigated whether competitiveness of *F. sanfranciscensis* strains depends on the presence/absence of *S. cerevisiae* or *K. humilis* when co-inoculated in the sourdough. Competitiveness of strains was monitored in rye sourdough using the CRISPR locus length polymorphism (CLLP)—PCR for strain differentiation. It was found that *F. sanfranciscensis* TMW 1.1150, TMW 1.1221 and TMW 1.1597 were dominant regardless of the presence/absence of both yeast species. Dominance of *F. sanfranciscensis* TMW 1.392, TMW 1.907 and TMW 1.2137 was significantly and diversely influenced by the presence of *S. cerevisiae* or *K. humilis*. *F. sanfranciscensis* TMW 1.2138 and TMW 1.726 were not able to compete against the other *F. sanfranciscensis* strains. It was possible to sort the eight strains into three different groups: 1. Strain competitiveness was independent of the presence/absence of yeasts; 2. Strain competitiveness was dependent on yeast species and 3. Strains were not competitive in the presence of strains belonging to group 1 or 2. Interestingly, in fermentations that were not inoculated with any yeast a spontaneous occurrence of *S. cerevisiae* or *K. humilis* was observed depending on the synergistic competitiveness of the respective *F. sanfranciscensis* used. Thus, the level of competitiveness was strain specific and, in some strains, dependent on the presence/absence of specific yeast species.

Keywords *Fructilactobacillus sanfranciscensis* · *Lactobacillus sanfranciscensis* · *Saccharomyces cerevisiae* · *Kazachstania humilis* · Sourdough fermentation · CRISPR locus length polymorphism PCR

Introduction

Sourdough

Sourdough is a specific ecosystem, which is mostly inhabited by lactic acid bacteria (LAB) and yeasts [1, 2]. It is applied as an ingredient to improve the flavor, nutritional features, rheology and shelf life of many baked goods, including bread, panettone, cake or pizza [1, 3, 4]. In bread baking with rye flour, it can be considered as essential to obtain baking ability, as sourdough improves the solubility of rye pentosans due to the acidification of the dough, which enhance water binding in the dough stage as rye flour is poor in gluten [5, 6]. There are different fermentation types of sourdoughs, which are classified due to the fermentation time, the acidification rate, the temperature and the handling of the sourdough, the used starter cultures and also due to occurrence of the dominant microbiota [7–9]. Sourdough of the fermentation type 1 is a propagated sourdough with rye or wheat flour, a fermentation time of 4–16 h at 25–35 °C and a medium acidification (pH 3.5–4) with a dry dough of

✉ Rudi F. Vogel
rudi.vogel@tum.de

[1] Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Gregor-Mendel-Straße 4, 85354 Freising, Germany

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a dough yield less than 200 [7, 9, 10]. The main microbiota in sourdoughs of this fermentation type are LAB, namely *Levilactobacillus brevis* (formerly *Lactobacillus brevis*), *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) or *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis)* and also the yeasts *Saccharomyces cerevisiae*, *Kazachstania exigua* and *Kazachstania humilis* [1]. The occurring LAB are in charge of acidifying of the sourdough, flavor formation and in a modest way to the leavening of the dough. Whereas, metabolites of the yeasts are leading on to leavening of the dough, and to flavor formation. Their interactions rely on the specialized use of carbohydrates, amino acids and the production of carbon dioxide [6].

F. sanfranciscensis a key organism in type one sourdough fermentation

Fructilactobacillus sanfranciscensis (formerly *Lactobacillus sanfranciscensis*) was recently re-classified by Zheng et al. [11] regarding their preference of using fructose as external electron acceptor. The heterofermentative *F. sanfranciscensis* was included into the Approved List of Bacterial Names by Weiss and Schillinger [12] as *L. sanfrancisco* and firstly characterized by Kline and Sugihara [13]. It dominates the microbiota in sourdough with type 1 fermentation, which represents the traditional sourdough fermentation [6, 14]. *F. sanfranciscensis* has a small genome of 1.2–1.3 kb, which is the smallest of all lactobacilli species but due to its high level of ribosomal operons per mbp, a rapid growth in sourdough is possible, which could be a crucial factor against sourdough competitors [2]. *F. sanfranciscensis* is encountered in sourdoughs not only from Germany [6, 15] but also from Italy [16], Greece [17], Belgium [18], Morocco [6, 19] and China [20] with a multitude of different strains [20–23]. Moreover, some of these strains can occur in one sourdough at the same time, which might affect the stability of the sourdough microbiota [20, 23–25]. *F. sanfranciscensis* contributes to the positive effects of sourdough in several different ways (e.g., increasing shelf life, texture and flavor), although some of these effects are strain-dependent properties [22, 26]. In addition, the competitiveness of these strains against competitors or other LAB in the sourdough is strain dependent [27]. The yeast as interaction partner of the LAB and, especially, *F. sanfranciscensis* are also influencing the ecosystem sourdough.

K. humilis and *S. cerevisiae* in sourdough fermentation

In the sourdough environment, yeasts are under the pressure of high acidity, high osmotic stress and low oxygen [28]. The yeasts are mainly responsible for the leavening of the dough. Furthermore, the quality of the flavor of the bread and also the aroma of the bread crumb increases [29]. In addition, yeast can also increase the food safety of the product as they are able to detoxify mycotoxins by integrating them in the yeast cell wall [30]. Some of these yeasts occurring are generalists like *S. cerevisiae* or sourdough specific like *K. humilis*. However, one sourdough mostly harbors only one yeast at a given time [31]. *Kazachstania humilis* as a maltose-negative yeast shares often its environment with the maltose-positive *F. sanfranciscensis.* Apart from suspected metabolic cooperation, this can basically be referred to their quite similar preferences of general growth parameters (e.g., temperature and preferred pH) [31–33]. The trophic relationship of these two species relies on a nutritional mutualism. The glucose released from maltose by *F. sanfranciscensis* is consumed by *K. humilis* in glycolysis. In turn, *K. humilis* degrades glucofructans in the dough, consumes the glucose and leaves the fructose for *F. sanfranciscensis*. Subsequently, *F. sanfranciscensis* uses fructose as an electron acceptor, reducing it to mannitol. This enables a metabolic switch from ethanol to acetate formation and generation of additional ATP. The acetate selects successively for acetate tolerant yeasts. In addition, *S. cerevisiae* is often found in sourdoughs. This appearance could be a result of not only contamination from the abundant addition of Baker's yeast for the leavening of the dough, but also to its ubiquitous occurrence and the adaptation of specific strains to the environmental conditions of the sourdough ecosystem.

The intra-species interactions of *F. sanfranciscensis* as well as strain-specific competitiveness in the sourdough are still not clear. Moreover, the impact of *S. cerevisiae* and *K. humilis* on *F. sanfranciscensis* competitiveness needs clarification.

Materials and methods

Strains and culture conditions

24 strains of *F. sanfranciscensis* out of the TMW strain collection were used (Table 1). The strains are stored at − 80 °C in glycerol stocks and were collected from different sourdoughs over the world. They are cultured on modified De Man, Rogosa, and Sharpe agar (mMRS) for up to 48 h at 30 °C [23]. In liquid culture, they were cultured anaerobically. The yeasts *K. humilis* TMW 3.1034 and *S. cerevisiae* TMW 3.1064 were isolated from rye sourdough with German origin and added to the TMW strain collection. They were cultured in yeast peptone glucose (YPG) media, aerobically at 30 °C for 16 h. The YPG media contained 1% w/v casein peptone (Roth, Karlsruhe, Germany), 0.5% w/v yeast extract (Roth) and 2% w/v glucose monohydrate (Merck,

Table 1 Bacterial strains and yeasts used in this study

Organism	Strain	Group	Accession No.	Isolation source	References
F. sanfranciscensis	DSM 20451^T		MIYJ00000000	Sourdough, USA	[13]
F. sanfranciscensis	TMW 1.54 (LTH 1729)		NZ_MIYE01000000	Rye sourdough, Germany	[34]
F. sanfranciscensis	TMW 1.392 (LTH 2590)	1	NZ_MIYH01000000	Sourdough, Germany	[8]
F. sanfranciscensis	TMW 1.640		SCEZ00000000	Wheat sourdough, Switzerland	[35]
F. sanfranciscensis	TMW 1.726	2	NZ_MIYD01000000	Sourdough, Italy	[36]
F. sanfranciscensis	TMW 1.897		SCEP00000000	Sourdough, Greece	In this study (2020)
F. sanfranciscensis	TMW 1.907	2	SCEY00000000	Sourdough, Greece	[23]
F. sanfranciscensis	TMW 1.936		SCEX00000000	Sourdough, Greece	In this study (2020)
F. sanfranciscensis	TMW 1.1150	1	NZ_MIYG01000000	Sourdough, USA	[23]
F. sanfranciscensis	TMW 1.1152		SCEV00000000	Sourdough, USA	[23]
F. sanfranciscensis	TMW 1.1154		SCEU00000000	Sourdough, USA	[23]
F. sanfranciscensis	TMW 1.1221	2	SCET00000000	Sourdough, France	[23]
F. sanfranciscensis	TMW 1.1304		SCES00000000	Rye sourdough, Germany	[2]
F. sanfranciscensis	TMW 1.1470		SCER00000000	Sourdough, Russia	[23]
F. sanfranciscensis	TMW 1.1597	2	NZ_MIYF01000000	Rye sourdough, Germany	[23]
F. sanfranciscensis	TMW 1.1730		SCEQ00000000	Sourdough, Germany	[23]
F. sanfranciscensis	TMW 1.2137[1] (LS3)	1	NZ_MIXX01000000	Sourdough, Italy	[37]
F. sanfranciscensis	TMW 1.2138[1] (LS12)	1	NZ_MIXY01000000	Sourdough, Italy	[37]
F. sanfranciscensis	TMW 1.2139[1] (LS27)		NZ_MIXZ01000000	Sourdough, Italy	[37]
F. sanfranciscensis	TMW 1.2140[1] (LS19)		NZ_MIYA01000000	Sourdough; Italy	[37]
F. sanfranciscensis	TMW 1.2141[1] (LS48)		NZ_MIYB01000000	Sourdough, Italy	[37]
F. sanfranciscensis	TMW 1.2142[1] (LS13)		NZ_MIYC01000000	Sourdough, Italy	[37]
F. sanfranciscensis	TMW 1.2314		SCEW00000000	Rye sourdough, Germany	[23]
F. sanfranciscensis	TMW 1.2323		VCSH00000000	Rye sourdough, Germany	In this study (2020)
K. humilis	TMW 3.1034			Rye sourdough, Germany	In this study (2018)
S. cerevisiae	TMW 3.1064			Rye sourdough, Germany	In this study (2018)
S. cerevisiae	TMW 3.970			Rye sourdough, Germany	In this study (2018)
S. cerevisiae	TMW 3.972			Rye sourdough, Germany	In this study (2018)
S. cerevisiae	TMW 3.971			Rye sourdough, Germany	In this study (2018)

Strains kindly provided by [1]Fabio Minervini and Maria de Angelis, Universita degli Studi Bari, Italy

Darmstadt, Germany) the pH was adjusted to 6.5. For YPG agar plates 1.5% w/v AgarAgar (Roth) was added.

Sourdough preparation and strain identification

For the rye sourdough, organic whole rye flour (*Secale cereal,* dm, Karlsruhe, Germany) together with tap water was mixed to a dough yield of 200. The sourdough was fermented for 24 h each and propagated by backslopping with 5% to the flour mass for 10 days. Samples were taken for DNA isolation over the whole experiment time from the fermented sourdough and directly after the start (day 0). Furthermore, the pH value was measured before and after propagation. For the examination of the colony forming units (cfu) samples were taken at day 0, 1, 5 and 10. In the sourdoughs with inoculation of only one *F. sanfranciscensis* strain an additional sample was taken at day 3. 1 g sourdough was mixed with 9 ml Ringer's solution (Merck) and a tenfold serial dilution was performed. These samples were plated out on YPG and mMRS agar plates to detect a broad range of microorganisms in the sourdough. For species identification, 96 isolates per sample were identified from colonies with matrix-assisted laser desorption ionization (MALDI)-time of flight (ToF) mass spectrometry (MS, Bruker, Billerica, USA).

DNA isolation and DNA amplification

The DNA isolation out of the sourdough was performed with the FastDNA™ Spin Kit for Soil (MP, Santa Ana USA). The CRISPR locus length polymorphism (CLLP) polymerase chain reaction (PCR) was accomplished with DNA from dough [23]. The detailed protocol has been described by Rogalski et al. [23]. For TMW, 1.1597 species-specific primer were created (1.1597_Mtf_for: 5′-GCAAACAAG CCAAGGGAAG-3′; 1.1597_Mtf_rev: 5′-CCATCCTCG

CCCATGTTATC-3′) as the strain is lacking a CRISPR locus. The same PCR protocol was performed as for the CLLP-PCR. The DNA of the *S. cerevisiae* colonies was extracted according to Cenis [38] with modifications. For the cell disruption, 0.5-mm glass beads (Roth) were added to the washed cell pellet, 300 µl extraction buffer was added and then cell disruption was performed with the FastPrep®-24 (MP Biomedicals) for 5 m/s for 45 s. The *S. cerevisiae* was characterized with the hypervariable microsatellite-based typing technique. Therefore, the loci SCYOR267C, YPL009c and C11 were applied as they are the most variable loci for strain differentiation [39]. The PCR was performed in a final volume of 50 µl containing 100 ng of yeast DNA, 5 µl 10 × PCR Reaction Buffer w/o $MgCl_2$ (MP Biomedicals), 200 µM of dNTPs (MP Biomedicals), 0.5 µM of each primer (Eurofins Genomics, Ebersberg, Germany), 1.25 U TAQ Polymerase (MP Biomedicals), 10 mM Tris pH 9.0 (GERBU Biotechnik GmbH, Heidelberg, Germany), 50 mM KCL (Roth), 1.0% Triton X100 (Sigma-Aldrich, St. Louis, USA) and 1.5 mM $MgCl_2$ (MP Biomedicals). The PCR amplification was carried out with a Mastercycler gradient (Eppendorf, Hamburg, Germany) using a 3-step temperature program: Step 1, 1 cycle: 1 cycle: 95 °C—4 min, step 2, 34 cycles: 94 °C—30 s, 53 °C—30 s, 72 °C—1 min, step 3, 1 cycle: 72 °C—10 min [39]. The fragments of the PCR were visualized with agarose gel electrophoresis.

Strain preparation for the lab-scale sourdough models

For the lab-scale sourdough models, eight *F. sanfranciscensis* strains were split into two groups. *F. sanfranciscensis* TMW 1.1150, TMW 1.392, TMW 1.2137 and TMW 1.2138 are in group 1 and *F. sanfranciscensis* TMW 1.1221, TMW 1.1597, TMW 1.907 and TMW 1.726 are in group 2 (Table 1). In these experiments each group was inoculated together with either *S. cerevisiae* TMW 3.1064 (+ *S. cerevisiae*) or *K. humilis* TMW 3.1034 (+ *K. humilis*) or without yeast inoculation (− yeast). Afterwards the same experiments were executed with only *F. sanfranciscensis* TMW 1.907 or TMW 1.392. For sourdough preparation, overnight cultures were set to an OD_{600} of 1 and 1 ml of each strain or the yeast was used for sourdough inoculation which yields in a total cell count of log 6 to 6.5 cfu/g in the sourdough.

Bioinformatic analyses

The strains for the sourdough trails were selected due to their genomic differences. The Average Nucleotide Identity (ANI) values of the genomes of 24 *F. sanfranciscensis* strains were calculated. Furthermore, the strains were clustered with neighbor-joining and the unweighted pair group method with arithmetic averages (UPGMA) in BioNumerics ver. 7.62. [40].

Statistical analysis and evaluation

All sourdough experiments were carried out in biological triplicates. Additional pH measurements, cfu/ml determination and MALDI-ToF MS analysis were performed in technical duplicates ($n = 6$). For statistical analysis of the results, a two-sided Student's t test was applied; results $p < 0.05$ were set as significant.

Results

Characterization of the competitiveness of *F. sanfranciscensis* strains

The competitiveness of eight strains separated in two different groups was examined (Table 1). The strains were chosen due to their genomic differences calculated with the ANI values of their genomes (Fig. 1). After the inoculation of the two groups of *F. sanfranciscensis* strains together with *S. cerevisiae* (+ *S. cerevisiae*) or *K. humilis* (+ *K. humilis*) or without yeast inoculation (− yeast) in the unfermented dough (day 0), the pH value was around 5.5–6. After 24 h of fermentation, the pH value was within a range of 3.5–4 (day 1). The pH value did not change during the entire experiment time for both groups and in all three conditions (1. + *S. cerevisiae* 2. + *K. humilis* 3. − yeast). In addition, the sourdoughs without yeast inoculation were significantly more acidic than the sourdoughs with yeast inoculation independently of the *F. sanfranciscensis* group (Fig. 2).

Furthermore, the log cfu/g of the LAB on the mMRS plates increases directly after 24 h from 6 to 6.5 at day 0 to 10 log cfu/g at day 1. The MALDI-ToF MS analysis showed that during the whole experiment, the bacteria on the mMRS plates were *F. sanfranciscensis*. On the YPG agar plates, the log cfu/g increases from 5.5 to 8 log cfu/g but only in the experiments were a yeast was inoculated in the beginning. Without yeast inoculation the log cfu/g was increasing slowly and only reaches 7 log cfu/g and 6 log cfu/g in groups 1 and 2, respectively (Fig. 2) MALDI-ToF MS analysis showed that in + *S. cerevisiae* and + *K. humilis,* only at day 0, colonies of *Pantoea spp* and *Ralstonia insidiosa* are present. After the first backslopping, they vanished and either *S. cerevisiae* or *K. humilis* were present on the YPG agar plates. In contrast, the main bacteria found on the YPG agar plates in − yeast were *Ralstonia insidiosa*. In group 1, − yeast *S. cerevisiae* appears in two biological replicates after 10 days of propagation. The microsatellite typing indicates that these two *S. cerevisiae* strains have the same pattern as *S. cerevisiae* TMW

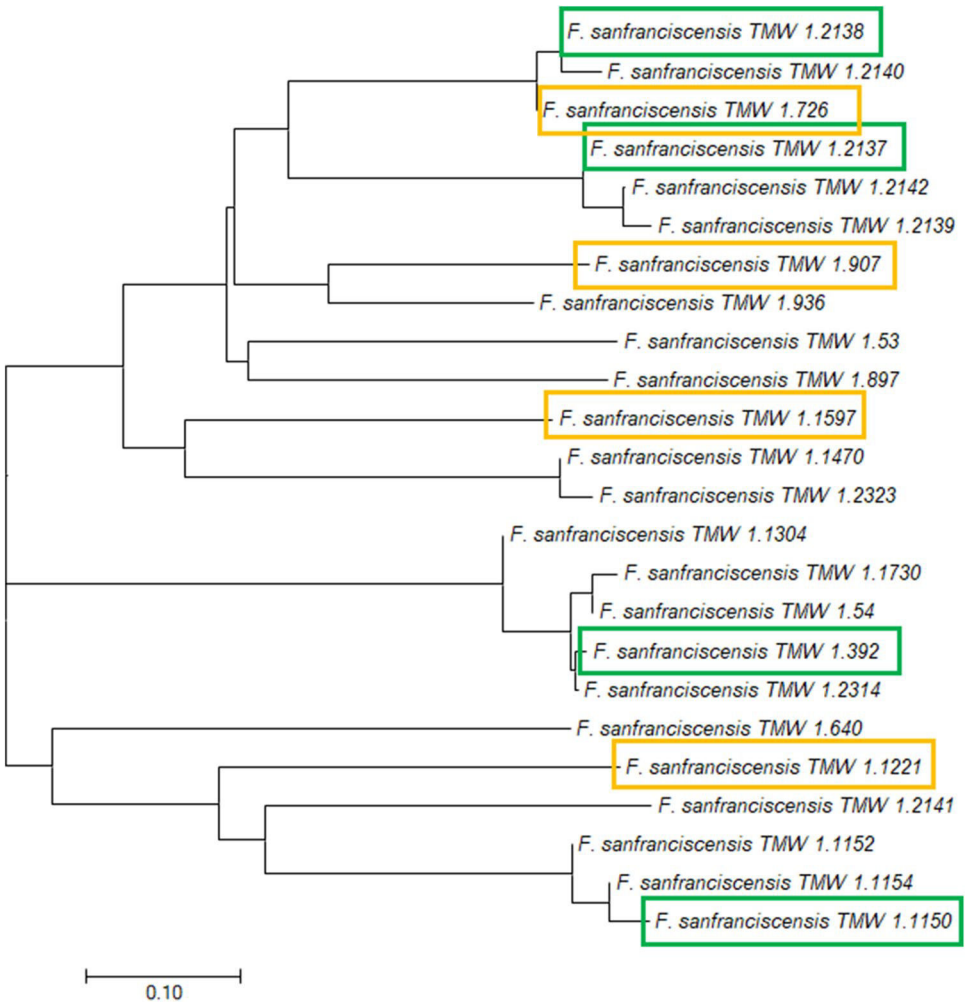

Fig. 1 UPGMA and neighbor-joining tree calculated with the ANI values of the different *F. sanfranciscensis* strains. The boxes mark the grouping of the different strains. Strains framed with a green box are in group 1 and strains framed with a yellow box are in group 2

3.1064. However, the *S. cerevisiae* strains only differ in one microsatellite locus (SCYOR267c) and not in the others tested (Fig. 3). In conclusion, it seems possible that similarities between the two exist.

The DNA samples of the sourdoughs were applied to perform the CLLP-PCR. With this PCR technique, the length polymorphism of the CRISPR locus is used for strain differentiation. In group 1, *F. sanfranciscensis* TMW 1.1150 was able to compete against the other three strains in all different conditions (+*S. cerevisiae*, +*K. humilis*, − yeast). In – yeast, *F. sanfranciscensis* TMW 1.392 was also dominant during the whole experiment time. The band intensity of TMW 1.392 seems to decrease when the band intensity of TMW 1.1150 increases. In the − yeast condition, this effect seems to be slowed down compared to the other two conditions (+*S. cerevisiae* and +*K. humilis*). Whenever the CLLP-PCR is not a quantitative PCR, the figures are representative of an analysis performed in triplicates (data not shown). Nevertheless, TMW 1.392 was able to persist in the sourdoughs at a detectable limit up to 6 days of backslopping. The strain TMW 1.2137 was only dominant in +*S. cerevisiae*. Furthermore, TMW 1.2138 was not found in the sourdoughs anymore after 1 day of propagation.

In contrast, TMW 1.392 and TMW 1.2137 were susceptible to the presence of yeasts in the sourdough in this strain combination. Regarding group 2, two strains are competitive in all three conditions; these are *F. sanfranciscensis* TMW 1.1221 and TMW 1.1597. The strain TMW 1.907 is only dominant in +*S. cerevisiae* but in − yeast, it is only visible at day 0 and directly eliminated after 24 h. The strain TMW 1.726 is able to compete only for at least 3 days in +*S. cerevisiae* and 2 days in − yeast. To conclude the strain, TMW 1.907 was susceptible to the presence of different yeast conditions in group 2 (Fig. 4).

Influence of *S. cerevisiae* and *K. humilis* on *F. sanfranciscensis* strains

To examine the dependence of *F. sanfranciscensis* strains on the presence/absence of yeasts, two susceptible strains of the former experiment were chosen. *F. sanfranciscensis* TMW 1.392 and TMW 1.907 were separately inoculated with *S.*

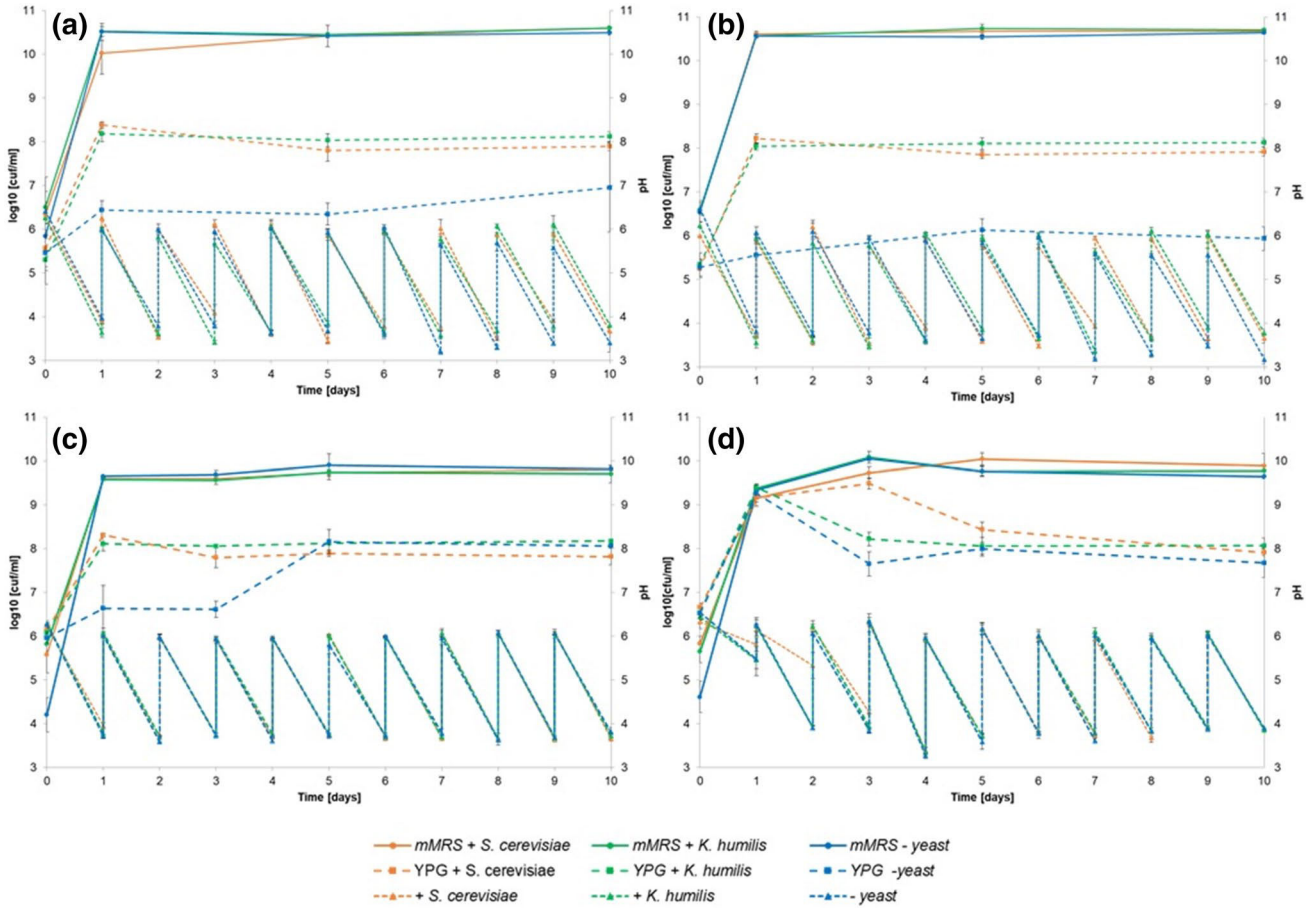

Fig. 2 Rye sourdough experiment of group 1 (**a**) and 2 (**b**) and the experiments only with *F. sanfranciscensis* TMW 1.392 (**c**) and TMW 1.907 (**d**) as LAB starter. Backslopping was performed every day for 10 days (1–10). Zero is the sample of the not fermented flour water mixture before fermentation which was inoculated with the F. *sanfranciscensis* groups or single strains and the yeast species. The log cfu/g of the colonies of the mMRS and YPG agar plates was measured at days 0, 1, 3, 5 and 10. The pH was measured before and after propagation. In orange the sourdough with *S. cerevisiae* inoculation (+*S. cerevisiae*), in green the sourdough with *K. humilis* inoculation (+*K. humilis*) and in blue the sourdough without yeast inoculation (− yeast) is shown

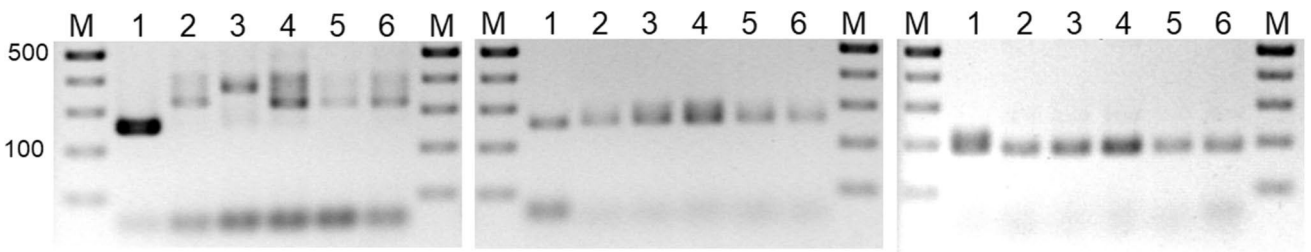

Fig. 3 Microsatellite loci typing of *S. cerevisiae*. With the microsatellite loci SCYOR267c (**a**), YPL009C (**b**) and C11 (**c**). Following *S. cerevisiae* strains were applied on the gel: TMW 3.970, TMW 3.972; TMW 3.971; TMW 3.1064; *S. cerevisiae* R1; *S. cerevisiae* R2 (lines 1–6). *S. cerevisiae* R1 and R2 were isolated out of the rye sourdough of group one without yeast inoculation. M: GeneRuler™ 100 bp plus DNA Ladder (Thermo scientific)

cerevisiae (+*S. cerevisiae)*, *K. humilis* (+*K. humilis)* or without yeast (− yeast) in a rye sourdough. In the dough with *F. sanfranciscensis* TMW 1.392 (+TMW 1.392), the acidification of the sourdough and the reaching of the max. log cfu/g in the sourdough proceeded as quickly as for the former experiments with four *F. sanfranciscensis* strains. The log cfu/g on the YPG agar plates was from day 1 to day 10 two log phases under the log cfu/g of the *F. sanfranciscensis*

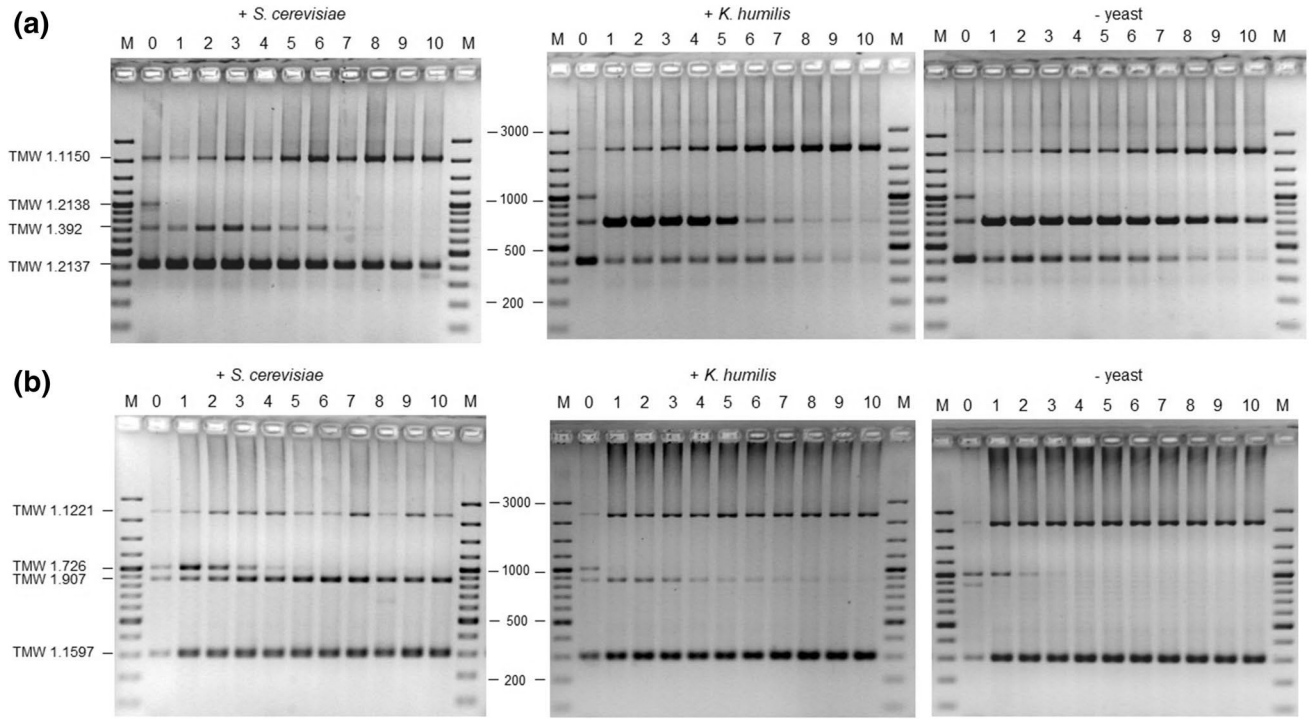

Fig. 4 The agarose gel electrophoresis of the CLLP-PCR of group 1 (**a**) and group 2 (**b**). The sourdoughs were also inoculated with *S. cerevisiae* (+*S. cerevisiae*) with *K. humilis* (+*K. humilis*) and without yeast inoculation (−yeast). The sourdoughs were propagated every 24 h for 10 days (line 0–10). Group 1 was inoculated with the *F. sanfranciscensis* strains TMW 1.1150 (2091 bp), TMW 1.2138 (1003 bp), TMW 1.392 (706 bp) and TMW 1.2137 (409 bp). Group 2 was inoculated with the *F. sanfranciscensis* strains TMW 1.1221 (2223 bp), TMW 1.726 (1003 bp), TMW 1.907 (872 bp) and TMW 1.1597 (302 bp). M: GeneRulerTM 100 bp plus DNA Ladder (Thermo scientific)

on the mMRS agar plates. In − yeast, the log cfu/g took up to 5 days to reach in two steps (from day 3 to day 5) the max. log cfu/g of approx. 8 (Fig. 2). During this experiment time, *K. humilis* appears in the sourdough without yeast inoculation (Fig. 5). In the sourdough with *F. sanfranciscensis* TMW 1.907 (+1.907), the acidification of the sourdough to 3.5–4 took up to 4 days (+*S. cerevisiae*) but minimum 3 days (+*K. humilis* and − yeast). Furthermore, the sourdough with the strain TMW 1.907 required up to 3 days to reach the max. log cfu/g of 10 (Fig. 2c, d). Whereas the microbiota, which was able to grow on the YPG agar plates, increases in all three yeast conditions (+*S. cerevisiae*, +*K. humilis*, − yeast) up to 9 log cfu/g after the first 24 h of fermentation. The log cfu/g in +*K. humilis* and in − yeast decreases after 3 days to 8 log cfu/ml. In contrast, in +*S. cerevisiae,* it takes till day 5 until the log cfu/g decreases to 8 log cfu/g (Fig. 2), which coincides with the slowly acidification of the sourdough in + TMW 1.907.

With MALDI-ToF MS, it was possible to monitor the occurrence of different species during the sourdough fermentation. On the mMRS agar plates, it was only possible for *F. sanfranciscensis* and other sourdough related LAB to grow. The YPG agar plates are more suitable for a wide range of bacteria and yeasts. In the sourdoughs + TMW 1.907, it was possible to find a broad range of bacteria on the agar plates. With the acidification of the sourdough, *F. sanfranciscensis* TMW 1.907 was able to persist against the natural microbiota of plant and grain endophytes (here referred to as "competitors") and finally was the dominant bacterial species in the sourdough together with the yeasts (*S. cerevisiae* or *K. humilis*). In all three doughs (+*S. cerevisiae*, +*K. humilis* and − yeast), TMW 1.907 was able to outcompete the competitors up to day 10. In contrast, TMW 1.392 was only able to outcompete the competitors in the sourdoughs with *K. humilis* and rising the presence of *K. humilis*. In the sourdough with *S. cerevisiae* were still competitors till the end of the experiment (Fig. 5). The competitors in all sourdoughs were only able to grow on YPG plates. From day 0 on, it was possible to find bacteria of the family Burkholderiaceae (*Ralstonia insidiosa*), Erwiniaceae (*Pantoea agglomerans, P. poae* and *P.* spp.). These bacteria species were outcompeted after the first days, due to the decrease of the pH. Subsequently, bacteria from the family of Enterobacteriaceae (*Escherichia vulneris*, *Citrobacter* sp., *Enterobacter cloacae* and *Cronobacter sakazakii*), Enterococaceae (*Enterococcus* sp.) and LAB like Leuconostaceae (*Leuconostoc citreum*) and Lactobacillaceae (*L. brevis*) were found. In + TMW 1.907, the differences in the log cfu/g

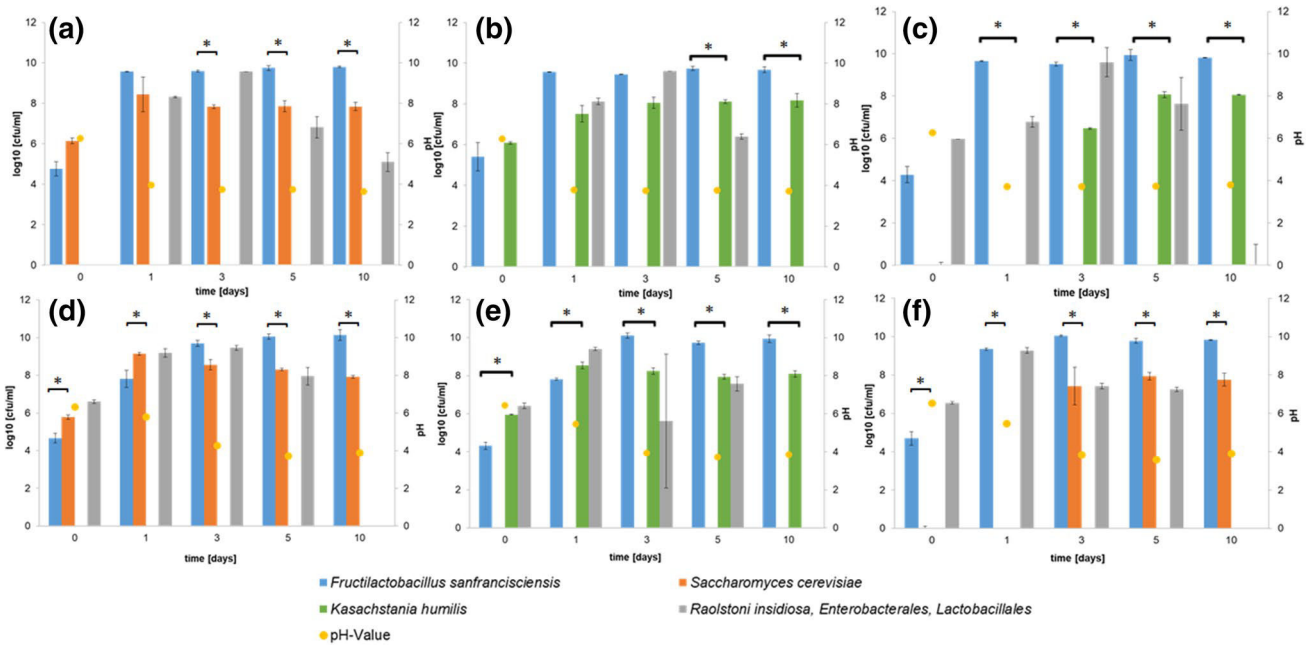

Fig. 5 The development of the log cfu/g and the pH (yellow) of *F. sanfranciscensis* TMW 1.392 (**a**–**c**) and TMW 1.907 (**d**–**f**) during the sourdough fermentation of 10 days. The colonies were determined with the MALDI-ToF MS and the occurrence of *F. sanfranciscensis* (blue), *S. cerevisiae* (orange), *K. humilis* (green) and other species [*Ralstonia insidiosa*, *Enterobacterales*, *Lactobacillales*] (gray) were pictured. The results are the product of three independent experiments. The statistical analysis was made with a student's *t* test and the asterisks mark significant differences between the log cfu/g of the microbiota

between the yeasts and *F. sanfranciscensis* were significant from day 0 on. Besides, in the sourdoughs with TMW 1.392, the significant differences appear only after a minimum of 3 days of propagation.

Discussion

Competitiveness of *F. sanfranciscensis* strains during sourdough development

The lab-scale rye sourdoughs were stable at the pH value and in the cell count after 5 days (Fig. 2). However, on strain level competitions up to the end of the experiment still existed (Fig. 4 + *K. humilis*, − yeasts). Strong strains were mostly dominant from the first backslopping on (TMW 1.1597 and TMW 1.1221); whereas, some weak strains were able to compete a couple of backslopping events until they vanished under the detection limit. Based on their competitiveness, it was possible to sort the eight strains into three different groups: A. Strain competitiveness was independent of the presence/absence of yeasts (TMW 1.1150, TMW 1.1221 and TMW 1.1597) B. Strain competitiveness was dependent on yeast species (TMW 1.392, TMW 1.907 and TMW 1.2137) and C. Strains were not competitive in the presence of strains belonging to group 1 or 2 (TMW 1.726 and TMW 1.2138). The origin of the strain had no impact on the competitiveness; the dominant strains were from USA (TMW 1.1150), France (TMW 1.1221) and Germany (TMW 1.1597, Table 1). TMW 1.2137 and TMW 1.2138 have also been applied as starters in a wheat sourdough trial. As in our study, they were also not able to persist in the wheat sourdough against the other microbiota [27]. The other strain from Italy (TMW 1.726) was also not able to dominate in the rye sourdough. *F. sanfranciscensis* strains TMW 1.907 and TMW 1.2137 were able to persist in their group in the rye sourdoughs when *S. cerevisiae* was inoculated. Whereas, TMW 1.392 was only able to persist in the sourdough when no yeast was inoculated.

Impact of *K. humilis* and *S. cerevisiae* on *F. sanfranciscensis* strains in rye sourdough

Furthermore, the impact of *K. humilis* and *S. cerevisiae* on the persistence of *F. sanfranciscensis* strains in rye sourdough was investigated. Therefore, two yeast-sensitive strains (TMW 1.392 and TMW 1.907) were inoculated in the sourdoughs without other *F. sanfranciscensis* competitors. *F. sanfranciscensis* TMW 1.392 was faster than TMW 1.907 in acidifying the sourdough and reaching the max log cfu/g (Fig. 2). However, TMW 1.907 was able to compete better against competitors (Fig. 5). One reason could be that *F. sanfranciscensis* TMW 1.392 is able to use more carbohydrate sources for growth than TMW 1.907, namely

sucrose (see below). Still, in the sourdoughs used in this work, sucrose is at a negligible level, and the alteration of the sucrose level decreases the dominance of TMW 1.392 against *L. plantarum* [41]. Lactobacilli, especially *F. sanfranciscensis,* are able to produce phenyl lactic acid (PLA). PLA is an antimicrobial agent which inhibits the growth of fungi and yeasts in the sourdough and is able to increase the shelf life of the bread [42]. Nevertheless, the concentration produced by *F. sanfranciscensis* is rather low to be the only reason for eliminating the competitors [42]. But *F. sanfranciscensis* is able to produce a lot more substance to inhibit the growth of competitors [43]. Whereas the metabolism of *F. sanfranciscensis* differs from stain to strain, it could be that TMW 1.907 is able to produce more anti-mould agents and is, hence, capable of eliminating the competitors better [22]. Still, TMW 1.392 only with *S. cerevisiae* was not able to outcompete the competitors. Metabolic changes in TMW 1.392 due to growth in the sourdough with *S. cerevisiae* could be the cause. However, differences in the general sugar utilization of the two different yeasts are unlikely to be the reason for this occurrence, as they show aside from maltose nearly the same fermentation profile [44].

Regarding the yeasts, it was possible to measure differences in the sourdough fermentation between *S. cerevisiae* and *K. humilis* when only one *F. sanfranciscensis* strain was inoculated. *S. cerevisiae* grows fast in the first 24 h to 9–10 log cfu/g. However, after a few backslopping events the log cfu/g of *S. cerevisiae* decreases 2 log units to approx. 8 log cfu/g. Whereas the *F. sanfranciscensis* strains increase their log cfu/g to 10. This is the normal LAB yeast ratio in the sourdough of 1:100 [31, 45]. It is possible that *F. sanfranciscensis* drives the cell count of the yeast in the sourdough. After *F. sanfranciscensis* was adapted to its environment the cell count of *S. cerevisiae* decreased. This observation fits to the results of Carbonetto et al. [46]. In their study, the cell count of *S. cerevisiae* and some *K. humilis* strains was compared in monoculture or in co-culture with *F. sanfranciscensis*. Together with *F. sanfranciscensis,* the cell count of the yeast species was decreased. It was also strain specific which yeast arises in the sourdough without yeast inoculation (− yeast). In the sourdough with *F. sanfranciscensis* TMW 1.392, *K. humilis* arises after 3 days; whereas in the sourdough with *F. sanfranciscensis* TMW 1.907, *S. cerevisiae* arises after 3 days. In conclusion, it is suggested that the yeast preference of *F. sanfranciscensis* is strain specific. *F. sanfranciscensis* TMW 1.392 is capable of metabolizing fructose, maltose, glucose and sucrose [47]. Furthermore, it is likely that it is able to utilize fructose as electron acceptor to regenerate NAD + with the NADH oxidase. This recycling makes it possible to produce more acetic acid instead of ethanol. *K. humilis* produces fructose as byproduct in cleaving glucofructans for using the glucose as carbohydrate source [48]. That would explain why TMW 1.392 prefers to coexist with *K. humilis*. This sourdough should have more acetic acid, which leads to a higher dough extensibility [49] and acts as a selective pressure on yeasts. In addition, *K. humilis* is more tolerant to acetic acid concentration compared to *S. cerevisiae* [46]. Nevertheless, also the carbohydrate metabolism can give a hint on the metabolic interactions of *S. cerevisiae*, *K. humilis* and *F. sanfranciscensis* strains. *F. sanfranciscensis* as autochthones sourdough organism utilize maltose as preferred carbohydrate source [22, 50]. The β-amylase of the rye or wheat flour releases maltose, which gets cleaved by *F. sanfranciscensis* in glucose-1-phosphate and glucose [51]. The glucose-1-phosphate is then utilized for the production of lactate, acetate, CO_2 and ethanol. The free glucose is then consumed by the maltose-negative *K. humilis* [6, 34]. The metabolism of *F. sanfranciscensis* is more challenged when living with the maltose-positive yeast *S. cerevisiae*. The cleavage of maltose is then increased, with the maltose-phosphorylase, and glucose and glucose-1-phosphate produced in a ratio of 1:1. Thus, the amount of glucose increases in the surrounding and this could lead to glucose repression in competitors like in the maltose-positive yeast [6, 34]. But still the question is, do we have here an interaction between *F. sanfranciscensis* and *K. humilis* or is it only a coincidence because of complementary requirements. Furthermore, the interaction of *F. sanfranciscensis* with *S. cerevisiae* can more be neutral/negative because of the metabolic pressures, which *F. sanfranciscensis* exerts on the yeast [46].

Conclusion

Similar to dairy and sausage fermentation, the quest for a sourdough starter attracts increasing attention [27]. To achieve this goal, first, a competitive *F. sanfranciscensis* strain or strain combination needs to be selected. In our study, the applied *F. sanfranciscensis* strains differed in their competitiveness against other strains and against competitors. Furthermore, it was shown that the preferred yeast as metabolic partner was strain specific and that the competitiveness of strains could depend on the presence or absence of yeasts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics requirements This article does not contain any studies with human or animal subjects.

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5.3 Intraspecies diversity and genome-phenotype-associations in *Fructilactobacillus sanfranciscensis*

Fructilactobacillus (F.) sanfranciscensis is an autochthones bacterium only isolated from sourdough. Still, metagenomic studies of insects and insect's frass revealed the presence of *F. sanfranciscensis*. Physiologic behavior of *F. sanfranciscensis* varies due to the occurrence of different strains, 24 *F. sanfranciscensis* strains were analyzed from different isolation sources. The genomic prediction was compared with physiological experiments, and the phenotype was examined. The core genome of these 24 strains comprises 43.14% of the pan genome with 0.87 Mbp out of 2.04 Mbp. It was possible to sort these strains into six different genetic clusters, since they differed in their response to carbon-sources and utilization of them as well as to the nucleotide metabolism, utilization of electron acceptors and EPS formation. Albeit all strains are well equipped in the exploitation of oxygen via NADH 2 oxidase, and in the thiol metabolism. The major differences of the strains were found in the sugar metabolism. As maltose is the main carbon source in sourdoughs all strains contain a maltose phosphorylase (*map*). However, only ten of 24 strains contain two maltose phosphorylases differentiated as *mapA* and *mapB* and *F. sanfranciscensis* TMW 1.897 contains only *mapA*. The strains of cluster 2 lack the mannitol dehydrogenase (*mdh*) gene applied for the regeneration of NAD out of fructose. Six strains were able to use fructose as electron acceptor and also to use fructose as carbon source, because of their functional fructokinase gene. Six strains with a functional levansucrase (*levS*) were able to grow with sucrose and seven were able to produce EPS out of sucrose. All strains have in common that they are not able to grow on pentoses like xylose, arabinose, or ribose. For the growth with ribose the ribose pyranase *rbsD* is needed for the formation of D-pyranoribose to D-furanoribose, which is missing in all strains. In conclusion the biodiversity of *F. sanfranciscensis* is greater than anticipated despite its small genome of 1.26 to 1.36 Mbp. They are well adapted to sugary and oxic environments and on the way to further adaption to the niche sourdough. Notwithstanding, some strains retained important properties to compete in intra-species competitions in the sourdough.

Author contributions: Esther Rogalski performed and planned the experiments as well as the data analysis and the genome analyses. She visualized and evaluated the data and wrote the original draft of the manuscript.

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Intraspecies diversity and genome-phenotype-associations in *Fructilactobacillus sanfranciscensis*

Esther Rogalski, Matthias A. Ehrmann, Rudi F. Vogel *

Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany

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ABSTRACT

In this study the intraspecies diversity of *Fructilactobacillus* (*F.*) *sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*) was characterized by comparative genomics supported by physiological data. Twenty-four strains of *F. sanfranciscensis* were analyzed and sorted into six different genomic clusters. The core genome comprised only 43,14 % of the pan genome, i.e. 0.87 Mbp of 2.04 Mbp. The main annotated genomic differences reside in maltose, fructose and sucrose as well as nucleotide metabolism, use of electron acceptors, and exopolysacchride formation. Furthermore, all strains are well equipped to cope with oxidative stress via NADH oxidase and a distinct thiol metabolism. Only ten of 24 genomes contain two maltose phosphorylase genes (*mapA* and *mapB*). In *F. sanfranciscensis* TMW 1.897 only *mapA* was found. All strains except those from genomic cluster 2 contained the mannitol dehydrogenase and should therefore be able to use fructose as external electron acceptor. Moreover, six strains were able to grow on fructose as sole carbon source, as they contained a functional fructokinase gene. No growth was observed on pentoses, i.e. xylose, arabinose or ribose, as sole carbon source. This can be referred to the absence of ribose pyranase *rbsD* in all genomes, and absence of or mutations in numerous other genes, which are essential for arabinose and xylose metabolism. Seven strains were able to produce exopolysaccharides (EPS) from sucrose. In addition, the strains containing *levS* were able to grow on sucrose as sole carbon source. Strains of one cluster exhibit auxotrophies for purine nucleotides. The physiological and genomic analyses suggest that the biodiversity of *F. sanfranciscensis* is larger than anticipated. Consequently, "original" habitats and lifestyles of *F. sanfranciscensis* may vary but can generally be referred to an adaptation to sugary (maltose/sucrose/fructose-rich) and aerobic environments as found in plants and insects. It can dominate sourdoughs as a result of reductive evolution and cooperation with fructose-delivering, acetate-tolerant yeasts.

1. Introduction

Fructilactobacillus (*F.*) *sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*) is a key species in traditional back-slopped type 1 sourdough fermentations (De Vuyst and Neysens, 2005; Van Kerrebroeck et al., 2017; Yang et al., 2017). Furthermore, it is the most frequently isolated species from rye and wheat sourdoughs. It dominates over heterofermentative and also homofermentative lactic acid bacteria (LAB) and is the major driver of the fermentation (Gobbetti and Corsetti, 1997; De Angelis et al., 2007). It was firstly isolated by Kline and Sugihara (1971) and validly published by Weiss and Schillinger (1984) as *Lactobacillus sanfrancisco*. Former studies, which reclassified the genus *Lactobacillus*, renamed it to *F.sanfranciscensis* for its preference to grow in presence of fructose as electron acceptor (Zheng et al., 2020). *F. sanfranciscensis* is autochthonous for the niche sourdough and has so far been solely isolated from this environment (De Angelis et al., 2007; Boiocchi et al., 2017). Still, metagenetic sequencing approaches delivered indications for its presence also in insect larvae (Boiocchi et al., 2017). Numerous studies were dedicated to the characterization of *F. sanfranciscensis* and its physiological properties due to its predominant role in the sourdough fermentation (Table A1)(Yazar and Tavman, 2012). Furthermore, *F. sanfranciscensis* is a heterofermentative lactic acid bacterium, which shares the sourdough environment with yeasts like *K. humilis* or *S. cerevisiae* (Gänzle et al., 1998; De Vuyst et al., 2017; Rogalski et al., 2020b).

Maltose, and to a lesser amount glucose, are continuously produced by flour amylases in sourdough, and consequently maltose is the most abundant sugar in this environment. *F. sanfranciscensis* effectively uses maltose as major carbohydrate source (Gobbetti et al., 1995; Corsetti and Settanni, 2007) and its metabolism is not repressed by glucose as

* Corresponding author at: Lehrstuhl für Technische Mikrobiologie, TU München, Gregor-Mendel-Straße 4, 85354, Freising, Germany.
E-mail address: rudi.vogel@tum.de (R.F. Vogel).

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reported for other lactobacilli. Whenever maltose is imported by a H^+-symport-driven maltose permease instead of a maltose PTS (Neubauer et al., 1994), its utilization via maltose phosphorylases is effective (Ehrmann and Vogel, 1998; Vogel et al., 2002). The utilization of fructose by *F. sanfranciscensis* is variable and can be metabolized enabling growth, or employing it as electron acceptor enhancing energy gain in the acetate kinase reaction, which accelerates growth (Ehrmann and Vogel, 2005; Gänzle et al., 2007). When fructose is used by *F. sanfranciscensis* as external electron acceptor it is reduced by mannitol dehydrogenase into mannitol enabling recycling of NAD (Stolz et al., 1995; Korakli and Vogel, 2003). Consequently, acetate is formed, instead of ethanol, together with one ATP. The combined use of maltose and fructose is a key function in the adaption of *F. sanfranciscensis* to the sourdough environment and its cooperation with *Kazachstania humilis*, which releases fructose from glucofructans in the sourdough (Vogel et al., 1999; De Vuyst and Neysens, 2005). Furthermore, sucrose metabolism has also been investigated in *F. sanfranciscensis* TMW 1.392 (Tieking et al., 2005b)(Table A1). In this strain the levansucrase LevS cleaves sucrose into fructose and glucose and uses the cleavage energy to form levan out of fructose (Tieking et al., 2005b).

Additionally, Buron-Moles et al. (2019) reported a positive API 50 CHL reaction in combination with D-xylose and L-arabinose in *F. sanfranciscensis* DSM 20451^T, and also a genetic pathway for ribose metabolism has been predicted for TMW 1.1304 (Vogel et al., 2011). Furthermore, Vogel et al. (2011) reported, that *F. sanfranciscensis* TMW 1.1304 lacks most of the enzymes required for purine metabolism.

Apart from the competition for sugars as energy source, life in sourdough offers numerous stresses, the response to which have been examined in several studies. Hörmann et al. (2006) provided an overview employing a proteomic study. Further studies include oxidative stress (Jänsch et al., 2011), acid stress (Serrazanetti et al., 2011) and thiol stress (induced by diamide) (Stetina et al., 2014)(Table A1). The latter report provided insight into the redox-regulation, which employs thiol metabolism and transport as well as glutathione-related functions, which underlines the adaptation to cereal environments in cooperation with yeasts.

The influence of different *F. sanfranciscensis* strains on baked goods is of interest for the baking industry, which develops and employs starter preparations harboring stable microbial consortia (Siragusa et al., 2009). Still, previous investigations were only carried out with a few specific strains of *F. sanfranciscensis*, while many different strains were isolated from different sources (Foschino et al., 2001; Kitahara et al., 2005; Picozzi et al., 2010; Lee et al., 2015; Lhomme et al., 2015, 2016; Yang et al., 2017). These strains differ in their physiology (Rogalski et al., 2020b), stress response and metabolism (Lhomme et al., 2015), just as their impact on persistence, cooperation and putative mutualism on sourdough microbiota (Siragusa et al., 2009), as well as the structural and sensorial quality of baked goods (Gänzle and Ripari, 2016). These differences should be reflected in their genomes.

In this study, we therefore aimed to differentiate intraspecies genomic differences of *F. sanfranciscensis* and cross-checked genomic predictions against selected physiological key functions of 24 representative *F. sanfranciscensis* strains isolated from different sourdoughs. This approach should enable a deeper insight into the lifestyle and niche adaptation and of *F. sanfranciscensis*.

2. Material and methods

2.1. Strains and culture conditions

Twenty-four *F. sanfranciscensis* strains out of the TMW strain collection were chosen according to previous studies as representatives of a wide strain selection (Table 1)(Rogalski et al., 2020a). The strains were collected from different sourdoughs and stored at −80 °C in 70 % glycerol. Furthermore, the strains were cultured in modified De Man, Rogosa and Sharpe medium with maltose, glucose and fructose as carbon sources (mMRS) for 24 h at 30 °C (Rogalski et al., 2020b). In liquid culture the strains were grown anaerobically in a static condition. For agar plates 15 g AgarAgar liter^{-1} (Roth, Karlsruhe, Germany) was added.

2.2. Growth experiments in the presence of sugars and external electron acceptors

To determine the carbohydrates, which can be utilized as sole carbon source by *F. sanfranciscensis,* growth experiments were performed. To this end, mMRS without any mono- and disaccharides was employed, and 2 % of either maltose (GEBRU Biotechnik GmbH, Heidelberg, Germany), glucose (GEBRU Biotechnik), fructose (Roth), sucrose (GEBRU Biotechnik), ribose (Roth), arabinose (Roth) or xylose (Sigma-Aldrich, St. Louis, USA) was added as sterile filtered solution to the autoclaved medium. The strains were pre-grown in mMRS as overnight cultures, washed in ¼ Ringer's solution (Merck, Darmstadt, Germany) and the OD_{600} of cell suspensions was adjusted to 0.05 in the selected media. The growth experiments were performed in 96 well plates (Sarstedt, Nümbrecht, Germany) and were monitored using a SPECTROstar^{nano} plate reader (BMG, Labtech, Ortenberg, Germany) for 48 h at 30 °C under oxic conditions. Moreover, the strains were grown in mMRS media as positive control. The impact on growth of malate and Na-gluconate as co-substrates for the carbohydrate metabolism, and citrate, fructose and oxygen as external electron acceptors, was determined in chemical defined media (CDM), which contained 2 % maltose under anoxic conditions. For anoxic growth the wells were covered with 100 µl of paraffin oil (Roth). The utilization of malate and Na-gluconate was additionally investigated under oxic conditions, because their conversion consumes NAD, which needs to be recycled via Nox2. The CDM was prepared in 1 l of autoclaved purified water (Morishita et al., 1981; Petry et al., 2000; Eckel and Vogel, 2020). The buffer solution for the CDM was prepared with 5.0 g of sodium acetate (Roth), 3.0 g of KH_2PO_4 (Merck), 3.0 g of K_2HPO_4 (Merck), 0.5 g of $MgSO_4 \cdot 7H_2O$ (Roth), 0.05 g of $MnSO_4 \cdot H_2O$ (Roth), 0.05 g of $FeSO_4 \cdot 7H_2O$ (Roth), 0.2 g of $CaCl_2$ (Roth) and 1 g of polysorbate 80 (GEBRU Biotechnik) and solved in purified water. The pH was adjusted to 6.5 and 50 ml of 2 M HCl (Roth) was added. For the vitamin solution a 50-fold stock was prepared in 80 ml of purified water with 5 mg of p-aminobenzoic acid (Sigma-Aldrich), 5 mg of folic acid (Roth), 20 mg of nicotinic acid (Sigma-Aldrich), 20 mg of Ca-pantothenate (Sigma-Aldrich), 10 mg of biotin (Roth), 20 mg of pyridoxal (Sigma-Aldrich), 20 mg of riboflavin (Sigma-Aldrich), 10 mg of vitamin B_{12} (AppliChem GmbH, Darmstadt, Germany) and 40 mg thiamine (Sigma-Aldrich). Furthermore, 8 ml of the vitamin mix were added to the buffer solution. In addition, all essential amino acid were solved in purified water with 0.4 g of cysteine (Roth), 0.3 g of aspartic acid (Sigma-Aldrich), 0.3 g of glutamic acid (Sigma-Aldrich), 0.2 g alanine (Sigma-Aldrich), 0.2 g arginine (Sigma-Aldrich), 0.2 g glycine (Gerbu Biotechnik), 0.2 g histidine (Roth), 0.2 g isoleucine (Merck), 0.2 g leucine (Merck), 0.2 g lysine (Roth), 0.2 g methionine (Roth), 0.2 g phenylalanine (Roth), 0.2 g proline (Merck), 0.2 g serine (Merck), 0.2 g threonine (Sigma-Aldrich), 0.2 g tryptophan (Sigma-Aldrich), 0.2 g tyrosine (Roth), 0.2 g valine (Sigma-Aldrich) and added to the buffer solution. Moreover, 20 g maltose (Merck) was added as carbon source. Two nucleotide solutions were mixed in 2 M NaOH (Roth), one with 40 mg of xanthine (Sigma-Aldrich) and 0.5 g of orotic acid (Sigma-Aldrich) and the second with 20 mg of adenine (SERVA Electrophoresis GmbH, Heidelberg, Germany) and 50 mg guanine (Sigma-Aldrich). The first was added to the buffer solution and the second was only omitted in experiments investigating the purine biosynthesis. In the end the pH of the CDM was adjusted to 5.4 and sterile filtered with 0.2 µm (Sarstedt, Darmstadt, Germany). In addition, 20 % stock solutions were prepared for citrate (Roth), fructose (Roth), malate (Sigma-Aldrich) or Na-gluconate (Roth) and sterile filtered. Prior to their use they were diluted to 2 % in CDM. To examine the functionality of the purine biosynthesis in *F. sanfranciscensis* CDM without purines (-P) was used

Table 1
Genetic properties of *F. sanfranciscensis* strains used in this study.

Organism	Strain	Accession No.	Isolation source	References	Genome size (bp)	GC content (%)	Total ORFs	CDS coding	Coding density	coding density/ genome size	Transposases	Pseudogenes	Phage proteins	Phage condition
F. sanfranciscensis	DSM 20541	MIYJ00000000	Sourdough, USA	Kline and Sugihara (1971)	1332599	33.80	1377	1221	88.67	0.62	34	83	11	incomplete
F. sanfranciscensis	TMW 1.54	NZ_MIYE01000000	Rye sourdough, Germany	Stolz et al. (1995)	1346022	33.66	1366	1225	89.68	0.62	29	76	8	incomplete
F. sanfranciscensis	TMW 1.392	NZ_MIYH01000000	Sourdough, Germany	Böcker et al. (1995)	1298193	33.53	1335	1185	88.76	0.64	21	78	12	incomplete
F. sanfranciscensis	TMW 1.640	SCEZ00000000	Wheat sourdough, Switzerland	Ehrmann and Vogel (2001)	1318743	34.28	1369	1243	90.80	0.65	29	66	9 10	incomplete incomplete
F. sanfranciscensis	TMW 1.726	NZ_MIYD01000000	Sourdough, Italy	Liske et al. (2000)	1288983	33.70	1322	1184	89.56	0.66	24	62	8	incomplete
F. sanfranciscensis	TMW 1.897	SCEP00000000	Sourdough, Greece	Rogalski et al. (2020a)	1270537	34.04	1303	1209	92.79	0.69	17	63	8	incomplete
F. sanfranciscensis	TMW 1.907	SCEY00000000	Sourdough, Greece	Rogalski et al. (2020b)	1302883	34.12	1349	1257	93.18	0.68	24	61	54	intact
F. sanfranciscensis	TMW 1.936	SCEX00000000	Sourdough, Greece	Rogalski et al. (2020a)	1263236	34.17	1290	1190	92.25	0.69	19	60	11	incomplete
F. sanfranciscensis	TMW 1.1150	NZ_MIYG01000000	Sourdough, USA	Rogalski et al. (2020b)	1318441	33.73	1340	1195	89.18	0.64	18	59	10	incomplete
F. sanfranciscensis	TMW 1.1152	SCEV00000000	Sourdough, USA	Rogalski et al. (2020b)	1273976	34.01	1299	1186	91.30	0.68	16	62	11	incomplete
F. sanfranciscensis	TMW 1.1154	SCEU00000000	Sourdough, USA	Rogalski et (al., 2020b)	1263243	33.95	1287	1191	92.54	0.70	17	59	10	incomplete
F. sanfranciscensis	TMW 1.1221	SCET00000000	Sourdough, France	Rogalski et al. (2020b)	1277804	33.92	1296	1203	92.82	0.69	18	53	11	incomplete
F. sanfranciscensis	TMW 1.1304	SCES00000000	Rye sourdough, Germany	Vogel et al. (2011)	1316865	34.21	1422	1280	90.01	0.64	35	77	13	incomplete
F. sanfranciscensis	TMW 1.1470	SCER00000000	Sourdough, Russia	Rogalski et al. (2020b)	1285197	33.99	1342	1211	90.24	0.66	30	65	10 22 32	incomplete questionable incomplete
F. sanfranciscensis	TMW 1.1597	NZ_MIYF01000000	Rye sourdough, Germany	Rogalski et al. (2020b)	1355924	33.85	1371	1232	89.86	0.63	17	64	10 30	incomplete intact
F. sanfranciscensis	TMW 1.1730	SCEQ00000000	Sourdough, Germany	Rogalski et al. (2020a)	1329696	34.03	1419	1279	90.13	0.64	32	76	12	incomplete
F. sanfranciscensis	TMW 1.2137	NZ_MIXX01000000	Sourdough, Italy	De Angelis et al. (2007)	1309145	33.76	1337	1210	90.50	0.66	25	53	8	incomplete
F. sanfranciscensis	TMW 1.2138	NZ_MIXY01000000	Sourdough, Italy	De Angelis et al. (2007)	1288546	33.71	1324	1190	89.88	0.66	24	61	8	incomplete
F. sanfranciscensis	TMW 1.2139	NZ_MIXZ01000000	Sourdough, Italy	De Angelis et al. (2007)	1367240	33.81	1392	1256	90.23	0.63	31	60	8	incomplete
F. sanfranciscensis	TMW 1.2140	NZ_MIYA01000000	Sourdough; Italy	De Angelis et al. (2007)	1330132	33.74	1360	1201	88.31	0.61	27	90		
F. sanfranciscensis	TMW 1.2141	NZ_MIYB01000000	Sourdough, Italy	De Angelis et al. (2007)	1347032	33.74	1377	1241	90.12	0.64	23	62	11 43 7	incomplete intact incomplete
F. sanfranciscensis	TMW 1.2142	NZ_MIYC01000000	Sourdough, Italy	De Angelis et al. (2007)	1341963	33.07	1342	1212	90.31	0.64	23	54	8	incomplete
F. sanfranciscensis	TMW 1.2314	SCEW00000000	Rye sourdough, Germany	Rogalski et al. (2020b)	1275060	33.90	1359	1230	90.51	0.67	24	64	13	incomplete
F. sanfranciscensis	TMW 1.2323	VCSH00000000	Rye sourdough, Germany	Rogalski et al. (2020a)	1283356	33.99	1342	1210	90.16	0.66	32	66	10 12	incomplete incomplete

and the growth experiments were carried out under oxic conditions. Moreover, the fermentation patterns of the strains were analyzed with the standard system API 50 CHL (BioMérieux, Marcy l'Etoile, France). With this test the fermentation of 50 carbohydrates was examined. The test was performed according to manual instructions (API systems, BioMérieux).

2.3. The production of exopolysaccharides

The production of exopolysaccharides (EPS) in *F. sanfranciscensis* was examined on agar plates with maltose mMRS (without glucose and fructose) with additional 50 g sucrose per liter for 24 h at 30 °C. In addition, the presence of a levansucrase gene (*levS*) was investigated with PCR. The primers for the PCR are Lev_1_f (5′-ATGACTAAAGAACATAAGAAAAATG-3′) and Lev_2_r (5′-CAAGAAACGTCGTAATGATTAA-3′). The PCR was performed within a 50 µL reaction volume containing 5 µL 10 × PCR-Mix (MP Biomedicals, Santa Ana, USA), 200 µM dNTPs (MP Biomedicals), 0.25 µM of each primer (Eurofins Genomics, Ebersberg, Germany), 1.5 U TAQ Polymerase (MP Biomedicals) and 10–50 ng of pure DNA isolated with the E.Z.N.A. ® DNA Kit from OMEGA Bio-tek (Norcross, USA). Besides, the PCR amplification was carried out with a Mastercycler gradient (Eppendorf, Hamburg, Germany). A standard PCR protocol for 16S RNA with the following settings was realized: 2 min. at 94 °C, then 32 cycles with 45 s at 94 °C, 1:30 min. at 52 °C, followed by 2 min. at 72 °C and in the end one time 5 min. at 72 °C.

2.4. Genome analyses

The genomes of the *F. sanfranciscensis* strains were sequenced and annotated as previously described by Rogalski et al. (2020b). In addition, the sequences were annotated also with the Rapid Annotation Subsystem Technology (RAST) server to obtain the Enzyme Commission (EC) numbers of the proteins and the localization in the subsystem of the genes and proteins (Aziz et al., 2008). The TIGR Annotation Engine was further applied to obtain the Gene Ontology (GO) terms and BLAST hits. The Average Nucleotide Identity (ANI) of the whole genome shotgun sequences (WGS) of the *F. sanfranciscensis* strains was calculated utilizing the ANIb algorithm with the JSpecies software tool vers. 1.2.1 (Richter and Rosselló-Móra, 2009; Hilgarth et al., 2018). The output was processed with the Molecular Evolutionary Genetics Analysis (MEGA) 7.2 tool to create a phylogenetic tree of the *F. sanfranciscensis* genomes. Besides, the BlAst Diagnostic Gene findEr (BADGE) was applied with default settings for the identification of the diagnostic marker genes (DMGs) of the different metabolic pathways of *F. sanfranciscensis* (Behr et al., 2016) and also for the prediction of the core-, pan- and accessory genome (Eisenbach et al., 2018). The predicted proteins of the analyzed metabolic pathways were controlled with smart Blast as well as with pBlast and nBlast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The occurrence of the predicted genes in all strains was verified with the CLC main workbench 8.0 (https://digitalinsights.qiagen.com/). Furthermore, the comparison of enzymes was carried out with Clustal Omega (Larkin et al., 2007) and visualized with Jalview 2.11 (Waterhouse et al., 2009). For predicted membrane proteins the TMHMM Server v. 2.0 was applied to analyze the membrane binding capacity (http://www.cbs.dtu.dk/services/TMHMM/). For the visualization of the BADGE results and the genomic comparison of the *F. sanfranciscensis* strains the Blast Ring Image Generator (BRIG) was applied with the pan-genome of the strains as reference (Alikhan et al., 2011). The occurrence of prophages as well as the production of bacteriocins was analyzed with web-based tools like PHASTER (Arndt et al., 2016, 2017) and BAGEL (de Jong et al., 2006). The results of the growth curves with different mono- and disaccharides as sole carbon sources were analyzed with the R (Vers.: i3863.3.1 × 64) package grofit (Kahm et al., 2010). For the visualization of the phylogenetic trees and tree diagrams Bionumerics V7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium) was applied and the unweighted pair group method with arithmetic mean (UPGMA) and the neighbor joining algorithm, or the similarity coefficient with categorical differences was used for the cluster analysis.

2.5. Statistical analysis

All experiments were performed in technical and biological triplicates (n = 3). A two-sided students *t*-test was performed and results with a p value < 0.05 was set as significant. Furthermore, the standard deviation and standard error was calculated for the results of the R analysis.

3. Results

3.1. Genomic relationship of the 24 F. sanfranciscensis strains

Based on their phylogenetic diversity calculated with the ANI values it was possible to sort the 24 *F. sanfranciscensis* strains into six different genetic clusters (Fig. 1 a). The cut off for a reasonable genetic cluster was set to 99.65 % identity. Still, five strains could not be grouped into any of these genetic clusters. Furthermore, with the BRIG analysis it was possible to examine the occurrence of similar open reading frames (ORFs) or the lack of ORFs. The core genome of the *F. sanfranciscensis* strains in this study comprises 43,14 % of the pan genome, i.e. 0.87 Mbp of 2.04 Mbp (Table 1). Strains of clusters 1, 2, 5 and 6 lack some group-specific genes and share some other exclusive genes (Fig. 1 b, 1–4). The calculation of the ANI values resulted in a similarity between 99.01 %–100 %. They all have a relatively small genome within a range of 1.26 Mbp to 1.36 Mbp, which results in a high coding density. The genomes of strains TMW 1.907 and TMW 1.2140 display the highest (93.81 %) and lowest (88.31 %) coding densities (Table 1). The ratio between coding density and genome size is similar between the strains. The otherwise reported effect that a smaller genome causes a higher coding density is therefore negligible within this species. The GC content ranges from 33.53 % to 34.28 % (Table 1). When the ORFs of the strains were sorted into SEED categories the distribution of the genes in the categories Amino Acids and Derivatives as well in Carbohydrates and DNA Metabolism is very similar (Figure A1). It should be noted that only genes with a proper annotation were sorted into these categories. Furthermore, it is noteworthy that TMW 1.1597 and TMW 1.2141 have also genes in the category Phages, Prophages, Transposable elements and Plasmids. This observation is in agreement with the analysis of PHASTER as these two strains and TMW 1.907 carry predicted intact prophages in their genomes (Table 1). Whereas the prophages' genomes of TMW 1.907 and TMW 1.2141 have a higher gene content, the genome of the TMW 1.1597 prophage is more condensed and the phage genes are located closely together. The number of the phage genes in the other strain was rather low and is listed in (Table 1).

All strains share all genes for the phosphoketolase pathway from maltose and all genes for the metabolism of cell wall compounds (Table A2-A3). Furthermore, strain TMW 1.2141 contains the genes required for the dTDP-rhamnose synthesis but lack the flippase gene, which should render them unable to produce heteropolysaccharides (Table A3). Oxidative stress is predictively alleviated via glutathione peroxidase (TMW11150_BGL46_00365 (24/24)), two cysteine transporters TcyB (TMW11150_BGL46_02635 (24/24), TMW11150_BGL46_01025 (24/24)) and thioredoxin reductase (TMW11150_BGL46_00775 (24/24)(Table A2-A3)). All strains lack the enzymes for the arginine deaminase (ADI) system or the glutamine/glutamate system with the glutamate decarboxylase and the formation of γ-aminobutyric acid (GABA). They also have in common numerous regulators and proteases against metabolic stress as well as DNA repair and mismatch repair proteins (Table A4). Moreover, it was not possible to detect any bacteriocin-producing operons with BAGEL. Focusing on strain specific differences revealed that genomic cluster 5 lacks all enzymes for the purine biosynthesis (Table A2-A3). Differences in the carbohydrate metabolism and the recycling of NAD were analyzed in

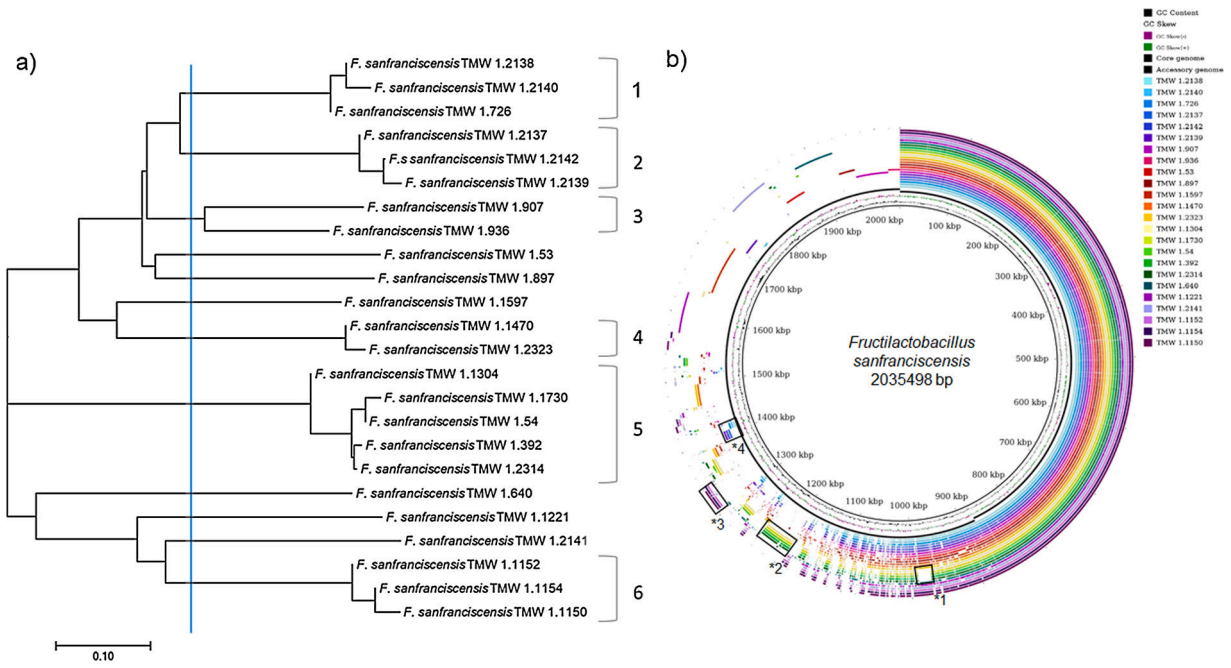

Fig. 1. Genetic similarities of the 24 *F. sanfranciscensis* strains. a: Neighbor joining tree calculated with the ANI values of the genome data of the strains, the strains were sorted into genetic clusters according to their genetic distance to each other (1–6). The blue line marks the grouping of the strains threshold for different groups is 99.65 % ANI similarity. b: BRIG analysis of the BADGE output of the *F. sanfranciscensis* strains. The squares are marking group specific ORFs missing/present (1–4). The different ring colors represent different *F. sanfranciscensis* strains.

detail.

3.2. Genomic prediction of carbohydrate utilization and NAD recycling

The clustering of the present genes encoding enzymes of the carbohydrate metabolism shows high similarities to the clustering of the ANI value tree. The genetic clusters are reflected in the tree diagram of the carbohydrate metabolism (Fig. 2). Only TMW 1.2140, which clustered together with group 1 in the ANI tree of Fig. 1, did not cluster into group 1. The predicted metabolic pathways of carbohydrate metabolism in *F. sanfranciscensis* are summarized in Fig. 5.

For the utilization and uptake of maltose most of the *F. sanfranciscensis* strains comprise two different systems (Fig. 5). All but TMW 1.897 carry the MFS maltose transporter gene *mpeB*

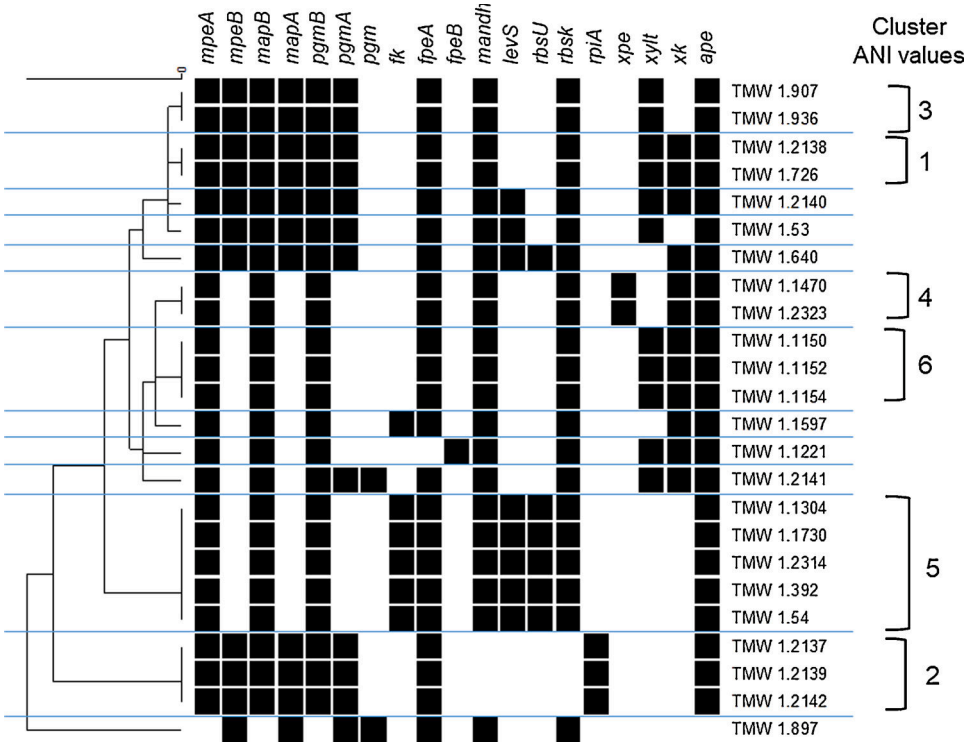

Fig. 2. Tree diagram clustered with UPGMA and the Similarity coefficient with Categorical differences. Clustering of the predicted functional genes involved in the metabolism of maltose, glucose, fructose, sucrose, ribose, arabinose and xylose. Non-functional or mutated genes are not listed. *mpe*, maltose permease; *map*, maltose phosphorylase; *pmg*, phosphoglucomutase; *fk*, fructokinase; *fpe*, fructose permease; *mandh*, mannitol dehydrogenase, *levS*, levansucrase; *rbsU*, ribose uptake protein; *rbsK*, ribokinase; *rpiA*, ribose-5-phosphate isomerase A; *xpe*, xylose permease; *xylt*, xylose uptake protein; *xk*, xylose kinase; ape, arabinose permease. Black squares represent present genes, white squares represent absence genes. The brackets with numbers describe the genetic clusters generated with the ANI values. For the visualization of the groups blue lines were inserted. There occurrence coincides with genes important for the carbohydrate metabolism except for TMW 1.2140 which is not sorted in cluster number 1.

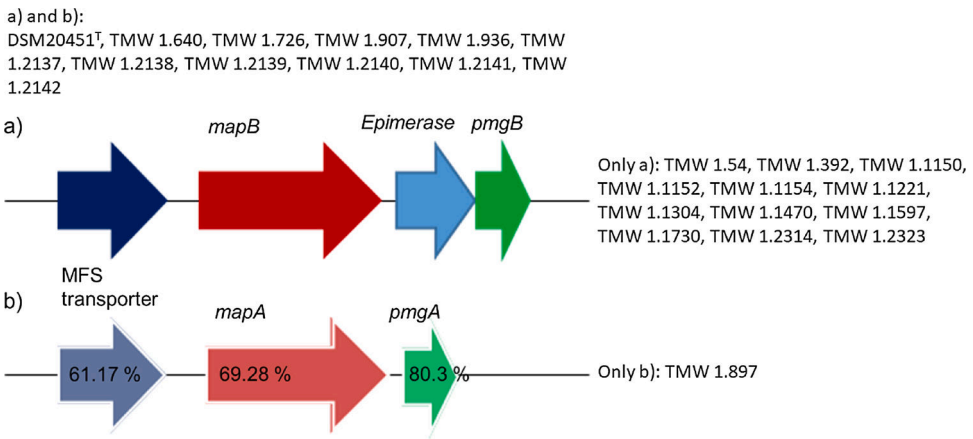

Fig. 3. Operon structure of genes involved in maltose metabolism. The different localization of the genes involved in the maltose metabolism in the operon of *mapA* and *mapB* are represented. Furthermore, similarity of the amino acid sequence of *map*, *pmg* and the MFS transporter of b) to a) is shown in percentage in b). Occurrence of the operons in the *F. sanfranciscensis* strains were included. *map*, maltose phosphorylase, *pmg*, phosphoglucomutase.

(TMW11150_BGL46_06310) and the maltose phosphorylase gene *mapB* (TMW11150_BGL46_06305) as well as the β-phosphoglucomutase β-*pmg*B (TMW11150_BGL46_06295). TMW 1.897 has only *mpe*A (TMW12137_BGL37_03210), *map*A (TMW12137_BGL37_03215) and β-*pmg*A (TMW12137_BGL37_03220) whereas strains TMW 1.2138, TMW 1.2137 and TMW 1.907 comprise both operons in their genome (Fig. 2, Table A2-A3). The maltose operons of *mapA* and *mapB* are not located in direct proximity. The operon of *mapB* contains an additional epimerase annotated as galactose mutarotase (TMW11150_BGL46_06300) between *mapB* and the phosphoglucomutase (*β-pmg*B) (TMW11150_BGL46_06295, Fig. 3). Furthermore, the MFS transporter, the *map* and the β-*pmg* of the two operons are different from each other with respect to their amino acid sequence (Fig. 3).

The ability to grow with sucrose as sole carbon source correlates with the occurrence of the levansucrase gene *levS* in the genome of *F. sanfranciscensis* (Fig. 5). By using genomic analysis, it was possible to find eight strains with *levS*. The different sizes of *levS* can be explained by different numbers of repeated sequences at the beginning and the end of the gene (Fig. 4). To exclude misarrangements of the repetitive sequences the genes were corroborated via PCR analysis. The middle part of LevS is conserved among 7 of the 8 strains. The amino acid sequence of TMW 1.640 contains several exchanges in the sequences, which can lead to protein dysfunctionalities. Furthermore, LevS is predicted as anchored to the cell wall by an LPQTG motiv. The Q at the variable X position of the LPXTG motive is conserved in the *levS* sequences of the *F. sanfranciscensis* strains (Fig. 4). Moreover, five of 24 strains contain genes coding for dextransucrases, but the amino acid sequence differs among the strains (Table A2-A3). However, in these strains the fructokinase is non-functional, predicting their inability to grow on fructose resulting from the dextransucrase reaction.

Fructose is frequently employed as external electron acceptor by *F. sanfranciscensis* to regenerate NAD via mannitol dehydrogenase (Fig. 5). However, some *F. sanfranciscensis* strains are predicted to utilize fructose as sole carbon source (Table 2). The reason for this ability is their functional fructokinase gene (Fig. 2). All strains contain at least parts of the fructokinase gene. In 18 strains including e.g. TMW 1.1150 (TMW11150_BGL46_01400) the enzyme lacks 58 amino acids in the middle of the enzyme predicting their inability to grow on fructose (Figure A2). Moreover, all genomes contain genes for isomerization of glucose-6-P to fructose-6-P and its subsequent conversion to acetyl-P and erythrose-P (Fig. 8).

Moreover, the uptake and catabolism of pentoses was investigated. Regarding ribose, the genetic analysis of the strains shows seven different ORFs encoding the ribose uptake protein *rbsU*. Nevertheless, five possible ORFs contain stop codons over the entire sequence. Two predicted functional RbsU transporters are listed in Fig. 2. At least 21 strains had the ribokinase (*rbsk*), which catalyzes the reaction from D-furanoribose to ribose-5-phosphate. Furthermore, there are two ORFs for the ribose-5-phosphate isomerase A, which drives the reaction from ribose-5-phosphate to ribulose-5-phosphate (Fig. 5). The first predicted functional RbsU was found in the genomes of all strains (TMW11150_BGL46_05195), while the second one was only found in genomes of cluster number 2 (TMW12137_BGL37_02905) (Fig. 2). Interestingly, all strains lack the ribose pyranase (*rbsD*). As this enzyme is essential for the conversion of D-pyranoribose to D-furanoribose, which is then used by *rbsk* (Fig. 5), strains remain predicted as unable to grow on ribose.

The occurrence of two different xylose transporters is listed in Fig. 2 (Table A2-A3). Nevertheless, xylose isomerase is missing, which is required for the processing of xylose to xylulose. The reaction from xylulose to xylulose-5-phosphate is catalyzed by the xylulokinase, which is found in the genomes of 12 strains (TMW11150_BGL46_05960). For the pentose arabinose the situation is similar. There are five different ORFs for arabinose permease genes, but only one ORF is predicted as functional (Fig. 2). The other two enzymes, which are important for the catabolism of arabinose, arabinose isomerase and the ribulokinase, are missing in all *F. sanfranciscensis* genomes (Fig. 5).

Additional molecules can be utilized to support the carbohydrate metabolism or for the recycling of electron acceptors. Na-gluconate and malate can be used to support the energy metabolism (Fig. 5) as long as NAD can be recycled, e.g via Nox2. With respect to utilization of Na-gluconate, only half of the strains contain a gluconate permease gene (TMW11470_EQU36_04615) but all of them carry a gluconokinase gene (TMW11150_BGL46_03360). There are three different ORFs coding for the malate permease. One of these is interrupted by stop codons in the sequence and is therefore predicted as non-functional. Yet, both the second one and the third ORF, which are found in 17 strains (TMW11150_BGL46_02590) and three strains (TMW12140_BGL40_06555), respectively, appear to be functional (Table A2-A3). Moreover, malate could be either oxidized to oxaloacetate by the malate dehydrogenase (TMW11150_BGL46_05870) and subsequently further oxidized to pyruvate (requiring NAD recycling), or converted to fumarate with the fumarate hydratase (TMW11150_BGL46_03575) (Fig. 5).

Fructose, citrate and oxygen can be utilized by *F. sanfranciscensis* as external electron acceptors as they enable recycling of NAD. All strains have the NADH oxidase 2, which catalyzes the reaction of O_2 to H_2O, and which is also able to eliminate reactive oxygen species. Twenty-one of 24 strains contain all genes important for the utilization of citrate (Table A2-A3), whereas all strains except those of cluster 2 carry the mannitol dehydrogenase (Fig. 2).

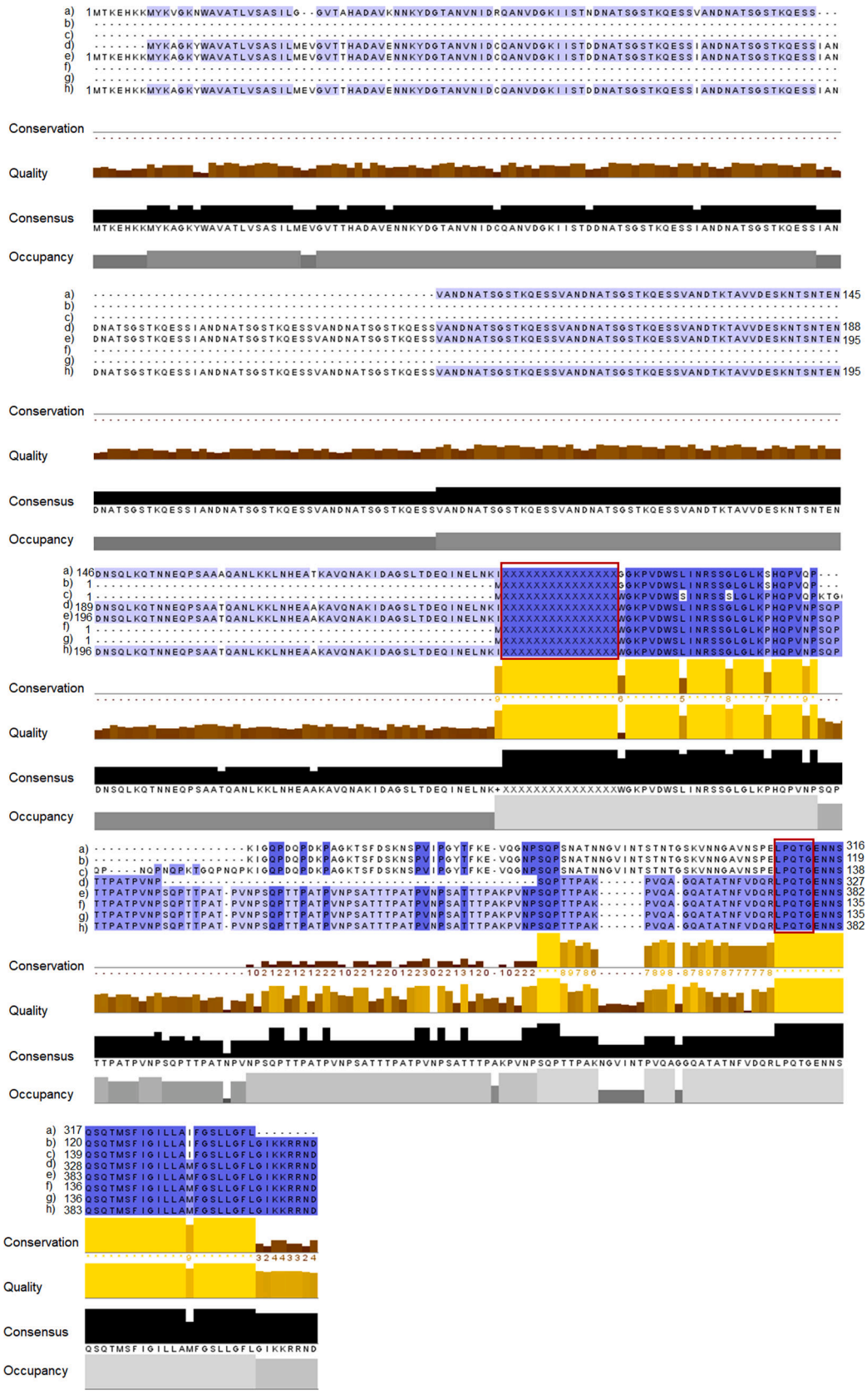

Fig. 4. Alignment of amino acid sequence of the levansucrase with Clustal Omega and Jalview. The amino acid sequence of *F. sanfranciscensis* TMW 1.2140, DSM 20451^{T}, TMW 1.640, TMW 1.1304, TMW 1.2314, TMW 1.392, TMW 1.54 and TMW 1.1730 was aligned (a–h). The red squares mark the middle part of the enzyme and the LPXTG anchor of the protein. The middle part is replaced by a placeholder as this part of the enzyme is similar in each strain. Furthermore, the amino acid at the X position of the anchor protein is glutamine (Q) in every strain.

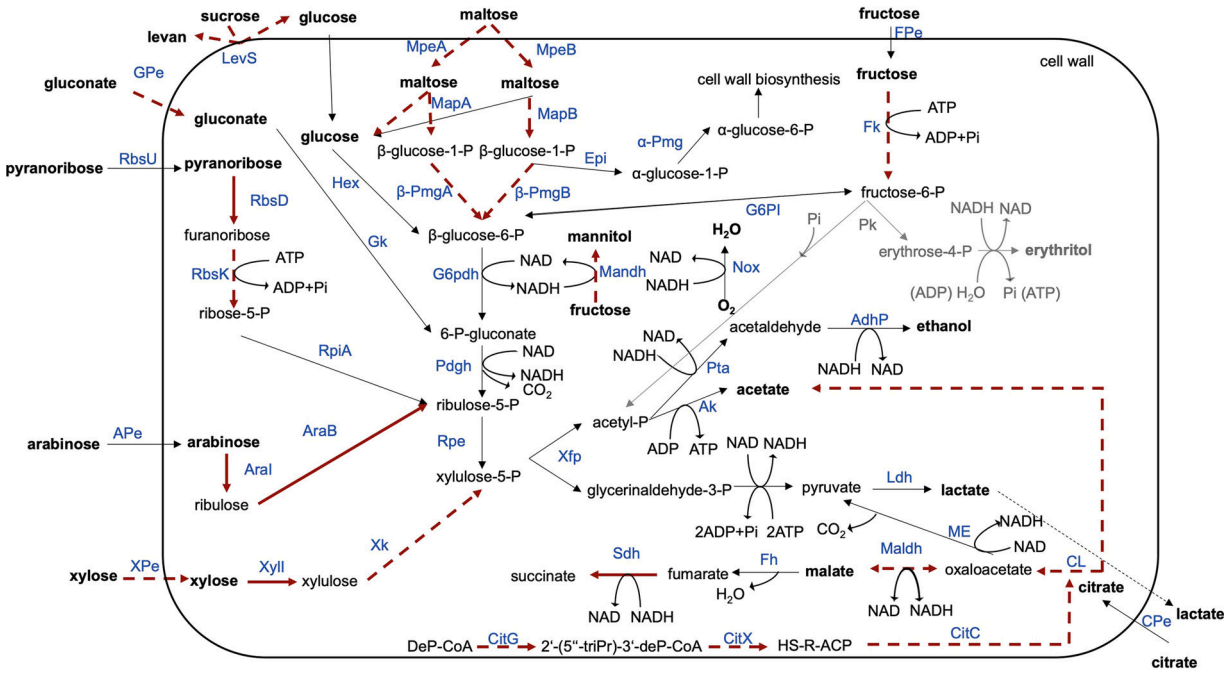

Fig. 5. Carbohydrate metabolism of *F. sanfranciscensis*. Red arrows are genes missing in all *F. sanfranciscensis* strains, red dashed arrows are genes missing in at least one *F. sanfranciscensis* strain. Grey parts refer to putative erythritol metabolism. The enzymes are written in blue. Black dashed and drawn through arrows are genes present in all *F. sanfranciscensis* strains. Bold compounds are putative substrates or end products of the carbohydrate metabolism. Fpe, fructose permease; MpeA and MpeB, maltose permease; MapA and MapB, maltose phosphorylase; LevS, levansucrase; GPe, gluconate permease; RbsU, ribose uptake protein; Ape, arabinose permease; XPe, Xylose permease; Pmg, phosphoglucomutase; Epi, epimerase; G6pdh, glucose-6-phosphate dehydrogenase; Pdgh, phosphate dehydrogenase; Rpe, ribulose-5-phosphate epimerase; Hex, hexokinase; Gk, gluconokinase; RbsD, ribose pyranase; RbsK, ribokinase; RpiA, ribose-5-phosphate isomerase A; AraI, arabinose isomerase; AraB, ribulokinase; XylI, xylose isomerase; Xk, xylose kinase; Mandh, mannit dehydrogenase, Nox, NADH oxidase; G5PI, glucose-6-phosphate isomerase; Fk, fructokinase; Pk, phosphoketolase; Xfp, xylulose-5-phosphate; Ak, acetate kinase; Pta, phosphotransacetylase; AdhP, alcohol dehydrogenase; Ldh, lactate dehydrogenase; CPe, citrate permease; CL, citrate lyase; ME, malic enzyme; Maldh, malate dehydrogenase; Fh, fumarate dehydrogenase; Sdh, succinate dehydrogenase; CitC, citrate ligase; CitX, holo-ACP synthase; CitG, ATP dephospho-CoA 5′-triphosphoribosyl transferase.

3.3. Physiological behavior of *F. sanfranciscensis*

The carbohydrates fermented by at least one of the *F. sanfranciscensis* strains in the API 50 CHL test are shown in Fig. 6. According to the API 50 CHL test all strains were able to use glucose and maltose as sole carbon sources. None of the strains showed a positive reaction with the pentoses ribose, xylose and arabinose after 48 h (Fig. 6).

Furthermore, the utilization of di- and monosaccharides as sole carbon source of *F. sanfranciscensis* was detected along growth experiments. Different strains displayed different growth behavior. The growth behavior characterized by μmax and lag phase of the *F. sanfranciscensis* strains was calculated and evaluated with grofit (Table 2).

It was not possible to detect any growth with ribose neither in the growth experiment as sole carbon source nor there was a positive signal in the API 50 CHL test (Fig. 6). The same was observed with arabinose or xylose as sole carbon source.

All strains from cluster number 5 and TMW 1.1597 (TMW11304_EQU35_01450) were able to grow with fructose as sole carbon source because of their functional fructokinase (Fig. 2, Figure A2, Table A2-A3).

All strains but DSM 20451^T and TMW 1.640, which carry *levS,* were able to grow with sucrose as sole carbon source (Fig. 2), by using the released glucose. Furthermore, these strains were also able to produce levan in mMRS with maltose and sucrose (Fig. 4, Fig. 7). TMW 1.1154 was able to produce EPS with its dextransucrase (Table A2).

The growth improvement through NAD recycling with external electron acceptors was investigated. All strains contain the NADH oxidase 2 in their genome. Furthermore, during growth experiments with CDM an increase of the growth rate was detected under oxic conditions (Fig. 8b). The genes for the citrate metabolism are found in 21 of the strains. Moreover, no increase of the growth rate was detected, when strains were growing on maltose with citrate as electron acceptor, and there were no growth differences in strains with and without the genes for citrate metabolism. The same observation was made with the addition of fructose as electron acceptor to maltose containing CDM.

Nevertheless, the addition of Na-gluconate to CDM under oxic conditions did increase the growth rate of *F. sanfranciscensis* TMW 1.1597, which contains the genes responsible for gluconate metabolism (Fig. 8c). It was not possible to observe this behavior in TMW 1.1150 as it lacks the gluconate permease (Table A2). The addition of malate did not influence the growth behavior of *F. sanfranciscensis* independently of the presence of the malate permease or the malate dehydrogenase. This did not change under oxic conditions or when citrate was added to the media.

Furthermore, the genomic analysis showed that the strains of cluster number 5 did not contain any genes for the purine biosynthesis (Table A2-A3). So, the growth of TMW 1.1304 (Cluster 5) and TMW 1.2323 (Cluster 4) was observed in CDM with and without purines. Interestingly, TMW 1.1304 was able to grow without purines but the addition of them positively influenced its growth behavior (Fig. 8a), whereas the growth of TMW 1.2323 was not influenced by the addition of purines.

4. Discussion

With a core genome comprising only 43.14 % of the pan genome, the biodiversity of *F. sanfranciscensis* is greater than anticipated. The physiological and comparative genomic analyses suggest that it can dominate sourdoughs as a result of domestication-driven reductive evolution from a life in sucrose/fructose-rich, oxic, plant and insect environments

Table 2
Growth behavior of *F. sanfranciscensis* strains.

Carbohydrate	mMRS*		Maltose		Glucose		Fructose		Sucrose	
Strain	μmax [OD/h]	lag phase [h]	μmax [OD/h]	lag phase [h]	μmax [OD/h]	lag phase [h]	μmax [OD/h]	lag phase [h]	μmax [OD/h]	lag phase [h]
TMW 1.53	0.16 ± 0.02	3.68 ± 1.55	0.10 ± 0.03	4.02 ± 1.84	0.03 ± 0.00	8.31 ± 7.12	0	0	0	0
TMW 1.54	0.30 ± 0.04	4.40 ± 0.24	0.46 ± 0.02	6.33 ± 0.36	0.39 ± 0.02	7.38 ± 0.27	0.10 ± 0.06	8.36 ± 4.83	0.36 ± 0.07	18.17 ± 5.82
TMW 1.392	0.34 ± 0.02	5.94 ± 1.08	0.33 ± 0.02	6.12 ± 1.17	0.33 ± 0.03	7.04 ± 1.48	0.21 ± 0.04	13.93 ± 1.40	0.29 ± 0.02	8.35 ± 2.34
TMW 1.640	0.27 ± 0.03	3.80 ± 0.10	0.31 ± 0.02	7.02 ± 1.29	0.18 ± 0.11	3.63 ± 2.10	0	0	0	0
TMW 1.726	0.48 ± 0.05	4.97 ± 0.38	0.43 ± 0.04	5.00 ± 0.44	0.25 ± 0.02	5.51 ± 0.57	0	0	0	0
TMW 1.897	0.22 ± 0.02	6.02 ± 1.35	0.18 ± 0.03	**24.97 ± 5.89**	0.11 ± 0.01	23.27 ± 5.93	0	0	0	0
TMW 1.907	0.19 ± 0.02	4.40 ± 0.57	0.03 ± 0.02	8.16 ± 4.71	0.06 ± 0.03	15.29 ± 7.14	0	0	0	0
TMW 1.936	0.16 ± 0.01	2.97 ± 1.29	0.05 ± 0.03	9.80 ± 5.66	0	0	0	0	0	0
TMW 1.1150	0.38 ± 0.04	7.29 ± 0.06	0.25 ± 0.03	8.75 ± 0.33	0.26 ± 0.02	7.90 ± 0.25	0	0	0	0
TMW 1.1152	0.43 ± 0.02	3.54 ± 0.42	0.39 ± 0.06	4.28 ± 0.92	0.41 ± 0.07	4.29 ± 0.66	0	0	0	0
TMW 1.1154	0.26 ± 0.03	2.80 ± 0.21	0.30 ± 0.06	4.44 ± 0.12	0.29 ± 0.06	4.76 ± 0.25	0	0	0	0
TMW 1.1221	0.23 ± 0.03	3.94 ± 0.39	0.27 ± 0.05	10.57 ± 4.10	0.24 ± 0.04	8.41 ± 2.38	0	0	0	0
TMW 1.1304	0.40 ± 0.04	4.31 ± 0.36	0.42 ± 0.02	5.38 ± 0.18	0.41 ± 0.00	5.91 ± 0.12	0.27 ± 0.01	31.67 ± 0.68	0.02 ± 0.01	9.72 ± 5.61
TMW 1.1470	0.20 ± 0.02	6.16 ± 0.27	0.38 ± 0.15	13.10 ± 1.59	0.32 ± 0.04	8.42 ± 0.50	0	0	0	0
TMW 1.1597	0.47 ± 0.05	5.54 ± 0.17	0.52 ± 0.01	6.60 ± 0.41	0.43 ± 0.03	7.73 ± 0.41	0.08 ± 0.01	10.95 ± 0.55	0	0
TMW 1.1730	0.35 ± 0.03	4.91 ± 1.87	0.44 ± 0.03	4.70 ± 1.20	0.43 ± 0.02	6.32 ± 1.56	0	0	0.26 ± 0.07	2.29 ± 0.58
TMW 1.2137	0.49 ± 0.12	3.19 ± 0.31	0.31 ± 0.05	3.44 ± 0.18	0.32 ± 0.04	3.09 ± 0.38	0	0	0	0
TMW 1.2138	0.39 ± 0.05	4.09 ± 0.09	0.26 ± 0.00	4.02 ± 0.46	0	0	0	0	0	0
TMW 1.2139	0.41 ± 0.01	5.55 ± 0.42	0.30 ± 0.06	6.29 ± 0.71	0.40 ± 0.01	7.28 ± 0.39	0	0	0	0
TMW 1.2140	0.28 ± 0.03	8.61 ± 2.20	0.24 ± 0.03	8.81 ± 1.19	0.31 ± 0.09	9.96 ± 2.60	0	0	0.18 ± 0.03	5.61 ± 0.87
TMW 1.2141	0.37 ± 0.01	2.93 ± 0.06	0.36 ± 0.02	3.20 ± 0.33	0.25 ± 0.02	4.97 ± 0.36	0	0	0	0
TMW 1.2142	0.40 ± 0.06	10.34 ± 3.15	0.33 ± 0.02	10.61 ± 3.29	0.32 ± 0.02	10.82 ± 2.95	0	0	0	0
TMW 1.2314	0.28 ± 0.02	2.69 ± 0.58	0.25 ± 0.04	22.99 ± 5.77	0.32 ± 0.09	26.44 ± 6.32	0	0	0.22 ± 0.07	7.18 ± 0.02
TMW 1.2323	0.26 ± 0.01	7.36 ± 0.78	0.28 ± 0.06	10.55 ± 0.24	0.18 ± 0.01	11.92 ± 1.92	0	0	0	0

* mMRS with maltose, glucose and fructose as C-source.

shared by acetic acid bacteria and yeasts. This is indicated by group- or strain specific differences (patchy metabolic pathways/non-functionality of genes) within the accessory genome referring to metabolic pathways for pentoses, citrate, malate, fructose, sucrose and EPS formation, and nucleotide biosynthesis. Within a small core genome strains are well equipped to metabolize maltose and regulate their redox household by a distinct thiol metabolism. Fructose metabolism is found in most strains except those of group 2 and should influence a competitive life in sourdough in cooperation with fructose-delivering, acetate-tolerant yeasts. Their pronounced equipment to cope with oxidative stress, e.g. via Nox2, may further relate to survival in insect vectors as conveyers between habitats.

The 24 *F. sanfranciscensis* strains in this study have a small genome from 1.26 Mbp to 1.36 Mbp and a high coding density of up to 93.18 %. The coding densities of other *lactobacilli* range from 80.4 %–87 % (Morita et al., 2008; Forde et al., 2011; Vogel et al., 2011). McCutcheon et al. (2009) found that insect endosymbionts have a high coding density up to 95.1 %. This is in agreement with the hypothesis that the origin of *F. sanfranciscensis* could be outside of the sourdough. In addition, in metagenome analyses *F. sanfranciscensis* sequences have been detected

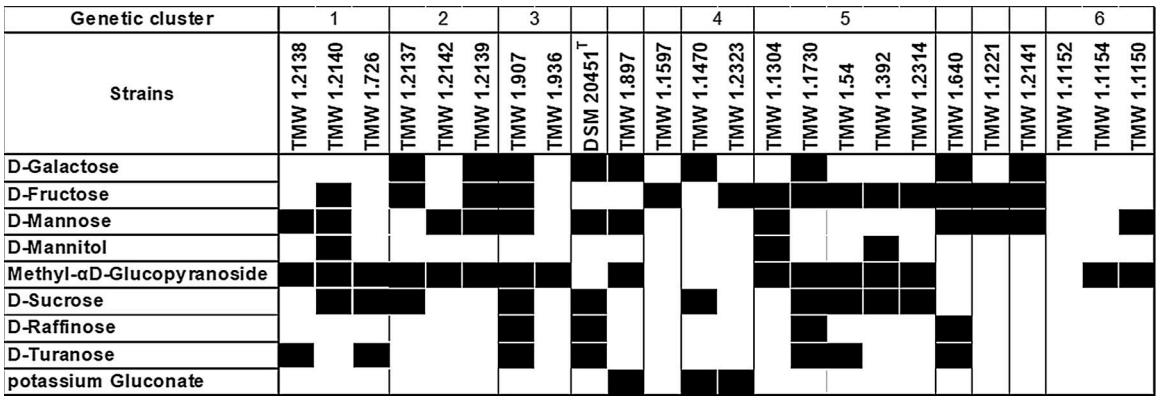

Fig. 6. Differential fermentation pattern analyzed by API 50 CHL test of the 24 *F. sanfranciscensis* strains after 48 h. Positive reactions on one carbon source were marked with black and negative reactions with white squares. The groups were made from the phylogenic characterization of the strains. Without all positive and negative reactions were all strains react equal.

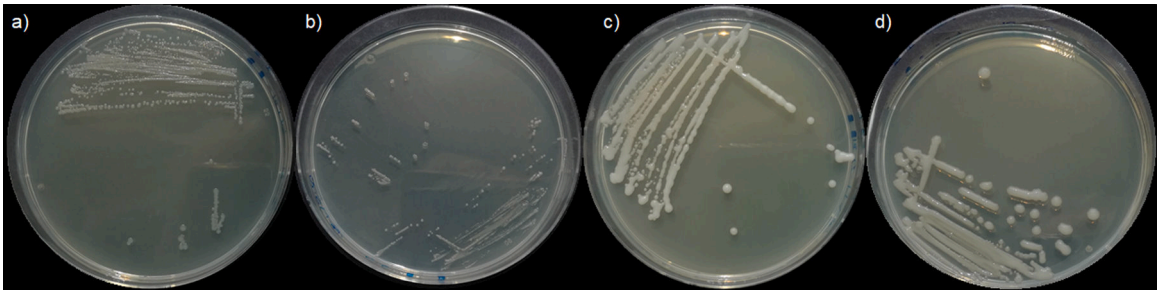

Fig. 7. Phenotype of EPS producing strains growing on mMRS agar plates with addition of sucrose. A) TMW 1.1597, b) DSM 20451^T, c) TMW 1.1304, d) TMW 1.1154. On agar plate c) and d) production of EPS is visible.

in insect frass and flower-associated insects, and it has also been associated with a plant origin (Groenewald et al., 2006; Gänzle and Ripari, 2016; Boiocchi et al., 2017). *F. sanfranciscensis* is genetically similar to *F. florum* and *F. fructivorans*. As a result they were recently grouped into the new genus *Fructilactobacillus* (Corsetti and Settanni, 2007; Gänzle and Zheng, 2019; Zheng et al., 2020). *F. florum* and *F. fructivorans* also have small genomes. They have been isolated from different food fermentations and are postulated to originate from the gut of fruit flies for their phylogenetic position (Zheng et al., 2020). Our study is supporting these findings, as it delivers arguments for the plant/insect relation of *F. sanfranciscensis*, derived from its genomic settings. Nonetheless, the origin of *F. sanfranciscensis* remains to be proven by isolation from such sources.

Genomic analyses predict strain-specific differences in carbohydrate metabolism, as a result of presence/absence of genes but also because of mutation or partial deletion of genes. Therefore, several approaches were employed to clarify physiological capabilities of the different *F. sanfranciscensis* strains. For an overview on fermentation capabilities API 50 CHL were performed. Still, these results could be misinterpreted especially when tests of different strains are performed with a read out at different time points (Kulwichit et al., 2007). This, as well as strain-specific differences, may explain varying results in different studies of *F. sanfranciscensis* (Vogel et al., 1994; Boyd et al., 2005; Buron-Moles et al., 2019). In growth experiments *F. sanfranciscensis* TMW 1.2138 was not able to grow with glucose as sole carbon source but showed a positive result in glucose fermentation in the API 50 CHL test (Table 2; Fig. 6). This finding is in agreement with the studies of Foschino et al. (2001) and Hammes et al. (1996), who classified *F. sanfranciscensis* strains into different groups due to their carbohydrate metabolism. They suggest that there are strains, which can use glucose and maltose (group 1) and strains, which are only able to use maltose (group 2). Still, the differences reported in these and our own studies, could be caused by long adaptation times to glucose of a strain adapted to growth on maltose (Stolz et al., 1993), because the genomic setting should allow utilization of glucose in all strains. The formation of "ropy" colonies of TMW 1.2141 as a result of the UDP-rhamnose operon was not observed, which matches the genomic prediction based on the lack of a flippase.

Ehrmann and Vogel (1998) discovered two different maltose phosphorylases in *F. sanfranciscensis* DSM 20451^T. These differ in their amino acid sequence and in their promoter (Fig. 3); *mapA* has an inducible promoter activated by maltose and *mapB*s promoter is constitutive (Vogel et al., 2002). This likely causes the differences in lag phases upon growth on maltose, i.e. strain TMW 1.897 with only *mapA* displays a very long lag phase. *F. sanfranciscensis* belongs to the few species, which utilizes the β-anomer of glucose-1-phosphate, which is used for the energy metabolism (Fig. 5), and is produced by both Maps of this species (Ehrmann and Vogel, 1998; Nilsson and Rådström, 2001; Andersson and Rådström, 2002), whereas the α-anomer of glucose-1-phosphate is utilized in different cellular processes like synthetic and degradative processes in the cell (Andersson and Rådström, 2002). The epimerase annotated as galactose mutarotase (TMW11150_BGL46_06300) downstream of *mapB* is assumed to perform the conversation from β-glucose-1-phosphate to α-glucose-1-phosphate. Moreover, the α-anomer is then introduced into the cell wall biosynthesis as it is transformed into α-glucose-6-phsophate by the α-phosphoglucomutase (TMW11150_BGL46_03695). Consequently, the maltose generated in strains harboring the epimerase in the *mapB* operon, is applied for the energy metabolism and the cell wall biosynthesis and found in many LAB. In contrast, the maltose metabolized by *mapA* is only used for the energy metabolism (Ehrmann and Vogel, 1998; Vogel et al., 2002).

Sucrose is often metabolized by dextran- and levansucrases under the production of EPS (Tieking et al., 2003). When metabolized by levansucrase the sucrose is cleaved into glucose, and a fructose oligomer (levan) is generated. The glucose can be used for energy metabolism (Fig. 5). Consequently, only strains with a functional LevS should be able to use sucrose as sole carbon source and produce EPS in the form of levan. DSM 20451^T and TMW 1.640, which harbor *levS* do not produce EPS and are unable to grow on sucrose (Table 2). This can be explained by the amino acid exchanges in the LevS core sequence of TMW 1.640. Sucrose is found in the grains of wheat and especially in rye, and the amount of sucrose in rye sourdough is between of 1.2–1.8%, rendering it a relevant carbon source besides maltose. Still, concentrations are too low to exploit any impact of EPS formation on bread properties (Tieking et al., 2003; Tieking and Gänzle, 2005; Tieking et al., 2005a; Galle et al., 2010; Galle and Arendt, 2014). Moreover, the sucrose in the sourdough is directly split by yeast invertase (Fujimoto et al., 2018; Loponen and Gänzle, 2018).

Fructose is metabolized by *F. sanfranciscensis* in different ways. It is utilized by all strains as external electron acceptor to recycle NAD except for the strains of cluster number 2. Furthermore, it can be used by strains of cluster number 5 and TMW 1.1597 as sole carbon source as they have a functional fructokinase (Fig. 2, Figure A2, Table A2-A3) (Gänzle et al., 2007). This may explain different behavior in competitive settings of different strains and even their varying preference for *Kazachstania* or *Saccharomyces* yeasts. These yeasts have different relations to sucrose and glucofructans, and may also differ in their tolerance to acetate, which results upon fructose reduction enabling the acetate kinase reaction (Rogalski et al., 2020a). In principle, the acetate reaction may also be fed in association with the metabolism of glucose to erythritol (Fig. 5) and, at first glance, all strains are predicted to enable this pathway. Still, current literature referring on erythritol metabolism in lactobacilli (Stolz et al., 1995; Kang et al., 2013) and ambiguous annotation of respective enzymes do currently not allow for an estimation of the contribution of this pathway to the lifestyle of *F. sanfranciscensis*.

As a heterofermentative species, *F. sanfranciscensis* should be able to use pentoses for energy metabolism since they could be fermented to ribulose- and xylulose-5-phosphate (Yazar and Tavman, 2012). However, in our study we could not observe growth on pentoses for any strain. This observation is supported by the genomic data. In *F. sanfranciscensis* the small ribose-pyranase (*rbs*D) is the only missing, but decisive enzyme for ribose fermentation. The D-ribose monomer

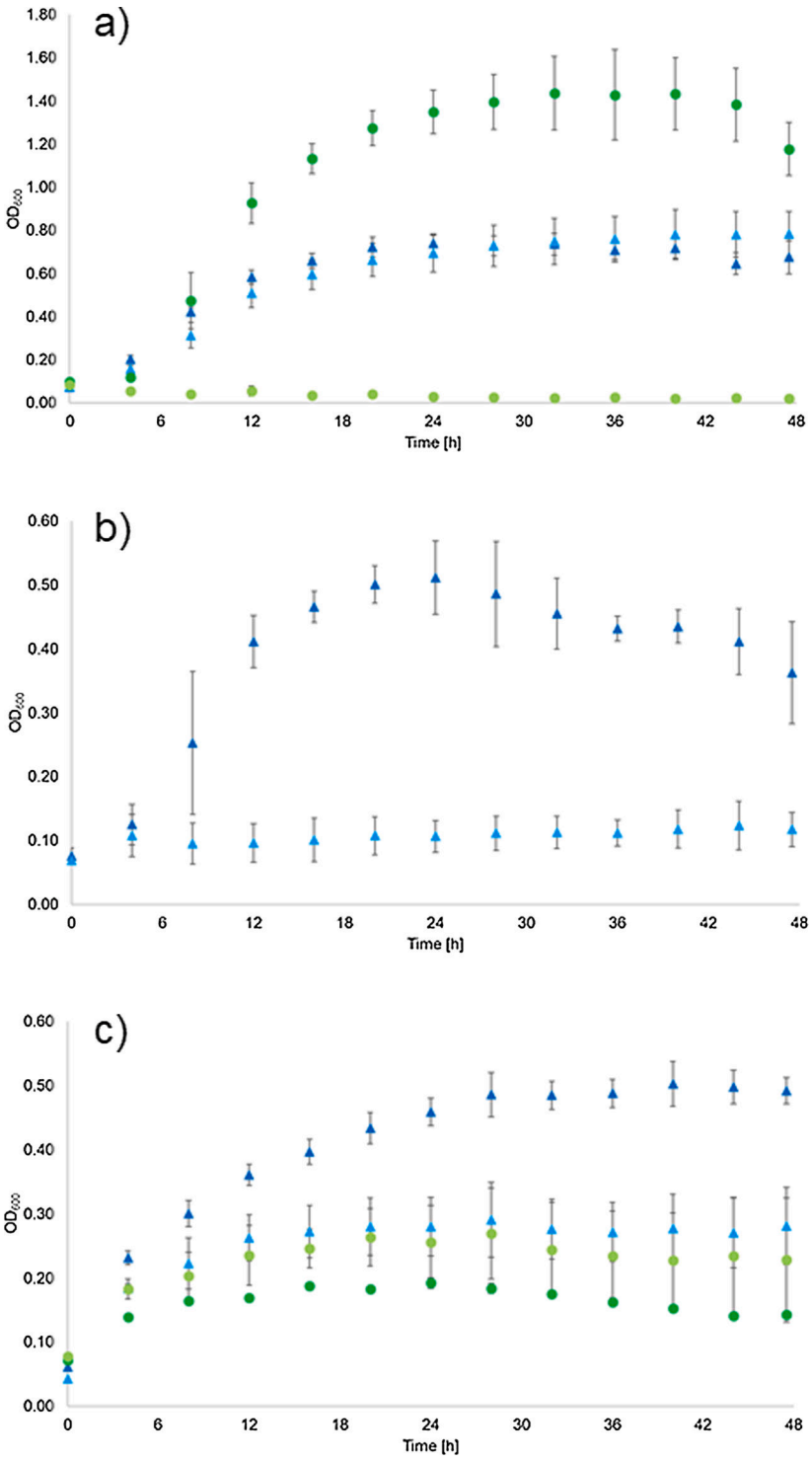

Fig. 8. Growth of *F. sanfranciscensis* strains in different CDMs measured in a plate reader for 48 h at 30 °C. The growth was detected with optical density values (OD_{600}). a) with (dark color) and without (light color) purines of TMW 1.1304 (orbital) and TMW 1.2323 (triangle) under oxic conditions; b) with (dark color) and without (light color) oxygen of TMW 1.392 (+/- O2); c) with (dark color) and without (light color) Na-gluconate of TMW 1.1597 (triangle) and TMW 1.1150 (orbital) under oxic conditions. The error bars were calculated with the standard deviation of the results of three independent experiments.

exists in four different forms in aqueous solutions: α-pyranose (22 %), β-pyranose (58 %), α-furanose (7 %), and β-furanose (12 %), with its open-chain form constituting less than 1 % (Ryu et al., 2004). Furthermore, with the ribose transporter *rbs*U only the β-pyranose anomer of ribose is imported into the cell. Whereas, the ribokinase (*rbs*k) merely binds to the α-ribofuranose. Therefore, the conversion of β-pyranoribose to α-ribofuranose is crucial for the ribose metabolism (Ryu et al., 2004). Moreover, known species like *Latilactobacillus sakei*, *Lactiplantibacillus (Lp.) plantarum, Levilactobacillus (Lel.) brevis* and *Latilactobacillus curvatus* (all formerly *Lactobacillus*, Zheng et al., 2020) with a functional ribose metabolism contain the *rbsD* in their ribose operon near *rbsK*, *rbsU* or the ribose operon repressor (Eisenbach et al., 2018). The missing *rbs*k is also the reason why the predicted ribose pathway in TMW 1.1304 is not functional (Vogel et al., 2011).

The addition of oxygen, fructose and citrate as external electron acceptors in CDM showed that oxygen is the favored electron acceptor. However, the niche sourdough is characterized by a low oxygen tension and in addition, oxygen needs to be shared with the yeast (De Vuyst

et al., 2016). Due to the refreshment and processing of the sourdough oxygen is introduced into the fermentation and can be used as electron acceptor again (Mihhalevski et al., 2011; De Vuyst et al., 2014). No growth improvement was observed upon addition of fructose and citrate to CDM despite genomic prediction (Fig. 8). One possible explanation for this effect could be that the CDM is the absolutely minimal medium for *F. sanfranciscensis* and a growth without oxygen is not possible. This hypothesis is supported by the observation that in mMRS *F. sanfranciscensis* is able to reach an OD_{600} max of 5 whereas in CDM only an OD_{600} of 0.3 is reached (Stetina et al., 2014). Moreover, it has previously been shown via HPLC analysis that the addition of fructose leads to an increase of mannitol in TMW 1.392, however, under different experimental conditions (Stolz et al., 1995; Korakli and Vogel, 2003). Apart from direct utilization of fructose as electron acceptor, its indirect use via fructose-6-phosphate should be possible, i.e. they predictively generate additional ATP via acetate kinase and erythrose kinase reactions in the presence of oxygen, which enables recycling of NAD (Fig. 8).

F. sanfranciscensis was unable to use Na-gluconate or malate, which originates from the metabolism of acetic acid bacteria, or from plants, respectively, as sole carbon source under anoxic conditions. Nevertheless, Na-gluconate increased growth on maltose of TMW 1.1597, which contains a functional gluconate permease in its genome. This is because NADH resulting from the phosphate dehydrogenase reaction can be re-oxidized to NAD by Nox2 in the presence of oxygen. In contrast, this was not detected for TMW 1.1150, which lacks the gluconate permease (Table A2-A3). Malate did not influence the growth of *F. sanfranciscensis* in CDM independently of the genomic settings of the tested strains. According to genomic data, *F. sanfranciscensis* is unable to use the fumarate respiration to regenerate NAD as it lacks the succinate dehydrogenase. As a consequence it cannot use malate for NAD recycling (Gänzle et al., 2007) despite residual properties for transport.

Genomic analysis revealed that the strains of cluster number 5 lack the enzymes for *de novo* purine biosynthesis from ribose-5-phosphate. The addition of adenine and guanine to CDM media enhanced the growth of these strains (Fig. 8a). Interactions with the yeasts or their partial cell lysis delivering RNA may help to fulfill its requirements for purines. Moreover, the energy, which is normally needed for the biosynthesis of purines could be used for an increased growth in the sourdough. This metabolite substitution was observed in other mixed-cultures of microorganisms in fermented food (Herve-Jimenez et al., 2009; Sieuwerts et al., 2010; Smid and Lacroix, 2013).

Interestingly none of the previously described mechanisms to cope with acid-stress like ADI, biogenic amine formation, or the glutamine/glutamate pathway (De Vuyst et al., 2009; Guan and Liu, 2020) were found in the annotated genomes of *F. sanfranciscensis*. Nevertheless, the genome of the *F. sanfranciscensis* strains harbor several proteases and DNA-repair proteins to combat metabolic stress evoked by pH-stress (Table A4). Hence, it may rather be truly tolerant to decreasing pH than employing (energy consuming) neutralization reactions. This may explain why *F. sanfranciscensis* is only acid tolerant to a pH of 3.6 (Kline and Sugihara, 1971; Gänzle et al., 1998), while other sourdough lactobacilli are acid tolerant up to a pH of 3.3 and lower like it occurs in sourdough type 2 fermentations (De Vuyst and Neysens, 2005).

In conclusion, adaptation to a competitive lifestyle in sourdough of *F. sanfranciscensis* appears to be based on different strategies. Firstly, *F. sanfranciscensis* has a very small genome and many metabolic pathways found in other heterofermentative lactobacilli are rendered non-functional, by mutation or loss. This saves a lot of metabolic energy, which were otherwise lost in the production of unnecessary “stand by” enzymes. Furthermore, analysis showed that *F. sanfranciscensis* strains are on the way to further adaption, as their small genome consists of a lot of incomplete pathways and mutated genes. In contrast, generalists like *Lel. brevis* or *Lp. plantarum* are able to utilize a broader spectrum of nutrients and persist in different environments. Secondly, its focus on the available substrates, namely maltose, sucrose and fructose from cooperation with the yeasts, and oxygen upon repeated refreshments, generally enhances its competitiveness in traditional sourdoughs. Thirdly, their adaption on the traditional sourdough in cooperation with yeasts is also reflected in an expressed thiol metabolism, with enzymes like the glutathione reductase (*gsh*), the cysteine transporter (*tcy*B) and the thioredoxin reductase (*trxR*) found in all strains (Table A4). The strain-specific differences found in the unexpectedly large accessory genome may further explain previously reported differences in their persistence and preference for cooperation with *Kazachstania* or *Saccharomyces*.

Ethics requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Esther Rogalski: Investigation, Methodology, Visualization, Writing - original draft. **Matthias A. Ehrmann:** Supervision, Project administration, Validation, Writing - review & editing. **Rudi F. Vogel:** Funding acquisition, Resources, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126625.

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5.4 Strain-specific interaction of *Fructilactobacillus sanfranciscensis* with yeasts in the sourdough fermentation

Fructilactobacillus (F.) sanfranciscensis is a key bacterium in traditional sourdough fermentations. It is mainly found in combination with *Saccharomyces* (*S.) cerevisiae* and *Kazachstania* (*K.) humilis*. In competition studies the behavior of eight different *F. sanfranciscensis* strains in combination without yeast, with *S. cerevisiae* or *K. humilis* was observed. It was possible to sort these strains into three different groups (see 5.2). Four strains of each group were chosen for further investigations. In metabolic studies as well as in sourdough trials the fermentation of these *F. sanfranciscensis* strains were observed. *F. sanfranciscensis* TMW 1.1150 was the strongest maltose and glucose fermenter, which explains its dominance in all sourdough fermentations. On the contrary, *F. sanfranciscensis* TMW 1.2138 was the weakest fermenter of maltose and additional not able to ferment glucose which explains why it was not competitive against the other strains at all. The cell sizes of these two strains can explain why the fermentation rate is different. *F. sanfranciscensis* TMW 1.1150 has significantly smaller cells than *F. sanfranciscensis* TMW 1.2138 and thus a higher surface to volume ratio, which predicts a higher metabolic rate. *F. sanfranciscensis* TMW 1.392 is only dominant in the competition studies when there was no yeast inoculated. In the metabolic studies it is the most diverse one. It is able to ferment maltose, glucose, sucrose and fructose to lactate, ethanol and in some cases acetate. Furthermore, its maltose fermentation is increased by external electron acceptors like fructose and oxygen. In combination with yeasts, it is in competition for the same substrates, as they are also depleted by the yeasts. The metabolism of *F. sanfranciscensis* TMW 1.907 was significantly increased by the presence of *S. cerevisiae*. There were more bacterial metabolites like lactate, acetate, and mannitol in the fermentation with *S. cerevisiae*. The mechanism for this finding is suggested to rely on stress-stimulation. The different behavior of the *F. sanfranciscensis* strains in the competition trials can be explained by the differences in their fermentation of carbohydrates and the use of electron acceptors. Consequently, there are different effects of yeasts on *F. sanfranciscensis* strains during the fermentation.

Author contributions: Esther Rogalski performed and planned the experiments as well as the data and genome analysis. She visualized and evaluated the data and wrote the original draft of the manuscript.

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ORIGINAL PAPER

Strain-specific interaction of *Fructilactobacillus sanfranciscensis* with yeasts in the sourdough fermentation

Esther Rogalski[1] · Matthias A. Ehrmann[1] · Rudi F. Vogel[1]

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Abstract
Fructilactobacillus (F.) sanfranciscensis is a key bacterium in traditional (type 1) sourdough fermentations. It typically occurs in combination with the sourdough yeast *Kazachstania (K.) humilis* or the generalist *Saccharomyces (S.) cerevisiae*. Previous studies revealed intra-species diversity in competitiveness or dominance in sourdoughs of *F. sanfranciscensis*, as well as preferences for a life with or without a specific yeast. In this study representative, differently behaving strains were studied in media with different sugars and electron acceptors, and in rye sourdough fermentations in the presence and absence of *K. humilis* or *S. cerevisiae*. Strain-specific differences were observed in sugar and organic acids spectra in media, and in sourdoughs with *F. sanfranciscensis* strains in combination with *K. humilis* or *S. cerevisiae*. *F. sanfranciscensis* TMW 1.1150 proved dominant in the presence and absence of any yeast because it most effectively used maltose. Its maltose fermentation was unaffected by electron acceptors. *F. sanfranciscensis* TMW 1.2138 was the weakest maltose fermenter and incapable of glucose fermentation, and evidently not competitive against the other strains. *F. sanfranciscensis* TMW 1.392 was the most versatile strain regarding the utilization of different carbohydrates and its ability to exploit electron acceptors like fructose and oxygen. In sourdoughs without yeasts, it outcompeted other strains. The metabolism of *F. sanfranciscensis* TMW 1.907 was stimulated in combination with *S. cerevisiae*. In competitive trials, it was assertive only with *S. cerevisiae*. The intra-species differences in carbohydrate metabolism can widely explain the differences in their behavior in sourdough fermentation. Interaction between *F. sanfranciscensis* and the yeasts was strain specific and supposedly commensal with *K. humilis* and rather competitive with *S. cerevisiae*.

Keywords *Kazachstania humilis* · *Saccharomyces cerevisiae* · Interaction · Electron acceptors · Sourdough fermentation · Competition · Carbohydrates · HPLC analysis

Introduction

The heterofermentative lactic acid bacterium (LAB) *Fructilactobacillus (F.) sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*) is a key species in traditional type-1-sourdough fermentations [1–4]. These fermentations last between 4 and 16 h and take place at medium temperatures and a pH between 3.7 and 4 [5, 6]. The fermentation conditions fit perfectly to the growth requirements of *F. sanfranciscensis* [7]. During fermentation, *F. sanfranciscensis* produces lactate, acetate, ethanol and carbon dioxide [8, 9].

✉ Rudi F. Vogel
rudi.vogel@tum.de

[1] Lehrstuhl Für Technische Mikrobiologie, Technische Universität München, Gregor-Mendel-Straße 4, 85354 Freising, Germany

The yeasts *Kazachstania (K.) humilis* (previously named *Candida humilis*) and *Saccharomyces (S.) cerevisiae* are also common inhabitants of sourdough [10, 11]. Whereas *K. humilis* is a typical sourdough yeast, which is only found in this niche, *S. cerevisiae* is a generalist with many biotypes [1, 12].

The microorganisms in the niche sourdough need to combat a stressful ecosystem. It is characterized by specialized offer of high and low molecular substrates and electron acceptors as well as high acidity and redox stress. Moreover, an adaption to the carbohydrates and nutritional options is required [2, 12–14]. *F. sanfranciscensis* is perfectly adapted to the sourdough surrounding. Maltose is its preferred carbohydrate, which is together with glucose constantly produced by the flour amylases from starch [15, 16]. Still, there are strain-specific differences in the utilization of carbohydrates between the *F. sanfranciscensis* strains

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[8, 17]. Sucrose, which is also present in the dough, and fructose (derived thereof) can be utilized in the metabolism by specific *F. sanfranciscensis* strains [8, 18–20]. Most of the *F. sanfranciscensis* strains are able to use fructose as an external electron acceptor for the recycling of NAD [4, 21]. Fructose is available in the sourdough due to the cleavage of glucofructans by specific yeasts like *K. humilis* and *S. cerevisiae* [22]. The yeasts, particularly *K. humilis* as maltose-negative yeast, use the resulting glucose for their metabolism [12, 23]. Consequently, there is no competition for the maltose in the sourdough between these two yeasts. As glucose can be released from the maltose phosphorylase reaction when maltose is abundant by *F. sanfranciscensis*, an often-found combination is *F. sanfranciscensis* and the yeast *K. humilis*, e.g. for strain TMW 1.392 (LTH 2590) [7, 21, 24, 25]. It, therefore, has been reasoned that this combination, which is often found in rye sourdough fermentations, is based on mutualism or may also result from indirect interactions based on glutathione and other thiol-metabolism, which act on the redox potential [1, 14, 23]. *S. cerevisiae* is often found in the bakeries surrounding and it is, therefore, assumed that it is also found in the sourdough [26, 27]. This yeast is a generalist as it can utilize maltose and glucose and various other sugars like sucrose as carbohydrate source [28]. Still, the *S. cerevisiae* sourdough isolates are acid resistant, which is not necessarily the case for the strains used for dough leavening [12, 29]. Otherwise, due to the usage of maltose by *S. cerevisiae*, *F. sanfranciscensis* is in general nutrient competition with the yeast. This stress is demonstrated by an increase of the maltose phosphorylase, which cleaves maltose in glucose-1-phosphate and glucose [21]. The glucose-1-phosphate is utilized in the metabolism of *F. sanfranciscensis* whereas the glucose is secreted in the abundance of maltose. The massive segregation of glucose leads to the glucose repression in many other LABs as well as in *S. cerevisiae* [28, 30]. This effect supposedly detains the *S. cerevisiae* from the uptake of maltose during the sourdough fermentation by glucose repression. In competition studies, it was found that the cell count of yeasts, especially *S. cerevisiae* and *K. humilis,* is always higher in the dough in the absence of LAB [23, 29]. This result illustrates a competitive influence of the LAB on the yeast. Furthermore, an intra-species competition between *F. sanfranciscensis* strains exists. It is possible that more than one strain of *F. sanfranciscensis* is present in a sourdough fermentation [31]. This phenomenon can be the result of a selection for strains in a distinct fermentation based on an intra-species competition or due to a coincidence by contamination form different sourdoughs. Moreover, in competition studies, a clear competition between strains in one sourdough fermentation was demonstrated [23, 32]. The strain-specific competition in the sourdough was independent or dependent on the yeast inoculated in the sourdough fermentation [23]. A genotype–phenotype study of *F. sanfranciscensis* showed that these strains have several differences in their carbohydrate utilization and their use of external electron acceptors [8, 17]. The present study was, therefore, dedicated to elucidate mechanisms of the strain-specific interaction between *F. sanfranciscensis* and yeasts in the sourdough fermentation by comparison of carbohydrate metabolism with their behavior in combination with yeasts in rye sourdough fermentations, and in previous competition studies.

Materials and methods

Strains and culture conditions

The *F. sanfranciscensis* strains TMW 1.1150, TMW 1.392, TMW 1.907 and TMW 1.2138 as well as the yeasts *K. humilis* TMW 3.1034 and *S. cerevisiae* TMW 3.1064 were chosen from the TMW strain collection based on their (different) competitiveness against other strains in the sourdough system and their genomic diversity [8, 23]. In the TMW strain collection, different yeast and lactic acid bacteria of different food fermentations were collected and stored. The strains were grown at 30 °C for 48 h in static conditions in modified DeMan Rogosa and Sharpe media (mMRS) [32]. The yeasts *K. humilis* TMW 3.1034 and *S. cerevisiae* TMW 3.1064 were grown overnight in yeast peptone glucose (YPG) media at 30 °C. For agar plates, 15% AgarAgar (Roth, Karlsruhe, Germany) was added to the media. For glycerol stocks, the overnight cultures were centrifuged, and the cell pellet was mixed with 70% glycerol and stored at − 80 °C.

Sourdough and sample preparation

Overnight cultures of the *F. sanfranciscensis* strains TMW 1.392, TMW 1.907, TMW 1.1150 and TMW 1.2138 were adjusted to an OD_{600} of 5 in 14 ml ¼ Ringer's solution and

were added to 100 g whole meal rye flour (dm, Karlsruhe, Germany) and 86 g tap water. For each strain, three separate sourdoughs were prepared, one without any yeast (− yeast) and one with the yeasts *K. humilis* TMW 3.1034 or *S. cerevisiae* TMW 3.1064. The yeast was added in a ratio of 1:100 to the bacterial cell count to the pre-fermented sourdough mixture simultaneously with the *F. sanfranciscensis* strain. The sourdough was propagated with 5% to the flour mass with a dough yield of 200. After three times of sourdough propagation, the sourdough was back slopped again for propagation and samples were taken after 0 and 24 h of the fermentation for DNA isolation, colony-forming units (cfu)/ml and high-throughput analysis matrix-assisted laser desorption/ionization (MALDI) time of flight (ToF) mass spectrometry (MS) measurements and HPLC analysis. During the whole fermentation process, the pH was measured before and after propagation. Furthermore, DNA isolation and the CLLP-PCR for strain identification were performed according to Rogalski et al. [32]. For the determination of the colony-forming units, 10 g of sourdough was mixed with 90 ml of ¼ Ringer`s solution (Merck, Darmstadt, Germany) and a tenfold serial dilution up to 10^{-7} was performed. Furthermore, the dilution steps were plated out on mMRS and YPG agar plates and incubated for 48 h at 30 °C aerobically. The colonies were counted and 48 of each plate were applied for MALDI ToF MS analysis (MS, Bruker, Billerica, USA).

Analytical analysis of carbohydrates and organic acids

Overnight cultures of the *F. sanfranciscensis* strains were prepared anaerobically in mMRS media under static conditions. The cultures were centrifuged at 7000*g* for 7 min, washed with ¼ Ringer's solution and adjusted to an OD_{600} of 5. Afterwards, the concentration was adjusted to 20 mM for maltose (GEBRU Biotechnik GmbH, Heidelberg, Germany), glucose (Merck), fructose (Omni Life Science GmbH & Co. KG, Bremen, Germany), sucrose (GEBRU Biotechnik) or ribose (Roth). To test the response of the strains to external electron acceptors, the combination of 20 mM maltose with 20 mM fructose, citrate (Roth), Na-gluconate (Roth) or malate (Sigma-Aldrich) was added to the cultures. To test the reaction with oxygen, cultures with 20 mM maltose were incubated in Erlenmeyer flasks at 150 rpm. The rest of the cultures was incubated at static conditions at 30 °C for 6 h. Samples were taken after 0 h and 6 h. Subsequently, the cultures were centrifuged for 10 min at 14,000 x*g* and the supernatant was filtered two times and added to HPLC vials for organic acid determinations (Phenomenex, Torrance, USA).

Also, sourdough samples were prepared for the HPLC analysis. Therefore, the sourdough samples were mixed 1:2 w/v in deionized water and centrifuged at 8000 ×*g*, 10 °C for 30 min. For the analysis of organic acids and ethanol, 5% perchloric acid (70%) was added and incubated overnight at 4 °C [33]. Afterwards, the supernatant was filtered with 2 µM membrane filters (Phenomenex, Germany). A different sample preparation was used for the analysis of carbohydrates. Here the centrifuged supernatant was incubated with 12.52 mM $ZnSO_4 \cdot 7H_2O$ (Carrez solution 2), 10 mM NaOH and 4.26 mM $K_4[Fe(CN_6)] \cdot 3H_2O$ (Carrez solution 1), centrifuged and also sterile filtered [33, 34].

Subsequently, for the analysis of organic acids and alcohols, a sulfonated styrene–divinylbenzene Rezex ROA column (Phenomenex), with 0.005 N H_2SO_4 as mobile phase, and for the analysis of sugars and sugar alcohols, a Rezex RPM column (Phenomenex) with deionized water as mobile phase were applied at 85 °C. Furthermore, an injection volume of 20 µl with a flow rate of 0.6 ml/min was chosen. The columns were coupled to a refractive index detection (RI) (ERC Refractomax 521, Thermo Fisher Scientific). The acids, sugars and sugar alcohols were identified and qualified with standards and the data were analyzed with Chromeleon™ software (Version 6.8, Dionex, Germany) [35]. Afterwards, the fermentation quotient (FQ) was calculated as the ratio of lactate to acetate for the sourdough samples as well as the ratio between lactate and ethanol [33]. The turnover of the metabolites during the 6 h of incubation was calculated. For the depletion of the substances, a ratio between the values of 6 h to 0 h of incubation time was calculated per g/cell dry mass. Furthermore, the production of the substances was calculated between the values of 0 h to 6 h of incubation time per g/cell dry mass. The uncalculated values were provided in the Figs. A1, 2, 3, 4 together with the standard deviation.

Determination of the cellular dry weight, morphology, and cell size

The cfu/ml and cellular dry weight of the *F. sanfranciscensis* strains were determined at an OD_{600} of 5. Therefore, overnight cultures were grown and set to an OD_{600} of 5 with ¼ Ringer`s solution. The determination of the cell count was performed as mentioned above. For the determination of the cellular dry weight, falcons were set in a desiccator for 1 h and weighed. The cultures with an OD_{600} of 5 were added to the pre-weighted falcons and centrifuged at 10,000 ×*g* for 10 min at RT. The cell pellet was dried for 24 h at 95 °C, cooled down for 1 h in a desiccator to RT and the falcon with the cell pellet was weighed again. For the measurements of the cell size, cells out of an overnight culture were examined under a light microscope (Axiostar Plus, Carl Zeiss AG, Oberkochen, Germany) and the cell size was determined with a 5 µm standard of the ZEN Blue Edition software (Carl Zeiss AG).

Statistical analysis

All experiments were performed in biological triplicates. In case of the determination of the cell count and the MALDI ToF MS analysis, technical duplicates were performed ($n = 6$). For analysis of the cell size, a two-sided Student's *t* test was applied. Furthermore, a one-way ANOVA was applied to analyze the metabolic differences in the sourdough when yeasts or no yeasts were inoculated. Therefore, only bacterial products like lactate, acetate and mannitol were calculated; results $p < 0.05$ were considered significant. The standard deviation was calculated for all analytical results. Outliers broader than 10% percent were ignored.

Results

Differences in cell morphology of *F. sanfranciscensis* strains

There are strain-specific differences in the cell morphology, cell size and cell weight of *F. sanfranciscensis* (Table 1; Fig. 1), which need to be considered in the comparison of metabolic turnover. TMW 1.392 and TMW 1.1150 have shorter/smaller cells than TMW 1.907 and TMW 1.2138. In the latter case, these two strains have also a broader variety in their cell morphology. The median cell size is about 5 µm with a large distribution in their cell size. Considering TMW 1.392 and TMW 1.1150, the cell size differs only slightly between the single cells (Fig. 1a, c). Furthermore, the cell sizes between the strains differed significantly from each other, with the exception of *F. sanfranciscensis* TMW 1.907 to TMW 1.2138 (Fig. 1). The differences in the cell size reflect the number of cells found in a solution of an OD_{600} of 5. The smaller the single cell, the higher the cell count of the strain in a defined solution (Table 1).

For *F. sanfranciscensis* strains TMW 1.907, TMW 1.2138 and TMW 1.1150, the measured cell size fits to the resulting cfu/ml and the cell dry weight at an OD_{600} of 5. *F. sanfranciscensis* TMW 1.907 and TMW 1.2138 had a high cell dry weight with a low cell count with larger cells. In addition, the *F. sanfranciscensis* strain TMW 1.1150 had the highest cell count, however, only at a medium cell dry weight because of its small cells. The cell dry weight differs from the cell size and the resulting cell count in *F. sanfranciscensis* TMW 1.392. *F. sanfranciscensis* TMW 1.392 had a cfu/ml of 6.16×10^9 in a culture with an OD_{600} of 5 although it had the lowest cell dry weight (Table 1).

The turnover of carbohydrates is strain dependent

The *F. sanfranciscensis* strains differ in their competitiveness in the sourdough and their genetic equipment [8, 23]. This should be reflected in the metabolism. Different sugars were chosen, which are common in sourdough fermentation, and the turnover is given in relation to the cell dry weight (Table 1; Fig. 2). Fermentation of a carbohydrate was recorded only when metabolites like lactate, acetate or ethanol were produced. Maltose was fermented by all strains within 6 h of incubation. *F. sanfranciscensis* TMW 1.1150 was the strongest maltose fermenter followed by TMW 1.392 (Fig. 2a). In the glucose fermentation, *F. sanfranciscensis* TMW 1.1150 showed the strongest turnover after 6 h compared to the other strains. In TMW 1.907 and TMW 1.392 a glucose turnover was recorded resulting in the production of lactate and ethanol, and very low amounts of acetate. Fructose and sucrose were degraded only by *F. sanfranciscensis* TMW 1.392. When fructose is degraded, lactate, mannitol, and acetate instead of ethanol was produced. The same turnover can be seen in the degradation of sucrose by *F. sanfranciscensis* TMW 1.392 (Fig. 2). There is no degradation of ribose. Only in *F. sanfranciscensis* TMW 1.1150, a turnover of ribose appears possible as with the degradation

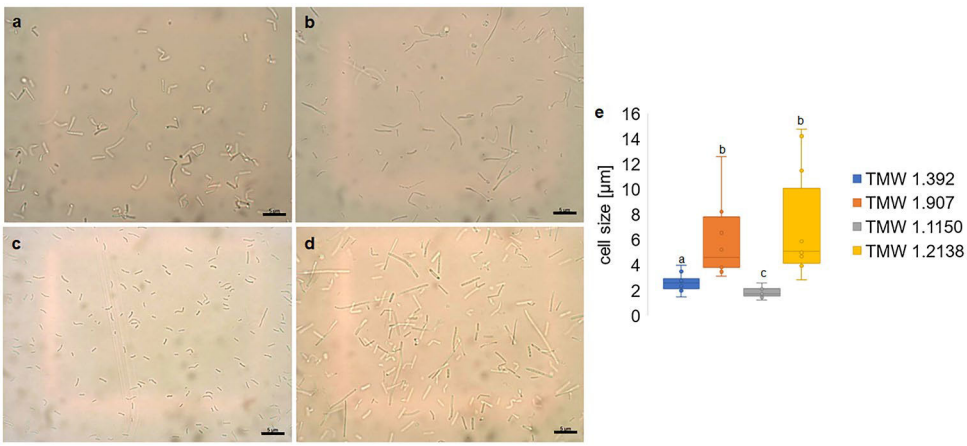

Fig. 1 Light electron microscopy image of *F. sanfranciscensis* strain **a** TMW 1.392, **b** TMW 1.907, **c** TMW 1.1150 and **d** TMW 1.2138. Size bars correspond to 5 µm, recordings are performed with ZEN Blue image software. The cell size in µm is illustrated in e) with the median and standard deviation. Bars with a different lowercase letter are differing statistically ($p < 0.05$) from each other

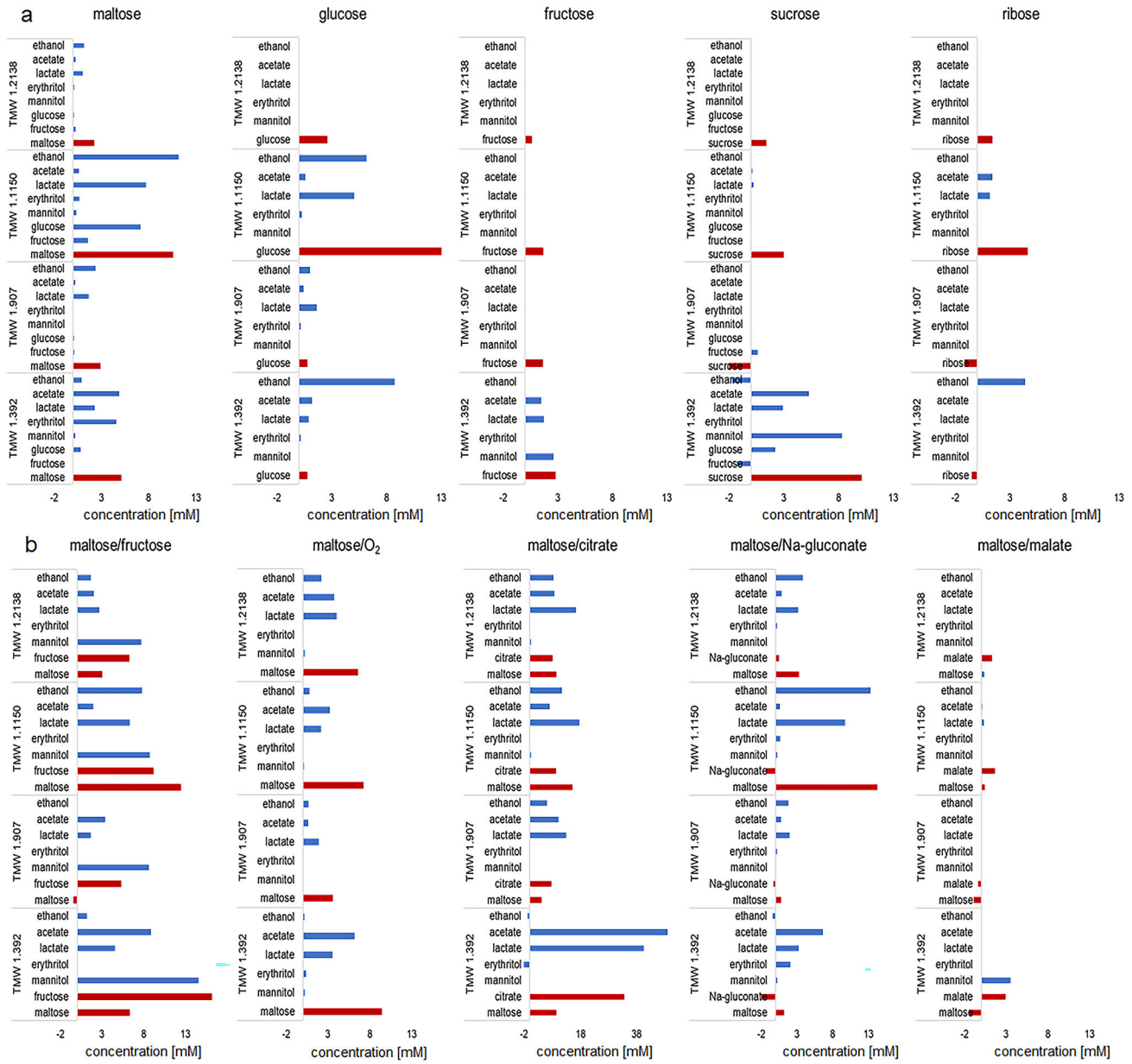

Fig. 2 Turnover of sugars and electron acceptors of *F. sanfranciscensis* TMW 1.392, TMW 1.907, TMW 1.1150, TMW 1.2138 in relation to the dry mass determined with HPLC analysis. The red bar indicates the consumption of the carbohydrate and the blue bar represents the production of the products during 6 h of incubation in Ringer's solution with 20 mM of each reagent. In **a** the fermentation with one sugar was presented and in **b** the fermentation with maltose and an additional electron acceptor was pictured

of ribose a production of acetate and lactate occurs (Fig. 2). In the fermentation of maltose and glucose, erythritol is produced every time in combination with acetate.

The enhancement of the maltose uptake in combination with fructose, oxygen, citrate, Na-gluconate and malate were determined (Fig. 2b). All strains produced more mannitol and less ethanol in combination with fructose, than solely with maltose (Fig. 2b). Moreover, no erythritol is produced in combination with external electron acceptors. When malate is added in combination with maltose, the maltose

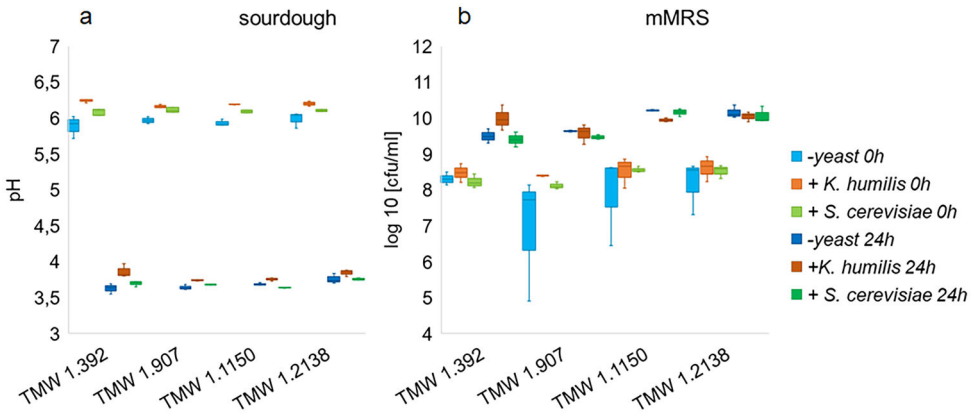

Fig. 3 Development of the pH values (**a**) and the cell count (**b**) of the sourdoughs between 0 and 24 h of fermentation. The sourdoughs were investigated from the *F. sanfranciscensis* strains TMW 1.392, TMW 1.907, TMW 1.1150 and TMW 1.2138 in combination without yeasts (−yeast), with *K. humilis* TMW 3.1034 (+*K. humilis*) or with *S. cerevisiae* TMW 3.1064 (+*S. cerevisiae*) respectively

uptake and turnover are decreased in all strains. The metabolism of *F. sanfranciscensis* TMW 1.392 was increased the most compared to the other strains by the addition of external electron acceptors like fructose, oxygen, and citrate. In combination with citrate, the production of lactate and especially acetate is increased the most, although the depletion of maltose is not increased (Fig. 2b). During the turnover of maltose in combination with Na-gluconate, mostly more lactate and ethanol were produced. Similar to the reaction with only maltose, erythritol and acetate were produced when Na-gluconate is added to the reaction.

The presence of yeasts influences the metabolic turnover in a sourdough fermentation by *F. sanfranciscensis*

All four *F. sanfranciscensis* strains were evaluated with regard to their metabolic performance in a rye sourdough fermentation in response to the presence of yeasts. For this purpose, the single strains were added together with no yeast (−yeast), *K. humilis* 3.1034 or *S. cerevisiae* 3.1064 in sourdough fermentation. No yeast growth was recorded in the samples without any added yeasts. The development of the pH was comparable in sourdoughs with the different strains alone. Still, the sourdough without any yeasts and the sourdough with *S. cerevisiae* 3.1064 was slightly more acidic than the sourdough with *K. humilis* 3.1034 (Fig. 3a). The development of the cfu/ml between 0 and 24 h was similar for the strains. It increases from around 8 to 10 log 10 [cfu/ml] within 24 h. In all −yeast fermentations a broad cfu/ml standard deviation at 0 h was observed, except for the sourdough with *F. sanfranciscensis* TMW 1.392 (Fig. 3b). In Fig. 4, the metabolites determined at 0 h and 24 h of fermentation of each strain and yeast combination are depicted. The same metabolites were detected with and without yeasts (Fig. 4). The amount of ethanol was higher in the presence of yeasts because of their alcoholic fermentation. Furthermore, in the presence of *S. cerevisiae* TMW 3.1064 and in combination with *F. sanfranciscensis* TMW 1.392 and TMW 1.2138 the ethanol concentration was significantly the highest. In the absence of yeast, the amount of ethanol was very similar and in combination with *F. sanfranciscensis* TMW 1.392 greater than with the other strains. Apart from the ethanol concentration, there were no significant differences between the sourdoughs with the two yeasts. It should be noted that it is not possible to measure maltose during the fermentation as it is depleted directly after its production (by amylases) and is, therefore, below the detection limit. Small amounts of glucose were determined during the fermentation. The highest amount of glucose can be measured in the fermentation −yeast with *F. sanfranciscensis* TMW 1.2138 (Fig. 4c).

The FQ as well as the ratio between lactate and ethanol are major factors to evaluate a sourdough fermentation as it delivers a main sensory characteristic (Table 2). A fermentation with a low FQ has a high amount of acetate compared to lactate. In most of the strains, except *F. sanfranciscensis* TMW 1.2138, the FQ is higher without any yeasts, which is the result of a low acetate concentration. In the *F. sanfranciscensis* strains TMW 1.1150, TMW 1.392 and TMW 1.907, the sourdough in combination with *S. cerevisiae* 3.1064 had the lowest FQ. The lowest FQ of all fermentations can be measured for the combination of *F. sanfranciscensis* TMW 1.2138 and *S. cerevisiae* TMW 3.1064 (Table 2). The ratio between lactate and ethanol was always higher in the absence of yeasts (lower amount of ethanol) and lower in the presence of yeasts. This observation is in line with the results above and the alcoholic fermentation of the yeasts (Fig. 3). In TMW 1.1150 and TMW 1.907, the differences in the lactate ethanol ratio were minor in the fermentation without yeasts and with *K. humilis* TMW

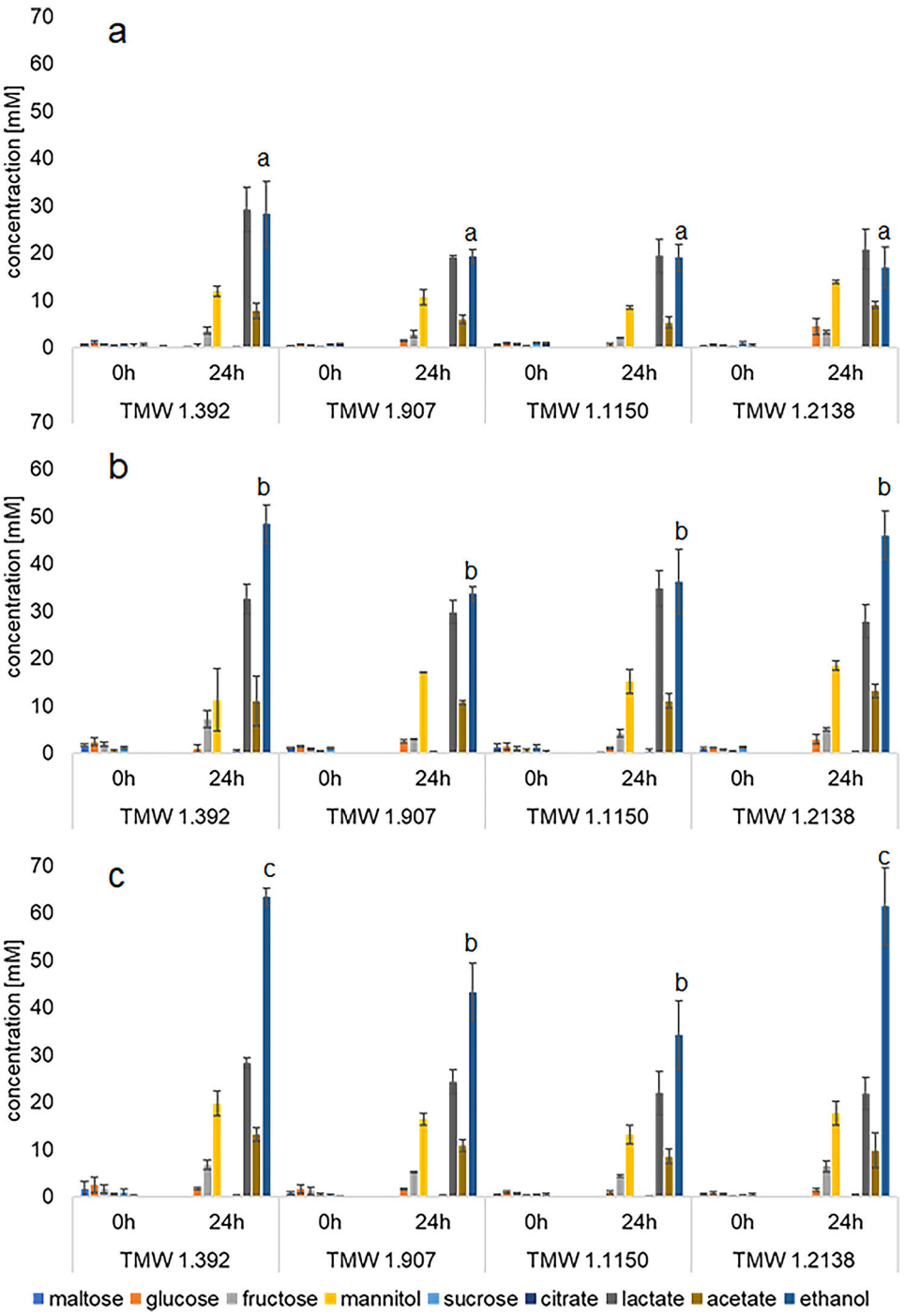

Fig. 4 HPLC analysis of the sourdough fermentation after 3 times of propagation at 0 h and 24 h. The *F. sanfranciscensis* strains TMW 1.392, TMW 1.907, TMW 1.1150 and TMW 1.2138 in combination without yeast (**a**) with *K. humilis* TMW 3.1034 (**b**) or *S. cerevisiae* TMW 3.1064 (**c**). Bars with a different lowercase letter are differing statistically ($p < 0.05$) from each other

3.1034 (Table 2). A one-way ANOVA analysis was applied to evaluate the statistical differences between the inoculation with and without yeasts (−yeast) for all *F. sanfranciscensis* strains. There were no differences between the inoculation of yeasts in the production of lactate and acetate in the *F. sanfranciscensis* strains TMW 1.2138 and in case of TMW 1.392 in the production of lactate and mannitol. Furthermore, the difference in acetate production was significant ($p < 0.05$) between the fermentation of *K. humilis* TMW 3.1034 and *S. cerevisiae* TMW 3.1064 of *F. sanfranciscensis* TMW 1.392, TMW 1.907 and TMW 1.1150. In *F. sanfranciscensis* TMW 1.1150, the acetate production in all yeast combinations was significantly

Table 1 Cell count (cfu/ml) and cell dry weight of the *F. sanfranciscensis* strains TMW 1.392, TMW 1.907, TMW 1.1150, TMW 1.2138 at an OD_{600} of 5

F. sanfranciscensis strain	cfu/ml	Cell dry weight (mg/50 ml)
TMW 1.392	$6.2\times10^{9}\pm0.7\times10^{9}$	30.6 ± 6.5
TMW 1.907	$9.7\times10^{7}\pm1.8\times10^{7}$	82.2 ± 24.8
TMW 1.1150	$1.2\times10^{10}\pm0.2\times10^{10}$	60.0 ± 12.4
TMW 1.2138	$1.2\times10^{8}\pm0.2\times10^{8}$	72.7 ± 8.2

Table 2 Relation between lactate to acetate (FQ) and lactate to ethanol after 24 h of sourdough fermentation for sourdoughs of the *F. sanfranciscensis* strains TMW 1.392, TMW 1.907, TMW 1.1150 and TMW 1.2138 in combination without yeast (−yeasts), with *K. humilis* TMW 3.1034 and *S. cerevisiae* TMW 3.1064

		FQ (lactate/acetate)	Lactate/ethanol
TMW 1.392	−Yeast	3.79	1.03
	+*K. humilis*	2.96	0.67
	+*S. cerevisiae*	2.17	0.45
TMW 1.907	−Yeast	3.25	0.99
	+*K. humilis*	2.78	0.89
	+*S. cerevisiae*	2.26	0.56
TMW 1.1150	−Yeast	3.71	1.02
	+*K. humilis*	3.17	0.96
	+*S. cerevisiae*	2.58	0.64
TMW 1.2138	−Yeast	2.32	1.23
	+*K. humilis*	2.11	0.60
	+*S. cerevisiae*	2.23	0.35

different, respectively. In TMW 1.907 only −yeast was different from the sourdoughs with yeast inoculation. The production of lactate was significant in the *F. sanfranciscensis* strains TMW 1.1150 and TMW 1.907 when inoculated with *K. humilis* TMW 3.1034.

Discussion

In former studies, the strain-dependent differences in the competitiveness in rye sourdough were investigated [23]. These competitive trials were also performed in the presence of the yeasts *K. humilis* TMW 3.1034 (+*K. humilis*) or *S. cerevisiae* TMW 3.1064 (+*S. cerevisiae*), or without any yeasts (−yeast). It was possible to categorize the eight tested *F. sanfranciscensis* strains into three different groups. *F. sanfranciscensis* TMW 1.1150 belonged to the group of dominating strains independently of yeast presence/absence. *F. sanfranciscensis* TMW 1.392 and TMW 1.907 belonged to the group for which the strain dominance was dependent on the presence/absence of yeast. *F. sanfranciscensis* TMW 1.907 prefers the presence of the yeast *S. cerevisiae* TMW 3.1064 whereas *F. sanfranciscensis* TMW 1.392 performed best with no added yeast in the fermentation. *F. sanfranciscensis* TMW 1.2138 belonged to the group of strains, which were not dominant in the sourdough competition independently of the presence or absence of yeasts [23]. In this study, the reasons for the different behavior in strain dominance were investigated along metabolic analyses. The metabolic turnover of *F. sanfranciscensis* strains was determined along the consumption of different carbohydrate sources and the concomitant production of acids and alcohols. The metabolite formation was normalized for comparison along the cell dry mass of the respective strain. This appeared as a better means compared to cell counts, because the cell size between strains varied significantly. This circumstance is apparently neglected in previous studies on the metabolism of *F. sanfranciscensis* strains but is considered as important. The metabolite analysis upon incubation with ribose or malate did not reveal any metabolic activity in any of the *F. sanfranciscensis* strains. The *F. sanfranciscensis* strain TMW 1.1150 was the most efficient per g/cell mass in maltose fermentation compared to the other four strains. This maltose fermentation or turnover within 6 h was neither influenced positively nor negatively by external electron acceptors. These results show that due to its rapid maltose fermentation this strain develops a rapid growth. Furthermore, the small cell structure is an advantage as cells with a higher surface to volume ratio are more effective [36]. In addition, this strain was also the most efficient in glucose fermentation, more efficient than any other strain (Fig. 2). Maltose and glucose are the main sugars in sourdough fermentation, as both are constantly produced by flour amylases [37]. A rapid fermentation of these sugars especially at the beginning of the fermentation leads to a growth benefit over the other strains. These other strains are not as fast as *F. sanfranciscensis* TMW 1.1150. This turnover can explain why *F. sanfranciscensis* TMW 1.1150 was dominant in all fermentations independently of the yeast co-inoculation. The addition of external electron acceptors did not influence the consumption of maltose, although an inoculation of yeasts in the sourdough increased its production of lactate, acetate and ethanol. In combination with *K. humilis* TMW 3.1034 the increase was more than with *S. cerevisiae* TMW 3.1064. Hence, there should be more factors above those ones determined in our study, which affect *F. sanfranciscensis* in the presence of yeasts. Indeed, stimulatory effects have been reported of nitrogen overflow, carbon dioxide or growth factors produced by *S. cerevisiae* on the survival of LAB in microbial communities in other LAB/yeast combinations [10, 38].

F. sanfranciscensis TMW 1.2138 (Ls12) was the weakest in the competition studies. It was unable to compete against the other strains in any combination [23]. Although, its isolation from a sourdough shows that *F. sanfranciscensis* TMW 1.2138 can compete in this environment. As it is a strain from a wheat sourdough, it possibly would better persist in other sourdough types against other strains of *F. sanfranciscensis* or other LAB. However, in competitive studies in wheat flour of Siragusa et al. [39], this strain was also outcompeted by the autochthones wheat microbiota. In our studies, it was the slowest in the consumption of maltose, as after 6 h of fermentation time only 2.2 mM of maltose were fermented (Fig. 2). Moreover, it was not able to ferment glucose, fructose or sucrose, which was also shown in previous studies [8]. A slow maltose fermentation and incapability of glucose fermentation explain why it is the weakest of the strains in sourdough fermentation. Furthermore, in the sourdough with *F. sanfranciscensis* TMW 1.2138, the presence or absence of the yeast did not alter the FQ as well as the production of the bacterial metabolites significantly. This result implies that the metabolic products of the yeasts do not affect the acetate level of *F. sanfranciscensis* TMW 1.2138 considerably (Table 2). The same observations were made before, as common electron acceptors like fructose and citrate did not alter the maltose fermentation of *F. sanfranciscensis* TMW 1.2138 significantly (Fig. 2) [8]. Moreover, only oxygen had a positive effect on maltose fermentation in the media fermentation. Though in combination with yeast in sourdoughs, the oxygen was consumed by the yeast's respiration [40]. In conclusion, these investigations explain why *F. sanfranciscensis* TMW 1.2138 belonged to the non-dominant strains in competitiveness trials.

The *F. sanfranciscensis* strains TMW 1.392 and TMW 1.907 were influenced either negatively or positively by the presence of the yeasts *K. humilis* TMW 3.1034 and *S. cerevisiae* TMW 3.1064. *F. sanfranciscensis* TMW 1.392 preferred the absence of the yeasts although it is often found in combination with *K. humilis* [41]. This observation can be explained by the metabolic versatility of *F. sanfranciscensis* TMW 1.392. Notwithstanding, that its maltose turnover was lower for every condition than the turnover of maltose by *F. sanfranciscensis* TMW 1.1150, it was able to alter the fermentation the most (Fig. 2). The turnover of maltose by *F. sanfranciscensis* TMW 1.392 increased the most with electron acceptors like fructose and oxygen. Furthermore, it was able to use fructose and sucrose also for its metabolism and a clear turnover was detectable. When yeasts are present in the sourdough fermentation, the advantage of sucrose fermentation by *F. sanfranciscensis* TMW 1.392 is neglectable as sucrose is directly cleaved by the yeasts invertase [42, 43]. Moreover, the production of acetate and thereby the recycling of NAD and the extraction of an extra ATP is increased significantly yeast-dependent with *S. cerevisiae* TMW 3.1064, more than with *K. humilis* TMW 3.1034 [37] (Table 2). Still, it appears that the advantage of an extra ATP through the acetate formation does not compensate for the lack of sucrose in sourdoughs with yeasts. Therefore, it can be explained why *F. sanfranciscensis* TMW 1.392 is not able to dominate in sourdoughs with yeasts together with *F. sanfranciscensis* TMW 1.1150 but without the yeasts [23].

Regarding *F. sanfranciscensis* TMW 1.907 it is difficult to explain why this strain is only dominant in combination with *S. cerevisiae* TMW 3.1046. In general, its turnover of maltose and glucose was better than in the weakest strain *F. sanfranciscensis* TMW 1.2138. It should be considered that their cell size and cell dry weight are similar (Table 1). Furthermore, its maltose turnover was increased by the presence of oxygen but not by fructose and citrate (Fig. 2). The production of the bacterial metabolites like lactate, acetate and mannitol was increased significantly in combination with yeasts. This effect can imply a stimulatory effect on the metabolism of *F. sanfranciscensis* TMW 1.907 by *S. cerevisiae* as well as *K. humilis* [10]. The higher production of ethanol in the sourdough fermentation is explained clearly by the alcoholic fermentation of the yeasts. An increase of ethanol formation is in sourdoughs with *F. sanfranciscensis* TMW 1.1150 and TMW 1.907 not as high as in sourdoughs with the other two strains (Fig. 4), although the ethanol concentrations are too low for inhibition of these species [7].

Taken together, the response of *F. sanfranciscensis* to the presence of yeasts is a strain- or group-specific trait. Generally, *K. humilis* revealed itself as a co-existing, i.e. commensal partner, which apparently neither elicit metabolic stress nor stimulation to *F. sanfranciscensis*, while the *S. cerevisiae* sourdough isolate rather showed competitive characteristics [23]. *F. sanfranciscensis* is in general nutrient competition with the *S. cerevisiae*, namely for maltose and sucrose, while the maltose-negative *K. humilis* prefers glucose [44]. Also, general mechanisms of redox-balance, e. g. thiol-metabolism, likely differ between yeast genera, and between *F. sanfranciscensis* strains, and may contribute to the strain-specific behavior observed [13, 14]. Depending on the *F. sanfranciscensis* partner the concomitant stress can therefore impose a negative effect on its competitiveness and metabolism in sourdoughs but can also be stimulatory. In this study, only *F. sanfranciscensis* TMW 1.907 was influenced positively by the presence of yeasts. In other combinations, the positive effects of the yeast interactions with *F. sanfranciscensis* are limited or absent, and the negative effects dominate, namely in combination with *S. cerevisiae*. Furthermore, the cell count of yeasts was decreased in combination with *F. sanfranciscensis* [23, 29], suggesting that these LAB also impose stress on the yeasts. This study, therefore, suggests that interactions of *F. sanfranciscensis* and the yeasts *S. cerevisiae* and *K. humilis* are competitive or commensal, respectively.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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6 Discussion

This thesis is dedicated to the interaction between *F. sanfranciscensis* and the sourdough yeasts *K. humilis* and *S. cerevisiae*. Therefore, the genomic diversity of *F. sanfranciscensis* was examined to explore strain-specific differences in the interaction with the yeasts. The knowledge on genomic equipment as well as phenotypic characteristics enable a better understanding of the fermentation, and in particular how the sourdough microbiota cope with the environmental parameters and partners sharing this niche. In competition trials intra-species diversity in the interaction with yeast partners was explored. Stable partnerships in the sourdough fermentation enable a stable sourdough microbiota through backslopping and are the prerequisite for a reproducible product. Furthermore, the characterization of the core *vs* accessory genomes can help to elucidate the origin of *F. sanfranciscensis*.

The former initial working hypotheses will be discussed in the following section (see 3.6).

a) The genomic setting of the different strains of *F. sanfranciscensis* refers to a sugary-oxic evolutionary origin of this species, with sourdough being a (late) special niche and insects being a putative connection between these habitats.

b) There are strain-specific differences namely in the sugar metabolism and use of electron acceptors by *F. sanfranciscensis* regarding the competitiveness and persistence in the sourdough system.

c) The strain-specific competitiveness of *F. sanfranciscensis* is either independent of the presence of a yeast, or it can be positively or negatively affected by presence/absence of sourdough yeasts.

d) The interaction between *F. sanfranciscensis* and the yeasts *K. humilis* and *S. cerevisiae* is commensal and competitive, respectively, rather than mutualistic with respect to the investigated compounds.

6.1 The genomic equipment of *F. sanfranciscensis* refers to a sugary-oxic evolutionary origin

The evolution of a new species takes up to millions of years and the development of sourdough is estimated to have occurred 5,000 years ago (Gänzle and Ripari, 2016). As a consequence the time frame for the development of a new species in the sourdough is too short (Gänzle, 2014). Still, adaptation of an existing species to a new habitat may occur in much shorter times. *F. sanfranciscensis* is until now only isolated from the sourdough and is believed to be autochthonous for this niche. Although, in metagenomic studies 16S rRNA sequences were found in insect larvae (Boiocchi *et al.*, 2017). Indeed, the genomic analysis of 24 *F. sanfranciscensis* strains showed patchy metabolic pathways relating to several carbohydrates and external electron acceptors, which are not common in the dough. The 24 *F. sanfranciscensis* strains showed a broad accessory genome with numerous fragments of metabolic pathways. Furthermore, there are strain heterogeneities in these pathways. Most of them use fructose as electron acceptors, while only strains from cluster number 5 are able use fructose as energy source. Whereas strains from cluster number 2 are not able to utilize fructose at all. Albeit fructose is metabolized by most *F. sanfranciscensis* strains as external electron acceptor to mannitol, which can be a relic of their former niche. The regeneration of NAD by reduction of fructose to mannitol is valuable in the sourdough environment but not crucial. *F. sanfranciscensis* strains like TMW 1.2137, which are unable to utilize fructose in any case are dominant in sourdough fermentations when *S. cerevisiae* TMW 3.1064 is present (Rogalski *et al.*, 2020c,a). In addition, there is a partial reduction of pathways for the metabolization of pentoses like xylose, arabinose, and ribose. The usage of pentoses appears not to be required for a life in the sourdough environment, which speaks for an evolutionary driven reduction of these pathways (Rogalski *et al.*, 2020a). The efficient utilization of maltose appears to be most crucial for inter- and intra-species competition in the sourdough. Dominant *F. sanfranciscensis* strains like TMW 1.1150 and TMW 1.1221 are unable to use other carbohydrates apart from maltose and glucose as energy sources. In addition, the dominant strains only have *mapB* with a constitutive promotor as maltose is constantly available in the sourdough fermentation. A second maltose operon with an inducible promotor like the one of *mapA* can be more an obstacle than an advantage in the fast conversion of maltose, because time and energy goes by in the induction of a second maltose promotor. This behavior can be seen in the strains, which are unable to compete in any sourdough fermentation (Rogalski *et al.*, 2020c,a).

F. sanfranciscensis is a facultatively aerobe bacterium. Thereby, it is tolerant to oxygen, which is advantageous in the sourdough as to the refreshment of the dough during backslopping oxygen is present (Mihhalevski *et al.*, 2011; De Vuyst *et al.*, 2014). The growth of *F. sanfranciscensis* in CDM was increased by an oxic environment (Rogalski *et al.*, 2020a). Furthermore, the utilization of maltose was increased in *F. sanfranciscensis* TMW 1.392 and TMW 1.2138 (Rogalski *et al.*, 2021), which is in case of *F. sanfranciscensis* TMW 1.392 an advantage in sourdoughs without yeasts. Although, in the sourdough with yeast the oxygen is rapidly depleted and *F. sanfranciscensis* TMW 1.392 is not dominant at all. Oxygen has no influence on the dominant *F. sanfranciscensis* strain TMW 1.1150, which again shows its perfect adaption to the niche (Rogalski *et al.*, 2021). As all *F. sanfranciscensis* strains contain the NADH oxidase *nox2* in their genome the metabolism of oxygen can be a useful relict out of an oxic ancestral origin (Rogalski *et al.*, 2020a).

The former niche of *F. sanfranciscensis* is predicted to be a sugary- especially maltose or fructose rich-oxic environment, which can be found in plants or insects. Still, the patchy metabolic pathways and the partial reduction of the pentose metabolism as well as restrictions in energy gain from hexose metabolism shows an ongoing adaption to the niche sourdough. For example, fumarate respiration no longer enabled, and the respective pathways in *F. sanfranciscensis* lack the succinate dehydrogenase. It is therefore impossible to recycle NAD out of malate, which may have been an important reaction in association with fruits or flowers, but rather useless in a sourdough system. When *F. sanfranciscensis* was found in spontaneous sourdough fermentation it only occurs when it had been inoculated with flowers (Gänzle and Ripari, 2016). Besides, other closely related members of the *Fructilactobacillus* group were found in insect frass and flowers, which gives a hint on the origination of *F. sanfranciscensis* (Zheng *et al.*, 2020). The oxygen tolerance can be an advantage when the sourdough is inoculated by fruit flies. These fruit flies can function as vector driven inoculation of sourdoughs from flowers. Actually, fruit flies are attracted by esters produced by yeast, as has been demonstrated in detail for yeasts in lambic beers, which harbor a consortium of LAB and yeasts (Christiaens *et al.*, 2014). Alternatively, its small genome of less than 2 Mbp and a GC content less than 40% and its extremely restricted metabolism fits to insect endosymbionts (Zheng *et al.*, 2015; Filannino *et al.*, 2016; Duar *et al.*, 2017; Gänzle and Zheng, 2019). In conclusion, the genomic equipment of *F. sanfranciscensis* displaying a relation to sugary-oxic environments matches the hypothesis that it is originating from flowers or insects.

6.2 There are strain-specific differences of *F. sanfranciscensis* regarding the competitiveness in the sourdough system

To monitor strain-specific differences of *F. sanfranciscensis* in the sourdough a strain-specific differentiation system was developed. It was important that the strain differentiation was also possible in the sourdough to monitor their behavior during the sourdough fermentation. One suitable approach was to use the length polymorphism of the CRISPR-locus of the 24 *F. sanfranciscensis* strains. 90.5% contain the CRISPR-Cas II-A system with 12 different genotypes. This property was suitable for a strain-specific differentiation system (Rogalski *et al.*, 2020b). For the *F. sanfranciscensis* strain TMW 1.1597, a strain without any CRISPR-System a strain-specific PCR set was developed. Based on this system the strain-specific competitiveness in the sourdough was monitored. Two groups with four different strains out of 24 were selected. The competitiveness of each strain in each group was detected regarding the presence of the yeasts *K. humilis* or *S. cerevisiae* or the absence of yeast. With this approach it was possible to sort the eight strains in three different groups:

Group A: The strains TMW 1.1150, TMW 1.1221 and TMW 1.1597 were dominant independently of the yeast's presence or absence.

Group B: The strains TMW 1.392, TMW 1.907 and TMW 1.2137 were only competitive with a specific yeast species.

Group C: Two strains the TMW 1.2138 and TMW 1.726 were not dominant at all independently of the presence or absence of a specific yeast (Rogalski *et al.*, 2020c).

This approach demonstrated that there are strain-specific differences regarding the competitiveness of the strains in the sourdough. These different interactions reside in the different genetic equipment of the *F. sanfranciscensis* strains.

The genome analysis of 24 *F. sanfranciscensis* strains showed an ANI value identity of at least 99.96%. Although only 43.14% of the pan genome belong to the core genome of the 24 strains, which is explained by a broad variability of strain- and group-specific genes (Rogalski *et al.*, 2020a). Sourdough is a stressful environment with a specific nutritional offer, low pH and variable oxygen availability (Jänsch *et al.*, 2007; Stetina *et al.*, 2014; De Vuyst *et al.*, 2016; Van Kerrebroeck *et al.*, 2017). A sourdough-adapted usage of available carbohydrates as well as properties to combat the redox stress are crucial to live in the niche sourdough. Therefore, species-specific differences were found firstly in the usage of carbohydrates, secondly in the usage of external electron acceptors, and in limited mechanisms to combat stress (Rogalski *et*

al., 2020a). There are no differences in the cysteine transporter or the glutathione reductase or the thioredoxin reductase (Table A 3; Table A 4). As there is no strain diversity in these systems, these mechanisms appear to be crucial for living in this niche. The eight *F. sanfranciscensis* strains of the competitive trials differ in their genomic equipment in the usage of carbohydrates for their metabolism. Genetic studies show a well-marked maltose metabolism with some strain-specific differences. The maltose phosphorylase B (*mapB*) is common to most of the strains. It has a constitutive promoter, and the maltose phosphorylase A (*mapA*) is only harbored by several strains, and most of them have this system in addition. The *mapA* has an inducible promoter. Although, two maltose phosphorylase systems do not guarantee a success in competitive trials against other strains. All strains from group A (dominant) have only *mapB*. The strains from group C have both maltose phosphorylase systems as well as the strains from group B, which are only dominant with *S. cerevisiae* (*F. sanfranciscensis* TMW 1.907 and TMW 1.2137) (Rogalski *et al*., 2020c,a). The metabolic turnover of four of these strains with at least one strain of each group was measured with different carbohydrate sources. It could be seen that *F. sanfranciscensis* TMW 1.1150 had the highest metabolic turnover of maltose of all tested strains followed by distance by *F. sanfranciscensis* TMW 1.392. Furthermore, *F. sanfranciscensis* TMW 1.1150 had the highest turnover of glucose (Rogalski *et al*., 2021). These two sugars are the most abundant in the sourdough fermentation, as they are constantly produced by the flour amylases (Corsetti and Settanni, 2007). This strong sugar metabolism can explain its dominance in all sourdough systems compared to the other tested strains. *F. sanfranciscensis* TMW 1.2138 was the slowest in the maltose fermentation and had a lack in the glucose consumption, which was observed by Rogalski *et al.* (2020a). During the backslopping in the sourdough fermentation only fast-growing strains were able to get into the new batch with high numbers to combat other *F. sanfranciscensis* strains and other members of the flour microbiota. *F. sanfranciscensis* TMW 1.2138 with its slow fermentation of maltose is then overgrown by the fast *F. sanfranciscensis* TMW 1.1150 strain. Although, *F. sanfranciscensis* TMW 1.2138 was isolated from a sourdough and thus it needs to be able to persist in this niche. As for many strains reported in literature it remains unknown whether it had reached high or relevant numbers in that respective dough. *F. sanfranciscensis* TMW 1.726 is genetically the closest to *F. sanfranciscensis* TMW 1.2138. This strain shows the same behavior in the sourdough like TMW 1.2138 and it is assumed that it is also slow in the maltose fermentation (Rogalski *et al*., 2020c).

One explanation for the different behavior of the strains related to the different cell sizes of the strains. The cells of *F. sanfranciscensis* TMW 1.1150 are significant smaller than of TMW 1.2138. Cells with a higher surface to volume ratio are more efficient in transport and subsequent metabolism, which can be one explanation (Harris and Theriot, 2018; Rogalski *et al.*, 2021). Furthermore, a different regulation of the maltose operon in the *F. sanfranciscensis* strains can be the reason for the consumption rate, but this regulation needs to be further examined e.g., by transcriptomics as expression rates may tell another story than just presence and absence of genes.

The usage of other carbon-sources for the metabolism like fructose and sucrose is not crucial for strain dominance in the sourdough. *F. sanfranciscensis* TMW 1.1150 and TMW 1.1221 were dominant in all sourdoughs without a functional fructokinase gene. The fructokinase gene is crucial for the fructose metabolism as it converts fructose into fructose-6-phosphate, which is part of the phosphoketolase pathway (Rogalski *et al.*, 2020a). Two strains with a functional fructokinase gene were applied in the competition trials in the sourdough. One of them, *F. sanfranciscensis* TMW 1.1597, was dominant in the sourdough trials whereas TMW 1.392 was only dominant without any yeast. The same occurrence was observed with the consumption of sucrose for the metabolism. *F. sanfranciscensis* TMW 1.392 was able to ferment sucrose but is not dominant in all sourdough fermentations. Whereas *F. sanfranciscensis* TMW 1.1150, TMW 1.1221 and TMW 1.1597 (all dominant) were not capable of the fermentation of sucrose. In conclusion, neither the usage of fructose nor sucrose for the energy metabolism is crucial for the strain dominance in the sourdough (Rogalski *et al.*, 2020c,a).

To sum it up, there are strain-specific differences in *F. sanfranciscensis* regarding the competitiveness in the sourdough. Moreover, the efficient consumption of maltose and eventually glucose is crucial for the strain dominance in the sourdough fermentation. The consumption rate of maltose is strain-specific and dependent on the cell size. There is a clear competition between the *F. sanfranciscensis* strains for the most common carbon-sources like maltose and glucose.

6.3 The strain-specific competitiveness of *F. sanfranciscensis* is influenced by the presences/absence of sourdough yeasts

The competitiveness of *F. sanfranciscensis* TMW 1.392, TMW 1.907 and TMW 1.2137 was influenced by the presence and absence of a specific yeast. *F. sanfranciscensis* TMW 1.392 was dominant together with TMW 1.1150 in the sourdough system without yeast inoculation for 10 days. Whereas TMW 1.907 and TMW 1.2137 were dominant in the sourdough with the yeast *S. cerevisiae* TMW 3.1064 (Rogalski *et al.*, 2020c).

In metabolic studies it was shown that *F. sanfranciscensis* TMW 1.392 is not as effective as TMW 1.907 and TMW 1.1150 in the metabolism of maltose. Still, it has the most diverse spectra in the fermentation of carbohydrates and external electron acceptors (Rogalski *et al.*, 2020a; Rogalski *et al.*, 2021). In intra-species competition without the presence of yeasts *F. sanfranciscensis* TMW 1.392 can use the different carbohydrates to compete with strains like *F. sanfranciscensis* TMW 1.1150 (as explained above). Although, in the presence of yeast, sucrose, fructose and oxygen is depleted by these and not further available for *F. sanfranciscensis* TMW 1.392, which is why it is not able to compete anymore in the sourdough (Rogalski *et al.*, 2021).

The situation is different for *F. sanfranciscensis* TMW 1.907. It was generally stimulated by the presence of yeasts especially from *S. cerevisiae* TMW 3.1064 (Rogalski *et al.*, 2021). Several studies assume that *F. sanfranciscensis* can by stimulated by carbon dioxide, a nitrogen flow or a general growth factor form the yeast (Ponomarova *et al.*, 2017; Sieuwerts *et al.*, 2018), which may explain this behavior.

These examples illustrate that the reasons for the strain-specific dependence on the presence/absence of yeasts are strain-dependent.

6.4 The interaction between *F. sanfranciscensis* and the yeasts *K. humilis* and *S. cerevisiae* is commensal or competitive, respectively

The interaction of *F. sanfranciscensis* with yeasts affects the environmental conditions of the sourdough through their metabolism, which exerts direct effects through consumption and formation of compounds. Firstly, there is a yeast-dependent competition for carbon-sources present in the sourdough (Stolz *et al.*, 1993). Secondly, metabolites of the yeasts could be used by *F. sanfranciscensis* strains and *vice versa* (Vrancken *et al.*, 2010; De Vuyst *et al.*, 2016). Thirdly, the different metabolic products of yeasts and *F. sanfranciscensis* influence the quality of the sourdough (Huys *et al.*, 2013). Subsequently, indirect effects including changes in the pH and redox potential can positively or negatively affect the behavior of yeasts and bacteria in the sourdough. It is therefore rather founded that interaction resides in different combinations of parameters than based on one mechanism. Figure 5a tries to summarize project these effects for specific *F. sanfranciscensis* strains and yeasts in a scheme, which is based on the interaction categories depicted in Figure 1. Still, from the data it becomes clear that the simplified categorization into such categories cannot hold true at strain level. Therefore, Figure 5b details the findings on the influence of bacterial and yeast partners by depicting estimated ranges of positive of negative interaction between strain-specific combinations. Some combinations exert solely negative effects on each other, which could be interpreted as competitions, as summarized in Figure 5a for *F. sanfranciscensis* TMW 1.392 and *S. cerevisiae*. The same *F. sanfranciscensis* strain finds a commensal partner in *K. humilis*, which leaves it nearly unaffected. *F. sanfranciscensis* TMW 1.1150 appears in a commensal relationship with both yeasts. *F. sanfranciscensis* TMW 1.907 even benefits from *S. cerevisiae*, which could be interpreted as commensalism if not parasitism, and *F. sanfranciscensis* TMW 1.2138 suffers from the presence of any of the yeasts.

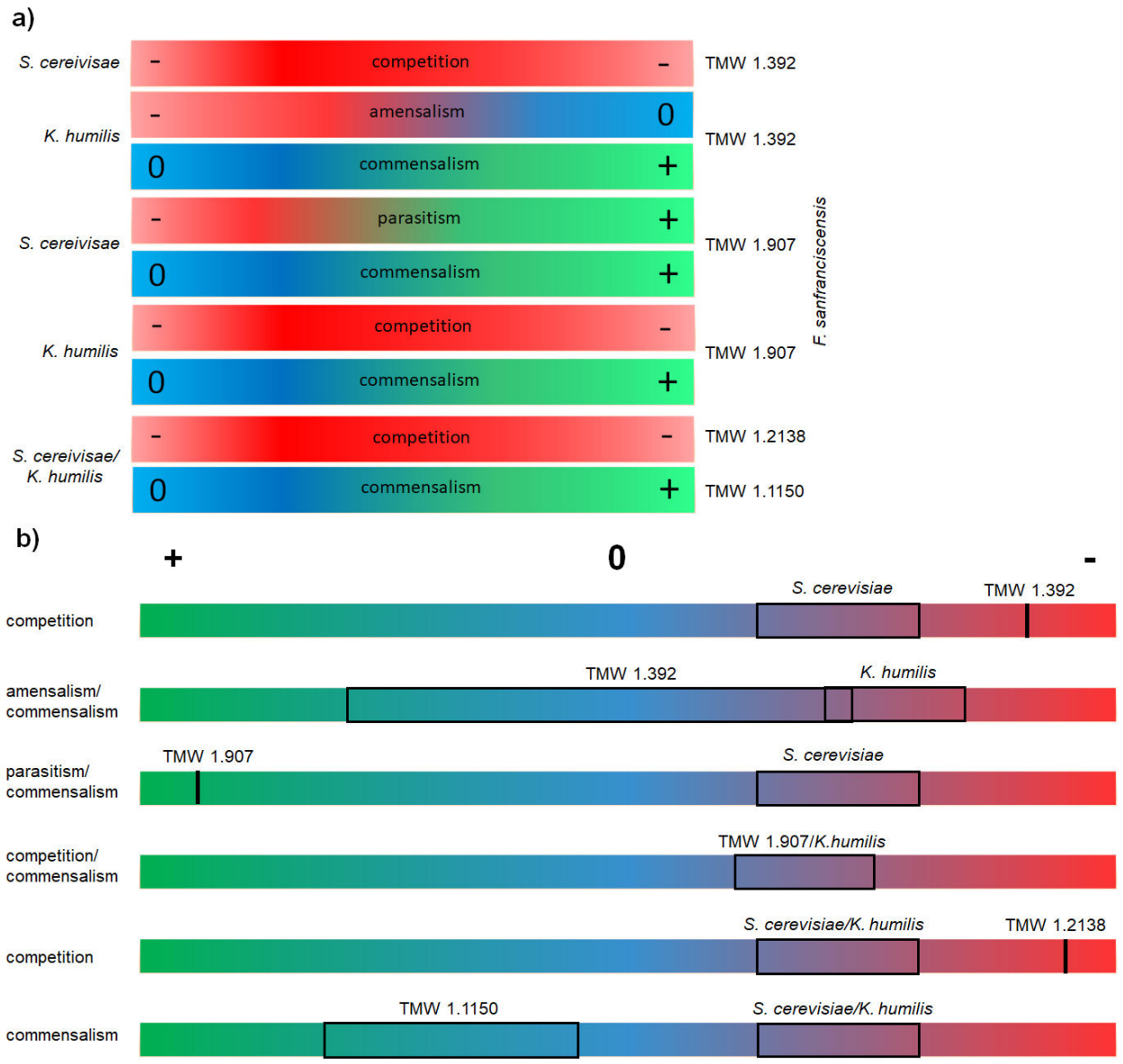

Figure 5: Strain-specific interactions of *F. sanfranciscensis* with the yeasts *S. cerevisiae* and *K. humilis*. The different forms of interactions between the *F. sanfranciscensis* strains TMW 1.907, TMW 1.392, TMW 1.1150 and TMW 1.2138 and the yeasts *K. humilis* and *S. cerevisiae* are displayed as bars or strokes. Plus: positive effect; Minus: negative effects; Zero: No effect.

In the following these interpretations are referred to the strain-specific metabolic differences of *F. sanfranciscensis* in relation to the two yeasts.

A broad variety of metabolic effects could lead to the assumption of a mutualism between *F. sanfranciscensis* and the yeasts *K. humilis* and *S. cerevisiae* in the sourdough. In a mutualism both species profit from each other. In sourdoughs with *F. sanfranciscensis* TMW 1.1150, TMW 1.907 or TMW 1.392 the acetate level was significantly higher with yeasts in the

sourdough and thus the FQ was lower (Rogalski *et al.*, 2021). This fact is explained by the cleavage of glucofructans by yeasts and the resulting free fructose. The fructose can so be used as external electron acceptor by *F. sanfranciscensis*. The fructose is converted to mannitol by the recycling of NAD^+. Therefore, the recycling of NAD^+ with the alcohol dehydrogenase is obsolete. Instead, one ATP is yielded by the formation of acetate with the acetate kinase (Stolz *et al.*, 1993; Vogel *et al.*, 2002; Rogalski *et al.*, 2020a). In turn, the yeasts, namely the maltose-negative *K. humilis* benefit from released glucose from the maltose phosphorylase reaction. However, this apparently clear mutualism is concomitant with the formation of acetate, which obviously counteracts these beneficial interactions. Other effects on the redox system of the dough may further contribute to the alleviation of any stimulatory effect. Taken together, the previously presumed mutualism exists but is not reflected in growth benefit upon strain-specific tracking and should rather be related to a commensal relationship of these specific strains to the yeasts. *F. sanfranciscensis* strains like TMW 1.907 and TMW 1.2137 profit from the combination with *S. cerevisiae* TMW 3.1064 (Figure 5b). They are now able to compete against other *F. sanfranciscensis* strains in the sourdough (Rogalski *et al.*, 2020c). Actually, recent studies showed that LAB can be positively influenced by *S. cerevisiae* with carbon dioxide, not classified growth factors or nitrogen (Ponomarova *et al.*, 2017; Sieuwerts *et al.*, 2018). In these combinations *F. sanfranciscensis* clearly profit from the combination with the yeasts. Although, there need to be positive benefits for the yeasts in the sourdough otherwise the interaction would be classified as commensalism or even parasitism (Figure 1; Figure 5). The maltose-positive *F. sanfranciscensis* is often found in the combination with the maltose-negative *K. humilis*. In this combination there is no competition for the carbohydrate maltose. Moreover, the cleavage of glucofructans by the yeast results in fructose (as mentioned above) and glucose. The glucose is then metabolized by *K. humilis*. Furthermore, the growth conditions of *F. sanfranciscensis* and *K. humilis* are similar (Gänzle *et al.*, 1998). The occurrence in the same sourdough can be a coincidence because of supplementary requirements. Furthermore, there is no clear benefit for the yeast although no negative effects, which speaks more for commensalism in this combination (Figure 5).

The yeasts *K. humilis* and *S. cerevisiae* are well adapted to the niche sourdough. They are pH- and acetic acid-tolerant and possess mechanisms to survive in the high acid and acetic acid surrounding produced by LAB especially by *F. sanfranciscensis*. They also need to deal with other anti-fungal substances like phenyl lactic acid (PLA) produced by *F. sanfranciscensis* (Vermeulen *et al.*, 2006; Axel *et al.*, 2016; Zhang *et al.*, 2019). In sourdough trials it can be observed that the interaction with *F. sanfranciscensis* leads to a decrease of the cell count of the

yeasts (Carbonetto *et al.*, 2020). In addition, when *F. sanfranciscensis* TMW 1.907 and *S. cerevisiae* TMW 3.1064 or *K. humilis* TMW 3.1034 were applied as starter in the sourdough the yeasts grow to the same cell count as *F. sanfranciscensis*. In normal sourdoughs the ratio between yeasts and LAB is between 1:10 and 1:100 (Ottogalli *et al.*, 1996; Rogalski *et al.*, 2020c). With the decrease of the pH and the adaption of *F. sanfranciscensis* to the sourdough environment the yeast cell counts decrease. Consequently, the cell count of the yeasts in the sourdough is controlled by *F. sanfranciscensis* (Carbonetto *et al.*, 2020; Rogalski *et al.*, 2020c). In the same setup with *F. sanfranciscensis* TMW 1.392 instead of TMW 1.907 the presence of *S. cerevisiae* leads to a worse competition against other sourdough microbiota and enterobacteria (Rogalski *et al.*, 2020c).

F. sanfranciscensis in combination with *S. cerevisiae* and other LAB compete for carbohydrates like maltose. In *F. sanfranciscensis* the maltose transported into the cell and cleaved by the maltose phosphorylase into glucose and glucose-1-phosphate. In stress situations the glucose is excreted in a 1:1 ratio (Stolz *et al.*, 1993; Rogalski *et al.*, 2020c). When maltose is depleted also by other organisms than *F. sanfranciscensis,* it is under stress. The massive excretion of glucose leads to the glucose repression in *S. cerevisiae* and other maltose fermenting LAB (Kayikci and Nielsen, 2015). Also, other metabolites like sucrose and oxygen are metabolized by *S. cerevisiae,* which are therefore not available for *F. sanfranciscensis*. In *F. sanfranciscensis* TMW 1.392 leads the lack of sucrose and oxygen to a decrease in its competitiveness against other *F. sanfranciscensis* strains (Pringle, 2016; Rogalski *et al.*, 2020c).

Both *F. sanfranciscensis* and the yeasts deal with the harsh sourdough environment, but they are apparently not depending on each other. Both species can compete alone in the dough even a dough without LAB, which is no sourdough (Brandt and Gänzle, 2006). The interaction of these species is facultative. The carbohydrate-dependent mutualism is more a commensalism as the yeast does not benefit from it (Figure 5)(Pringle, 2016). Still some *F. sanfranciscensis* strains profit from the stress induced by *S. cerevisiae* as they are able to compete in the sourdough against other strains. Although, benefits are limited and negative effects of the combination are well reported (Carbonetto *et al.*, 2020; Rogalski *et al.*, 2020c; Rogalski *et al.*, 2021). In conclusion, rather a commensalism/competition exists between *F. sanfranciscensis* and the yeasts *K. humilis* and *S. cerevisiae* than a mutualism as the negative effects and stress predominates (Figure 5). Although it should be noted that stress is useful for some strains in an intra-species competition.

7 Conclusion and outlook

In conclusion, the interactions between *F. sanfranciscensis* and the yeast *S. cerevisiae* and *K. humilis* are highly complex. The present study shows that the strains of *F. sanfranciscensis* are divers in their carbohydrate and redox metabolism and that these differences influence their competitiveness in combination with the occurrence of yeasts like *S. cerevisiae* and *K. humilis*. Furthermore, the genetic equipment of *F. sanfranciscensis* relates to a sugary-oxic origin as it is found in/on flowers and insects. Further studies are needed to find the origin of this species. It may be searched for insect origin and flowers. It is important to isolate live strains and not only rely on sequencing studies. Furthermore, it is interesting how *F. sanfranciscensis* is able to colonize an existing dough with an existing stable microbiota and how long it takes for the different strains to be dominant in this microbiota, and how many cells of the strains are important for the strain to colonize. Furthermore, this thesis gives a hint on the nature of the interaction/symbiosis of these species. The results of this thesis reveal a rather commensal or competitive than mutualistic interaction of these species. These could also be proven in further experiments like transcriptomic studies where the expression of specific genes can be observed in combination with yeasts. These studies might also explain why specific strains are more dominant in the sourdough than others, or why maltose metabolism is highly effective with only MapB. For example, the genetic equipment in the metabolism of maltose does not give a hint on why one strain is faster and more effective in the turnover of maltose. The tolerance against pH and acetate could further highlight intra-species competition of *F. sanfranciscensis* because *F. sanfranciscensis* does not contain any usual mechanisms against pH stress like the GABA and glutamine/glutamate or ADI pathway it is highly acid tolerant down to pH 4.

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Parts of this work were published in peer-reviewed journals. The details are listed in the Chapter List of publications and student's projects.

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9 List of publications and student's projects

9.1 Publications

Rogalski, E., Vogel, R.F., Ehrmann, M.A., 2020. Monitoring of *Lactobacillus sanfranciscensis* strains during wheat and rye sourdough fermentations by CRISPR locus length polymorphism PCR. International journal of food microbiology 316, 108475.

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9.2 Presentations at academic symposia

Rogalski, E. (2019). CRISPR-tracking von *Lactobacillus sanfranciscensis* Stämmen in Weizen- und Roggensauerteigen. 20.5-21.5.2019, GDL-Forum Sauerteig VI, Minden. Oral presentation.

Rogalski, E. (2019). Probing the performance of *Lactobacillus sanfranciscensis* strains in wheat and rye sourdough by using CRISPR-locus length polymorphism. 26.05-30.05.2019, BAGECO, Lisbon. P72. Poster presentation.

9.3 Student's projects

Gerhard Schwaiger – Internship: Physiologische Charakterisierung von *Lactobacillus lindneri*. 23.04.2018 - 28.05.2018.

Patrick Auer – Internship: Interaktionen zwischen *Lactobacillus sanfranciscensis* und den Hefen *Saccharomyces cerevisiae* und *Kazachstania humilis*. 03.02.2020 - 28.02.2020.

10 List of abbreviations

a-	arabinose
-a	without acetate
AFLP	amplified fragment lenght polymorphism
ANI	average nucleotide identity
approx.	approximately
ATP	adenosine triphosphate
B.C.	Before Christ
BADGE	BlAst Diagnostic Gene findEr
BLAST	Basic Local Alignment Search Tools
BMEL	German Ministry of Food and Agriculture
BRIG	Blast Ring Image Generator
-c	without citrate
C.	*Candida*
Cas	CRISPR associated genes
CDM	chemical defined media
cfu	colony forming units
Cl.	*Companilactobacillus*
CLLP	CRISPR locus length polymorphism
cr	CRISPR
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DGGE	denaturation gradient gel electrophoresis
DMGs	diagnostic marker genes
DY	dough yield
EC	enzyme commissions
EPS	exopolysaccharides
EU	European Union
f	fructose
F.	*Fructilactobacillus*
FQ	fermentation quotient
g	glucose
gc	Na-gluconate
GI	glycemic index

GO	Gene Ontology
HCCA	α-cyano-4-hydroxycinnamidacid
HPLC	high performance liquid chromatography
K.	*Kazachstania*
KONSTABLE	Use of biological diversity for establishment of stable sourdough consortia
L.	*Lactobacillus*
LAB	lactic acid bacteria
Lc.	*Lacticaseibacillus*
Le.	*Levilactobacillus*
levS	levansucrase
Li.	*Limosilactobacillus*
Lp.	*Lactiplantibacillus*
Lt.	*Lentilactobacillus*
m	maltose
MALDI	matrix-assisted laser desorption/ionization
map	maltose phoysphorylase
mdh	mannitol dehydrogenase
MEGA	Molecular Evolutionary Genetics Analysis
mfg	maltose fructose glucose
MLST	multi locus sequence typing
mMRS	modified DeMan Rogosa and Sharpe
MS	mass spectrometry
mt	malate
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogen
NCBI	National center for Biotechnology Information
nt	nucleotide
-p	without pyrimidines
P.	*Pediococcus*
PAM	protospacer adjacent motif, protospacer adjacent motif
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PLA	phenyl lactic acid

r	ribosomal, ribose
RAPD	randomly amplified fragment lenght polymorpic DNA
RAST	Rapid Annotation Subsytem Technology
rbsD	ribose-pyranase
s	sucrose
S.	*Saccharomyces*
T.	*Torulaspora*
ToF	time of flight
tracrRNA	trans activating CRISPR RNA
UPGMA	unweighted pair group method
W.	*Weissella*
x	xylose
YPG	yeast peptone glucose

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12 Appendix

12.1 Publication 1

S 1: Genomic context of the CRISPR-Cas system Type II-A in *L. sanfranciscensis* strains.

	Position of CRISPR-Cas system						
Strain	**contig**	**start**	**contig**	**end**	**orf downstream**	**dir.**	**CRISPR-Cas system**
DSM20451	1	161028	1	167909	nicotionamid mononucleotide transporter	+	Type II-A/a
TMW 1.54	1	163708	1	169939	citrate:sodium symporter	+	Type II-A/b
TMW 1.392	1	161175	1	167472	citrate:sodium symporter	+	Type II-A/b
TMW 1.640	2	109516	2	116680	nicotionamid mononucleotide transporter	+	Type II-A/a
TMW 1.726	11	3028	11	9982	nicotionamid mononucleotide transporter	+	Type II-A/a
TMW 1.907	19	11089	19	17912	nicotionamid mononucleotide transporter	+	Type II-A/a
TMW 1.1150	1	160724	1	168405	citrate:sodium symporter	+	Type II-A/b
TMW 1.1152	1	66167	1	58750	citrate:sodium symporter	-	Type II-A/b
TMW 1.1154	1	66427	1	58746	citrate:sodium symporter	-	Type II-A/b
TMW 1.1221	3	65270	45	3798	citrate:sodium symporter	+	Type II-A/b
TMW 1.1304	1	61024	1	54787	citrate:sodium symporter	-	Type II-A/b
TMW 1.1470	1	159948	1	168097	citrate:sodium symporter	+	Type II-A/b

TMW 1.1730	1	160468	1	166705	citrate:sodium symporter	+	Type II-A/b
TMW 1.2137	1	72097	1	65737	nicotionamid mononucleotide transporter	-	Type II-A/a
TMW 1.2138	13	8332	13	1378	nicotionamid mononucleotide transporter	-	Type II-A/a
TMW 1.2139	1	71971	1	65677	nicotionamid mononucleotide transporter	-	Type II-A/a
TMW 1.2140	13	3028	13	9982	nicotionamid mononucleotide transporter	+	Type II-A/a
TMW 1.2142	1	72097	1	65737	nicotionamid mononucleotide transporter	-	Type II-A/a
TMW 1.2314	1	160468	1	166705	citrate:sodium symporter	+	Type II-A/b

S 2: Comparison of Cas and Csn2 protein sequences of related CRISPR type II-A systems.

Organism	**GenBank accession no.**	**Similarity (%)**			
		Cas9	Cas1	Cas2	Csn2
L. sanfranciscensis TMW 1.1304	AEN99270.1	100	100	100	100
L. sanfranciscensis	WP_103423054.1	98.95	99.34	99.01	99.55
Lactobacillus lindneri TMW 1.2007	GCA_002907115.1	79.00	75.42	88.12	55.00
Lactobacillus buchneri NRRL B-30929	CP002652.1	65.70	69.40	70.00	38.00
Lactobacillus florum	WP_035421986.1	59.21	73.09	85.15	49.09

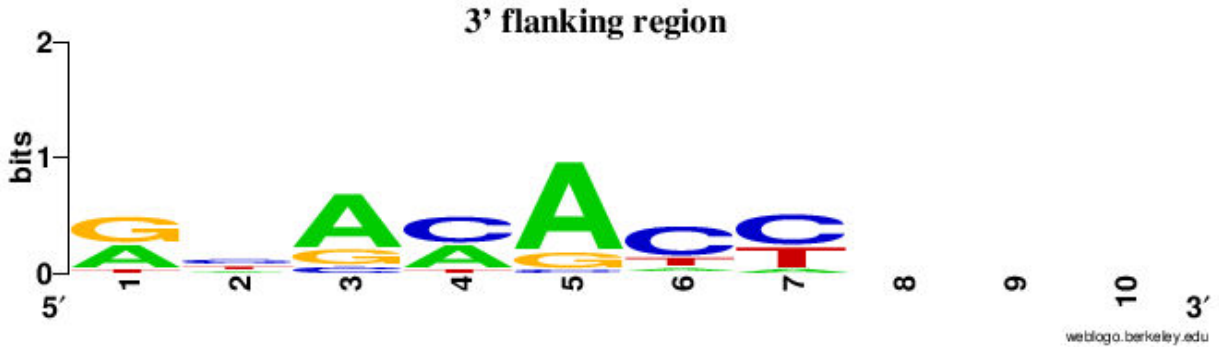
S 3: Protospacer sequences of *L. sanfranciscensis* spacer which match with virus sequences.

Strain	**Spacer**	**Coverage**	**Protospacer match (accession-nr.)**	**Organism**	**Annotation**	**Sequence (5'-3')**		
						5' flanking sequence	Protospacer	3' flanking sequence
1.53	1	29/29	LN885237.1	*Lactobacillus phage EV3*	putative endolysin	TCAGACCAAA	TTTATCAAGGTCAAAATATGGGATTAAGA	GAATGCACGA
1.53	10	29/30	CP031181.1	*L. brevis*	plasmid mobilization relaxosome protein MobC	ACCAATAAAA	ACGGTGCTAGCGCCGCTAGTGCGTTGAACT	GGCTCACGTA
1.54	2	29/31	AB024514.1	*L. plantatum plasmid pLTK2*	hypothetical protein	CTTTATCAAA	CGTGGTTCTGTAAGAGTCGGCTATGTCCTTT	GTGCAACTGG
1.640	4	29/30	LN885237.1	*Lactobacillus phage EV3*	putative head-tail joining protein	TAGCGTTATC	TTCACGGGTGTGTTCAACGAAGTGATCGCC	ACGCATCTTC
1.726	3	29/30	LN885237.1	*Lactobacillus phage EV3*	phage minor tail protein	TTAGTTAAGG	TTGAAGCCACTGGCCTGAACCAGCTAAGCA	AAGCACTGGA
1.726	10	30/30	LN885237.1	*Lactobacillus phage EV3*	phage integrase	TTTTGGTGTG	CTTTTGGTGTGTCAACTTTTAAACTTCTAT	AAACACAGAC
1.726	12	30/30	LN885237.1	*Lactobacillus phage EV3*	hypothetical protein	ATGGTATTGA	AAGTTATCAAAACAGCAATCAAGTTAATTC	AAACACTAAT

1.907	2	30/30	LN885237.1	*Lactobacillus phage EV3*	No ORF	TGTCTGTGTT	TATAGAAGTTTAAAAGTTGACACACCAAAA	GCACACCAAA
1.907	6	29/30	LN885237.1	*Lactobacillus phage EV3*	prophage tail, putative lysozyme	TTCATCTGAA	ATAGTTGGAAGTTGATGTGGAATTCCTTTA	GTCCACTTCT
1.907	10	30/30	LN885237.1	*Lactobacillus phage EV3*	hypothetical protein	CCTATCGCAT	TACTAAAAGAACTGCCAACAAAATATAAGG	TTACACCCAG
1.1150	3	30/30	LN885237.1	*Lactobacillus phage EV3*	phage tail protein	TGATTCAGTA	ACTAAAACAACCGTCTTGCTATTATCATTA	ACAAATTCCG
1.1150	5	28/30	CP014907.1	*L. lindneri*	phage major capsid protein	TCAACTAATG	TTGGAGCTGGTGCTTTTGAAAATGATACTA	ATAAAGTACG
1.1150	10	29/30	LN885237.1	*Lactobacillus phage EV3*	phage terminase large subunit	ATTCTCAACT	TTGCCAAGTTAGTGCCGGATGTGGATACTG	GTAAACCGGT
1.1221	23	30/30	CP031181.1	*L. brevis plasmid pUCCLB556_G*	relaxase	ACGCATCACG	CAATCTTTTGCCTTGAACGAGTTAGACCCT	ACGAATCCAT
1.1470	6	29/30	LN885237.1	*Lactobacillus phage EV3*	putative endolysin	TGCACGATTA	TATTTGGTTAGAAACAGGCGCAGACCAAGC	GCAAACTAAA
1.1470	8	28/30	LN885237.1	*Lactobacillus phage EV3*	phage minor tail protein	ACCCCTTTAG	TCAGGATTTGTGTCTGATTGACACTATGCG	TCAAGCCATA

1.1470	23	29/30	LN885237.1	*Lactobacillus phage EV3*	phage integrase	ACTTCAAACG	CAGTAAATAAGGTTCTGAGAGAAAGTTTAA	GCAAGCTTAA
1.1470	25	29/30	LN885237.1	*Lactobacillus phage EV3*	putative helicase	AAAGATGGTT	TAGATATTGTTTATGAAGATATGGTGGAAG	GTAAATCTGT
1.2137	4	29/30	LN885237.1	*Lactobacillus phage EV3*	putative head-tail joining protein	ACGACGGCAC	CAAGAAGATGCGTGGCGATCACTTCGTTGA	ACACACCCGT

S 4: PAM sequence of the CRISPR-Cas Type II-A system of *L. sanfranciscensis*. 10 nt of the 3'-flanking region of 19 protospacers from viruses were aligned and visualized with WebLogo (Crooks *et al.*, 2004).



S 5: Nucleotide sequences and structural details of important elements in the CRISPR-Cas system Type II-A. Exemplarily the repeat spacer array of *L. sanfranciscensis* TMW 1.1150 spacer 10 was pictured with the corresponding protospacer (bold, center). The PAM sequence of the protospacer was in the 3'-flanking region. The predicted tracrRNA with structural details binds to the repetitive sequence of the array (bottom).

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Table A 1: Overview of research of properties of *F. sanfranciscensis.*

	Metabolism	**Reference**	**Proteins involved**	**Occurrence in Strains**
General properties	NaCl tolerance	(Gänzle *et al.*, 1998)		
	Acidification for activity of cereal enzymes	(Gänzle, 2014)		
	CO_2 production	(Brandt *et al.*, 2004)		
	Reduction of flavor active aldehydes	(Gänzle, 2014)		
Carbohydrate metabolism	Sugar metabolism in general	(Vogel *et al.*, 2011)	Maltose, Fructose, Ribose, Na-gluconate	
	Fermentation of Maltose	(Ehrmann and Vogel, 1998; Foschino *et al.*, 2001)	Maltosephosphorylase, Maltose MFS transporter, Phosphoglucomutase	24/24
	Fermentation of Glucose	(Foschino *et al.*, 2001)	Hexose	23/24
	Fermentation of Xylose	(Gobbetti *et al.*, 1999)		0/24
	Sucrose metabolism	(Korakli *et al.*, 2001; Tieking *et al.*, 2005b)	Levansucrase	8/24
	Production of Fructan	(Korakli *et al.*, 2003)	s.o.	6/24
	Malate metabolism	(Corsetti and Settanni, 2007)	Malate dehydrogenase	21/24
Cofactor recycling	Cofactor regeneration	(Hansen and Schieberle, 2005)		
	Mannitol metabolism	(Korakli *et al.*, 2000; Korakli	Mannitdehydrogenase	21/24

		and Vogel, 2003)		
	Citrate metabolism	(Corsetti and Settanni, 2007)	Citrate lyase, oxaloacetate-decarboxylase	22/24
	Electron acceptor alpha-Ketoglutarate	(Tanous *et al.*, 2005; Zhang *et al.*, 2010)	Glutamate dehydrogenase	0/24
			Citrate Permease	24/24
			Citrate lyase	21/24
			Oxalacetate decarboxylase	21/24
			Aspartate Aminotransferase	24/24
	Electron acceptor Oxygen	(Jänsch *et al.*, 2011)	NADH Oxidase	24/24
Stress metabolism	Generation of aroma compounds after stress exposure	(Guerzoni *et al.*, 2007)		
	Thiol exchange reactions	(Jänsch *et al.*, 2007)	Gluthatione reductase	22/24
	Stress response with cystine	(Stetina *et al.*, 2014)	Cystine transporter	24/24
	Stress response with thioredoxine	(Stetina *et al.*, 2014)	Thioredoxine, Thioredoxine reductase	24/24
	Leucin catabolism	(Serrazanetti *et al.*, 2011)		
	Peptidase activity	(De Angelis *et al.*, 2007)	PepT, PepQ, PepI, PepX, PepR, PepN, PepV	
	Synthesis of cyclopropane fatty acids	(Montanari *et al.*, 2010)		
	Acid stress response	(Serrazanetti *et al.*, 2011)	Leucin decarboxylation	
Amino acid metabolism	Phenylalanine metabolism and formation of PLA	(Vermeulen *et al.*, 2006)	Alpha-ketoglutarate aminotransferase, Dehydrogenase, Decarboxylase, Multi-enzyme complexes	

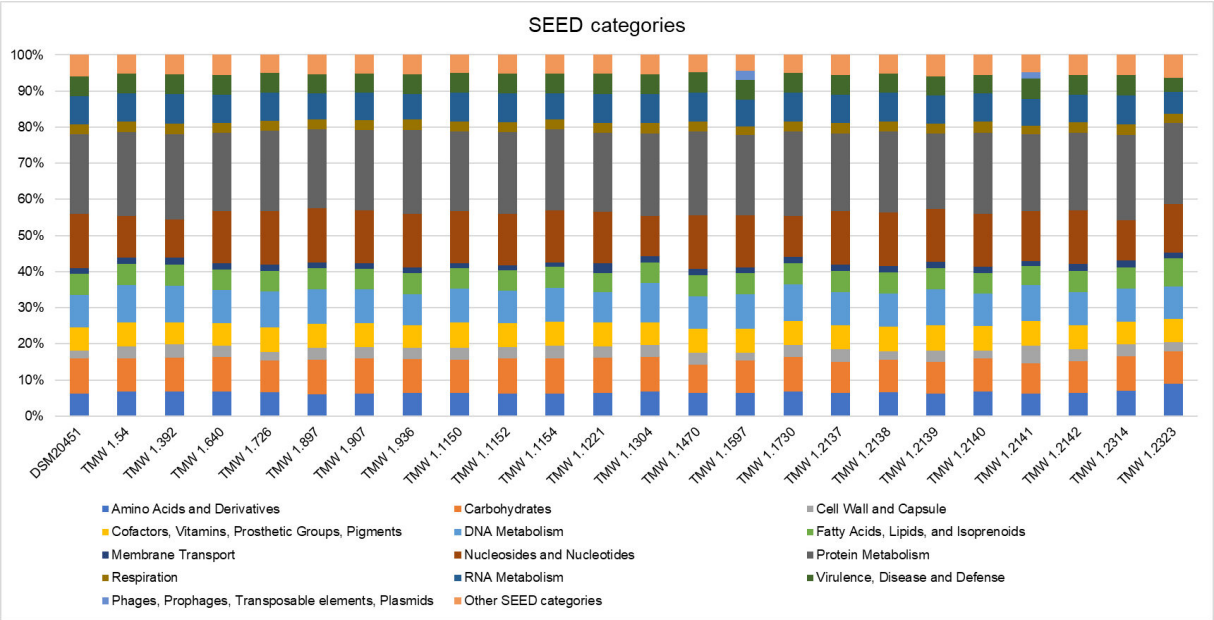

Figure A 1: The SEED categories calculated by RAST of the different *F. sanfranciscensis* strains.

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TMW 1.1304    1 MLYGSIEAGGTKFVCAVGDENFKVIDQTQFPTTTPDETLAKVIRYFNKFD     50
                :|||||||||||||||.|||||||||||||||||||||.|||
TMW 1.1150    1 -----MEAGGTKFVCAVGDENFNVIDQTQFPTTTPDETLAKVIRYFKKFD     45

TMW 1.1304   51 INAFGIASFGPIDVDKNSETYGWIIKTPKKGWSNIDFLGKMKESFNVPML    100
                |:|||||||||||||||||||||||||||||||
TMW 1.1150   46 IDAFGIASFGPIDVDKNSETYGWIIKTPKKGWSNI---------------     80

TMW 1.1304  101 WTTDVNGSAYGEYISAKKNDENVKSVAYITIGTGIGMGGVINGDFLGVMG    150
                                                       |||||||
TMW 1.1150   81 ----------------------------------------DFLGVMG     87

TMW 1.1304  151 TPEFGHIKVKRHRDDLDFKGICPWHGDCLEGVASGPTFEARDGIEGRQTP    200
                ||||||||||||||||||||||||||||||||||||||||||||||||||
TMW 1.1150   88 TPEFGHIKVKRHRDDLDFKGICPWHGDCLEGVASGPTFEARDGIEGRQTP    137

TMW 1.1304  201 INDPKWNIIAYYVAQAVVDLTVTFRPNKVVLGGGVCTPEFIAKVRAQFTL    250
                ||||||||||||||||||||||||||||||||||||||||||||||||||
TMW 1.1150  138 INDPKWNIIAYYVAQAVVDLTVTFRPNKVVLGGGVCTPEFIAKVRAQFTL    187

TMW 1.1304  251 LFNNYLSVGSLEKYITAPEIAHNGSATFGDFVLAKKALDEKDNI    294
                |||||||||||||||||||||||||||||||||||||||||||
TMW 1.1150  188 LFNNYLSVGSLEKYITAPEIAHNGSATFGDFVLAKKALDEKDNI    231
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Figure A 2: Comparison of the fructokinase enzyme of *F. sanfranciscensis* TMW 1.1150 and TMW 1.1304 with Clustal Omega.

Table A 2: Overview of genes involved in the energy-, cell wall and capsule-, carbohydrate-, organic acids-, nucleotides and nucleosides- and phosphorous metabolisms in *F. sanfranciscensis* strains.

metabolism	gene	amount	TMW 1.1150	TMW 1.1152	TMW 1.1154	TMW 1.1221	TMW 1.1304	TMW 1.1470	TMW 1.1597	TMW 1.1730	TMW 1.2137	TMW 1.2138	TMW 1.2139	TMW 1.2140
phosphoketolase pathway	glucose-6-phosphate dehydrogenase (g6pdh)	24	BGL46_04600	EQU37_04675	EQU01_03970	EQU42_02980	EQU35_04120	EQU36_05460	BGL45_05860	EQU06_03760	BGL37_04385	BGL38_03545	BGL39_04305	BGL40_05365
	6 phosphogluconate dehydrogenase (6pg-dh)	24	BGL46_02030	EQU37_01175	EQU01_01175	EQU42_02430	EQU35_02080	EQU36_01700	BGL45_00525	EQU06_02080	BGL37_02125	BGL38_01710	BGL39_02055	BGL40_00950
	phosphoketolase	24	BGL46_02550	EQU37_06075	EQU01_05795	EQU42_04125	EQU35_02565	EQU36_04495	BGL45_05065	EQU06_02625	BGL37_05365	BGL38_06140	BGL39_05150	BGL40_05935
	ribulose-phosphate 3-epimerase (rpe)	24	BGL46_01855	EQU37_01360	EQU01_01360	EQU42_01260	EQU35_01910	EQU36_01875	BGL45_00695	EQU06_01910	BGL37_01955	BGL38_01540	BGL39_01885	BGL40_01120
	Alcoholdehydrogenase (adhP)	24	BGL46_03625	EQU37_03485	EQU01_05040	EQU42_04190	EQU35_05190	EQU36_02655	BGL45_04655	EQU06_05105	BGL37_04905	BGL38_04755	BGL39_04825	BGL40_04800
	pyruvate kinase	24	BGL46_04095	EQU37_02650	EQU01_02200	EQU42_00685	EQU35_02865	EQU36_01415	BGL45_00200	EQU06_04680	BGL37_03635	BGL38_03210	BGL39_03545	BGL40_03185
	pyruvate oxidase	24	BGL46_05500	EQU37_04045	EQU01_03610	EQU42_01845	EQU35_04380	EQU36_06260	BGL45_05630	EQU06_04145	BGL37_04840	BGL38_04260	BGL39_04605	BGL40_04630
metabolism of cell wall compounds	alpha-phosphoglucomutase (pmg)	24	BGL46_03695	EQU37_03415	EQU01_04970	EQU42_04260	EQU35_05120	EQU36_02725	BGL45_04725	EQU06_05035	BGL37_04980	BGL38_04685	BGL39_04900	BGL40_04730

	glucose-6-phosphate isomerase (g6pi)	24	BGL46_01405	EQU37_01810	EQU01_01810	EQU42_05905	EQU35_01455	EQU36_02350	BGL45_01160	EQU06_01455	BGL37_01500	BGL38_01075	BGL39_01430	BGL40_01585
	glutamine-fructose-6-phosphate aminotransferase	24	BGL46_04850	EQU37_04955	EQU01_04260	EQU42_03680	EQU35_01300	EQU36_03760	BGL45_03875	EQU06_01300	BGL37_03910	BGL38_01810	BGL39_03820	BGL40_01780
	phosphoglucosamine mutase	24	BGL46_04870	EQU37_04935	EQU01_04240	EQU42_03695	EQU35_01290	EQU36_03750	BGL45_03865	EQU06_01290	BGL37_03920	BGL38_01820	BGL39_03830	BGL40_01790
	UDP-N-acetylglucosamine diphosphorylase	24	BGL46_06090	EQU37_05130	EQU01_04495	EQU42_01985	EQU35_04830	EQU36_06405	BGL45_05395	EQU06_04750	BGL37_05520	BGL38_05345	BGL39_05440	BGL40_05060
	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	24	BGL46_00565	EQU37_00705	EQU01_00705	EQU42_05145	EQU35_00685	EQU36_00540	BGL45_01860	EQU06_00550	BGL37_00735	BGL38_00580	BGL39_00735	BGL40_00545
	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	24	BGL46_06125	EQU37_05165	EQU01_04460	EQU42_02020	EQU35_04795	EQU36_06440	BGL45_05360	EQU06_04715	BGL37_05515	BGL38_05380	BGL39_05475	BGL40_05095
	UDP-N-acetylenolpyruvoylglucosamine reductase	24	BGL46_04890	EQU37_04915	EQU01_04220	EQU42_03715	EQU35_01270	EQU36_03730	BGL45_03845	EQU06_01270	BGL37_03940	BGL38_01840	BGL39_03850	BGL40_01810
dTDP-rhamnose synthesis / rhamnose containing glycans	**alpha-D-glucose-1-phosphate thymidylyltransferase**	3	0	0	0	0	0	0	0	0	0	0	0	BGL40_05640
	dTDP-glucose 4,6-dehydratase	1	0	0	0	0	0	0	0	0	0	0	0	0
	dTDP-4-dehydro-6-deoxy-D-glucose 3,5-epimerase	1	0	0	0	0	0	0	0	0	0	0	0	0
	dTDP-beta-L-	1	0	0	0	0	0	0	0	0	0	0	0	0

	rhamnose:NADP+ 4-oxidoreductase													
sucrose metabolism	**levansucrase (levS)**	8	0	0	0	0	EQU35_05860	0	0	EQU06_05785	0	0	0	BGL40_06685
	dextransucrase	5	BGL46_04455	0	EQU01_06390	0	0	0	BGL45_06010	0	0	0	0	0
glucose metabolism	**hexokinase/ glucokinase**	24	BGL46_01730	EQU37_01485	EQU01_01485	EQU42_01385	EQU35_01785	EQU36_02000	BGL45_00820	EQU06_01785	BGL37_01830	BGL38_01415	BGL39_01760	BGL40_01245
maltose metabolism	**maltose/H+ symporter A (mpeA)**	11	0	0	0	0	0	0	0	0	BGL37_03210	BGL38_02780	BGL39_03115	BGL40_02750
	maltose/H+ symporter B (mpeB)	23	BGL46_06310	EQU37_05930	EQU01_05745	EQU42_04750	EQU35_06020	EQU36_05970	BGL45_06500	EQU06_06010	BGL37_06200	BGL38_05830	BGL39_06295	BGL40_05660
	maltose phosphorylase (mapB)	23	BGL46_06305	EQU37_05935	EQU01_05740	EQU42_04745	EQU35_06015	EQU36_05965	BGL45_06505	EQU06_06015	BGL37_06195	BGL38_05825	BGL39_06290	BGL40_05655
	maltose phosphorylase (mapA)	11	0	0	0	0	0	0	0	0	BGL37_03215	BGL38_02785	BGL39_03120	BGL40_02755
	beta-phosphoglucomutase (pgmB)	23	BGL46_06295	EQU37_05945	EQU01_05730	EQU42_04735	EQU35_06005	EQU36_05955	BGL45_06515	EQU06_06025	BGL37_06185	BGL38_05815	BGL39_06280	BGL40_05645
	beta-phosphoglucomutase (pgmA)	12	0	0	0	0	0	0	0	0	BGL37_03220	BGL38_02790	BGL39_03125	BGL40_02760
fructose	**fructokinase (fk)**	18	BGL4	EQU3	EQU0	EQU4	0	EQU3	0	0	BGL3	BGL3	BGL3	BGL4

metabolism			6_01400	7_01815	1_01815	2_05910		6_02355			7_01495	8_01070	9_01425	0_01590
	fructokinase (fk)	6	0	0	0	0	EQU35_01450	0	BGL45_01165	EQU06_01450	0	0	0	0
	fructose permease (fpe)	24	BGL46_02330	EQU37_03080	EQU01_02650	EQU42_00215	EQU35_02345	EQU36_04250	BGL45_04050	EQU06_02845	BGL37_02900	BGL38_02465	BGL39_02805	BGL40_02435
	glucose-6-phosophate isomerase (g6pi)	24	BGL46_01405	EQU37_01810	EQU01_01810	EQU42_05905	EQU35_01455	EQU36_02350	BGL45_01160	EQU06_01455	BGL37_01500	BGL38_01075	BGL39_01430	BGL40_01585
	mannitol dehydrogenase (Mandh)	21	BGL46_02325	EQU37_03085	EQU01_02645	EQU42_00220	EQU35_02340	EQU36_04245	BGL45_04055	EQU06_02850	0	BGL38_02470	0	BGL40_02440
	ribose uptake protein (RbsU)	6	0	0	0	0	EQU35_03245	0	0	EQU06_02365	0	0	0	0
	ribose transproter (RbsU)	24	BGL46_02355	EQU37_03055	EQU01_02675	EQU42_00145	EQU35_02380	EQU36_04285	BGL45_04015	EQU06_02810	BGL37_02865	BGL38_02430	BGL39_02770	BGL40_02400
ribose metabolism	**ribokinase (rk)**	21	BGL46_02320	EQU37_03090	EQU01_02640	EQU42_00225	EQU35_02335	EQU36_04240	BGL45_04060	EQU06_02855	0	BGL38_02475	0	BGL40_02445
	ribose 5-phosphate isomerase A (rpiA)	24	BGL46_05195	EQU37_03745	EQU01_03470	EQU42_02215	EQU35_03395	EQU36_03270	BGL45_03395	EQU06_02515	BGL37_05840	BGL38_02295	BGL39_06155	BGL40_02260
	ribose 5-phosphate isomerase A (rpiA)	3	0	0	0	0	0	0	0	0	BGL37_02905	0	BGL39_02810	0
	ribulose-phosphate 3-	24	BGL4	EQU3	EQU0	EQU4	EQU3	EQU3	BGL4	EQU0	BGL3	BGL3	BGL3	BGL4

	epimerase		6_01855	7_01360	1_01360	2_01260	5_01910	6_01875	5_00695	6_01910	7_01955	8_01540	9_01885	0_01120
	ribose-phosphate pyrophosphokinase	24	BGL46_00295	EQU37_00970	EQU01_00975	EQU42_03320	EQU35_00965	EQU36_00270	BGL45_01580	EQU06_00270	BGL37_01015	BGL38_00300	BGL39_01015	BGL40_00265
	ribose-phosphate pyrophosphokinase	24	BGL46_06095	EQU37_05135	EQU01_04490	EQU42_01990	EQU35_04825	EQU36_06410	BGL45_05390	EQU06_04745	BGL37_05525	BGL38_05350	BGL39_05445	BGL40_05065
	sugar porter family MFS transporter (xpe)	2	0	0	0	0	0	EQU36_03035	0	0	0	0	0	0
xylose metabolism	**D-xylose proton-symporter (xylT)**	11	BGL46_05905	EQU37_04430	EQU01_04685	EQU42_03975	0	0	0	0	0	BGL38_04475	0	BGL40_04255
	xylulokinase (xk)	12	BGL46_05960	EQU37_04485	EQU01_04740	EQU42_04030	0	EQU36_02980	BGL45_03265	0	0	BGL38_04415	0	BGL40_04195
arabinose metabolism	**predicted Arabinose ABC transporter permease (ape)**	23	BGL46_06325	EQU37_05900	EQU01_05770	EQU42_04775	EQU35_06050	EQU36_05995	BGL45_06470	EQU06_05980	BGL37_06230	BGL38_05860	BGL39_06325	BGL40_05690
gluconate metabolism	**gluconat permease (gpe)**	12	0	0	0	0	0	EQU36_04615	BGL45_06240	0	BGL37_02240	0	BGL39_02170	BGL40_06715
	gluconokinase (gk)	24	BGL46_03360	EQU37_05585	EQU01_06090	EQU42_03805	EQU35_06605	EQU36_04640	BGL45_06275	EQU06_06580	BGL37_02270	BGL38_05665	BGL39_02200	BGL40_05605
citrate metabolism	**citrate-sodium symporter**	24	BGL46_00990	EQU37_00280	EQU01_00280	EQU42_06235	EQU35_00260	EQU36_00975	BGL45_02265	EQU06_00975	BGL37_00285	BGL38_03795	BGL39_00285	BGL40_04110
	citrate-sodium symporter	21	BGL4	EQU3	EQU0	EQU4	EQU3	0	BGL4	EQU0	BGL3	BGL3	BGL3	BGL4

			6_05865	7_04390	1_04645	2_03935	5_05020		5_03170	6_04900	7_06060	8_04515	9_05715	0_04295
	citrate lyase subunit alpha (CitF)	22	BGL46_05890	EQU37_04415	EQU01_04670	EQU42_03960	EQU35_04995	0	BGL45_03195	EQU06_04925	BGL37_06085	BGL38_04490	BGL39_05740	BGL40_04270
	citrate (pro-3S)-lyase subunit beta (CitE)	21	BGL46_05885	EQU37_04410	EQU01_04665	EQU42_03955	EQU35_05000	0	BGL45_03190	EQU06_04920	BGL37_06080	BGL38_04495	BGL39_05735	BGL40_04275
	citrate lyase subunit beta (CitE)	0	0	0	0	0	0	0	0	0	0	0	0	0
	citrate lyase subunit gamma (CitD)	21	BGL46_05880	EQU37_04405	EQU01_04660	EQU42_03950	EQU35_05005	0	BGL45_03185	EQU06_04915	BGL37_06075	BGL38_04500	BGL39_05730	BGL40_04280
	citrate (pro-3S)-lyase ligase (CitC)	21	BGL46_05875	EQU37_04400	EQU01_04655	EQU42_03945	EQU35_05010	0	BGL45_03180	EQU06_04910	BGL37_06070	BGL38_04505	BGL39_05725	BGL40_04285
	holo-ACP synthase (CitX)	22	BGL46_05895	EQU37_04420	EQU01_04675	EQU42_03965	EQU35_04990	0	BGL45_03200	EQU06_04930	BGL37_06090	BGL38_04485	BGL39_05745	BGL40_04265
	triphosphoribosyl-dephospho-CoA synthase (CitG)	22	BGL46_05900	EQU37_04425	EQU01_04680	EQU42_03970	EQU35_04985	0	BGL45_03205	EQU06_04935	BGL37_06095	BGL38_04480	BGL39_05750	BGL40_04260
	malate permease	17	BGL46_02590	EQU37_05415	EQU01_05575	EQU42_04080	EQU35_05890	EQU36_05705	BGL45_05105	EQU06_05755	BGL37_05315	0	BGL39_05200	0
malate metabolism	**malate permease**	3	0	0	0	0	0	0	0	0	0	0	0	BGL40_06555
	malate dehydrogenase (maldh)	21	BGL46_058	EQU37_043	EQU01_046	EQU42_039	EQU35_050	0	BGL45_031	EQU06_049	BGL37_060	BGL38_045	BGL39_057	BGL40_042

			70	95	50	40	15		75	05	65	10	20	90
	NAD-dependent malic enzyme (me)	24	BGL46_02595	EQU37_05410	EQU01_05580	EQU42_04075	EQU35_05895	EQU36_05710	BGL45_05110	EQU06_05750	BGL37_05310	BGL38_05500	BGL39_05205	BGL40_05860
	class II fumarate hydratase (fh)	24	BGL46_03575	EQU37_03540	EQU01_05500	EQU42_05830	EQU35_05230	EQU36_02600	BGL45_04610	EQU06_05235	BGL37_06360	BGL38_04805	BGL39_06455	BGL40_04850
acetate metabolism	**acetate kinase (ak)**	24	BGL46_00850	EQU37_00420	EQU01_00420	EQU42_01130	EQU35_00400	EQU36_00835	BGL45_02145	EQU06_00835	BGL37_00450	BGL38_00865	BGL39_00450	BGL40_00835
oxygen depletion	**NADH-Oxidase (NOX)**	24	BGL46_04800	EQU37_05005	EQU01_04310	EQU42_03630	EQU35_01360	EQU36_03815	1	EQU06_01360	BGL37_03860	BGL38_01760	BGL39_03770	BGL40_01730
	amidophosphoribosyltransferase	19	BGL46_01325	EQU37_01895	EQU01_01895	EQU42_04965	0	EQU36_02475	BGL45_01235	0	BGL37_01360	BGL38_01020	BGL39_01360	BGL40_01640
	phosphoribosylamine-glycine ligase	19	BGL46_01345	EQU37_01875	EQU01_01875	EQU42_04945	0	EQU36_02455	BGL45_01215	0	BGL37_01380	BGL38_01040	BGL39_01380	BGL40_01620
de novo purine biosynthesis	**phosphoribosylglycinamide formyltransferase**	19	BGL46_01335	EQU37_01885	EQU01_01885	EQU42_04955	0	EQU36_02465	BGL45_01225	0	BGL37_01370	BGL38_01030	BGL39_01370	BGL40_01630
	phosphoribosylformylglycinamidine synthase (purS)	19	BGL46_01310	EQU37_01910	EQU01_01910	EQU42_04980	0	EQU36_02490	BGL45_01250	0	BGL37_01345	BGL38_01005	BGL39_01345	BGL40_01655
	phosphoribosylformylglycinamidine synthase (purQ)	19	BGL46_01315	EQU37_01905	EQU01_01905	EQU42_04975	0	EQU36_02485	BGL45_01245	0	BGL37_01350	BGL38_01010	BGL39_01350	BGL40_01650
	phosphoribosylformylglycinamidine synthase	19	BGL46_013	EQU37_019	EQU01_019	EQU42_049	0	EQU36_024	BGL45_012	0	BGL37_013	BGL38_010	BGL39_013	BGL40_016

	(purL)		20	00	00	70		80	40		55	15	55	45
	phosphoribosylformylglycinamidine cyclo-ligase	19	BGL46_01330	EQU37_01890	EQU01_01890	EQU42_04960	0	EQU36_02470	BGL45_01230	0	BGL37_01365	BGL38_01025	BGL39_01365	BGL40_01635
	phosphoribosylaminoimidazolesuccinocarboxamide synthase	19	BGL46_01305	EQU37_01915	EQU01_01915	EQU42_04985	0	EQU36_02495	BGL45_01255	0	BGL37_01340	BGL38_01000	BGL39_01340	BGL40_01660
	adenylosuccinate lyase	24	BGL46_01355	EQU37_01865	EQU01_01865	EQU42_04935	EQU35_01415	EQU36_02445	BGL45_01205	EQU06_01415	BGL37_01390	BGL38_01050	BGL39_01390	BGL40_01610
	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase	19	BGL46_01340	EQU37_01880	EQU01_01880	EQU42_04950	0	EQU36_02460	BGL45_01220	0	BGL37_01375	BGL38_01035	BGL39_01375	BGL40_01625
	cytidine deaminase	3	0	0	0	0	EQU35_04255	0	0	EQU06_03625	0	0	0	0
	guanine deaminase	19	BGL46_05820	EQU37_04345	EQU01_04600	EQU42_06260	0	EQU36_03070	BGL45_03130	0	BGL37_06020	BGL38_04555	BGL39_05675	BGL40_04335
depletion	**guanine deaminase**	5	0	0	0	0	EQU35_05065	0	0	EQU06_04855	0	0	0	0
	cytosine deaminase	5	0	0	0	0	EQU35_05400	0	0	EQU06_05325	0	0	0	0
	adenine deaminase	5	0	0	0	0	EQU35_06170	0	0	EQU06_06060	0	0	0	0
polyphosphat	**exopolyphosphatase**	24	BGL4	EQU3	EQU0	EQU4	EQU3	EQU3	BGL4	EQU0	BGL3	BGL3	BGL3	BGL4

e metabolism			6_06025	7_05065	1_04560	2_01920	5_04895	6_06340	5_05460	6_04815	7_05455	8_05280	9_05375	0_04995
	exopolyphosphatase	3	0	0	0	0	0	0	0	0	0	0	BGL39_05985	BGL40_06410
	exopolyphosphatase	4	0	0	0	0	0	0	0	0	0	0	BGL39_05995	BGL40_06400
	polyphosphat kinase	4	0	0	0	0	0	0	0	0	0	0	BGL39_05990	BGL40_06405

Table A 3: Continuation of Table A2: Overview of genes involved in the energy-, cell wall and capsule-, carbohydrate-, organic acids-, nucleotides and nucleosides- and phosphorous metabolisms in *F. sanfranciscensis* strains.

metabolism	**gene**	**amount**	**TMW 1.2141**	**TMW 1.2142**	**TMW 1.2314**	**TMW 1.2323**	**TMW 1.392**	**TMW 1.53**	**TMW 1.54**	**TMW 1.640**	**TMW 1.726**	**TMW 1.897**	**TMW 1.907**	**TMW 1.936**
phosphoketolase pathway	**glucose-6-phosphate dehydrogenase (g6pdh)**	24	BGL41_05380	BGL42_04740	EQT93_04220	FG115_05620	BGL47_04110	BHU32_05355	BGL44_03300	EQU33_04840	BGL43_04015	EQT97_04020	EQT98_03885	EQU27_04135
	6 phosphogluconate dehydrogenase (6pg-dh)	24	BGL41_00750	BGL42_02125	EQT93_02560	FG115_03920	BGL47_01540	BHU32_02030	BGL44_02565	EQU33_00010	BGL43_01710	EQT97_01470	EQT98_00755	EQU27_01995
	phosphoketolase	24	BGL41_01315	BGL42_04385	EQT93_03575	FG115_03615	BGL47_02460	BHU32_02190	BGL44_04425	EQU33_03375	BGL43_06090	EQT97_05725	EQT98_05945	EQU27_01240
	8x lactate dehydrogenase (ldh)	24												

	ribulose-phosphate 3-epimerase (rpe)	24	BGL41_00580	BGL42_01955	EQT93_02390	FG115_04095	BGL47_01710	BHU32_01850	BGL44_02395	EQU33_00180	BGL43_01540	EQT97_01295	EQT98_00585	EQU27_02170
	Alcoholdehydrogenase (adhP)	24	BGL41_03820	BGL42_05260	EQT93_05300	FG115_02540	BGL47_05060	BHU32_04840	BGL44_04870	EQU33_05650	BGL43_05230	EQT97_05100	EQT98_04830	EQU27_05420
	pyruvate kinase	24	BGL41_01030	BGL42_03640	EQT93_01425	FG115_01410	BGL47_01340	BHU32_04145	BGL44_01465	EQU33_02250	BGL43_03210	EQT97_02080	EQT98_02155	EQU27_02805
	pyruvate oxidase	24	BGL41_05675	BGL42_05040	EQT93_04480	FG115_06085	BGL47_04335	BHU32_05065	BGL44_03860	EQU33_03970	BGL43_05060	EQT97_02845	EQT98_03260	EQU27_04590
metabolism of cell wall compounds	**alpha-phosphoglucomutase (pmg)**	24	BGL41_03750	BGL42_05335	EQT93_05230	FG115_02610	BGL47_05130	BHU32_04915	BGL44_04940	EQU33_05720	BGL43_05160	EQT97_05030	EQT98_04895	EQU27_05495
	glucose-6-phosphate isomerase (g6pi)	24	BGL41_00130	BGL42_01500	EQT93_01935	FG115_04570	BGL47_02160	BHU32_01390	BGL44_01945	EQU33_00640	BGL43_01075	EQT97_04205	EQT98_00115	EQU27_06215
	glutamine-fructose-6-phosphate aminotransferase	24	BGL41_03405	BGL42_03915	EQT93_01780	FG115_05320	BGL47_02305	BHU32_05915	BGL44_01800	EQU33_02505	BGL43_01810	EQT97_03295	EQT98_04585	EQU27_05125
	phosphoglucosamine mutase	24	BGL41_03390	BGL42_03925	EQT93_01770	FG115_05310	BGL47_02315	BHU32_05930	BGL44_01790	EQU33_02515	BGL43_01820	EQT97_03285	EQT98_04575	EQU27_05135
	UDP-N-acetylglucosamine diphosphorylase	24	BGL41_06250	BGL42_05670	EQT93_04950	FG115_06280	BGL47_04820	BHU32_05660	BGL44_04635	EQU33_03420	BGL43_05490	EQT97_03530	EQT98_04105	EQU27_04785
	UDP-N-acetylglucosamine 1-	24	BGL41_025	BGL42_007	EQT93_005	FG115_005	BGL47_005	BHU32_005	BGL44_005	EQU33_009	BGL43_005	EQT97_004	EQT98_031	EQU27_007

	carboxyvinyltransferase		70	35	50	35	80	80	80	65	80	40	50	15
	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	24	BGL41_06285	BGL42_05635	EQT93_04915	FG115_06245	BGL47_04785	BHU32_05695	BGL44_04600	EQU33_05455	BGL43_05525	EQT97_03495	EQT98_04140	EQU27_04750
	UDP-N-acetylenolpyruvoylglucosamine reductase	24	BGL41_03370	BGL42_03945	EQT93_01750	FG115_05290	BGL47_02335	BHU32_02900	BGL44_01770	EQU33_02535	BGL43_01840	EQT97_03265	EQT98_04555	EQU27_05155
dTDP-rhamnose synthesis / rhamnose containing glycans	**alpha-D-glucose-1-phosphate thymidylyltransferase**	3	BGL41_06590	0	0	0	0	BHU32_06065	0	0	0	0	0	0
	dTDP-glucose 4,6-dehydratase	1	BGL41_06580	0	0	0	0	0	0	0	0	0	0	0
	dTDP-4-dehydro-6-deoxy-D-glucose 3,5-epimerase	1	BGL41_06585	0	0	0	0	0	0	0	0	0	0	0
	dTDP-beta-L-rhamnose:NADP+ 4-oxidoreductase	1	BGL41_06515	0	0	0	0	0	0	0	0	0	0	0
sucrose metabolism	**levansucrase (levS)**	8	0	0	EQT93_05640	0	BGL47_02400	BHU32_04320	BGL44_06625	EQU33_06790	0	0	0	0
	dextransucrase	5	BGL41_05530	0	0	0	0	0	0	0	0	EQT97_06400	0	0
glucose metabolism	**hexokinase/ glucokinase**	24	BGL41_00455	BGL42_01830	EQT93_02265	FG115_04220	BGL47_01835	BHU32_01725	BGL44_02270	EQU33_00305	BGL43_01415	EQT97_01170	EQT98_00460	EQU27_02295
maltose	**maltose/H+ symporter A**	11	0	BGL4	0	0	0	BHU3	0	EQU3	BGL4	EQT9	EQT9	EQU2

metabolism	**(mpeA)**			2_03210				2_02740		3_05270	3_02780	7_04565	8_01555	7_01845
	maltose/H+ symporter B (mpeB)	23	BGL41_06615	BGL42_06270	EQT93_05795	FG115_06500	BGL47_05640	BHU32_06045	BGL44_05515	EQU33_06645	BGL43_05805	0	EQT98_06300	EQU27_05785
	maltose phosphorylase (mapB)	23	BGL41_06610	BGL42_06265	EQT93_05790	FG115_06505	BGL47_05645	BHU32_06050	BGL44_05520	EQU33_06650	BGL43_05800	0	EQT98_06295	EQU27_05780
	maltose phosphorylase (mapA)	11	0	BGL42_03215	0	0	0	BHU32_02745	0	EQU33_05275	BGL43_02785	EQT97_04570	EQT98_01550	EQU27_01850
	beta-phosphoglucomutase (pgmB)	23	BGL41_06600	BGL42_06255	EQT93_05780	FG115_06515	BGL47_05655	BHU32_06060	BGL44_05530	EQU33_06660	BGL43_05790	0	EQT98_06285	EQU27_05770
	beta-phosphoglucomutase (pgmA)	12	BGL41_06865	BGL42_03220	0	0	0	BHU32_02750	0	EQU33_05280	BGL43_02790	EQT97_04575	EQT98_01545	EQU27_01855
fructose metabolism	**fructokinase (fk)**	18	BGL41_00125	BGL42_01495	0	FG115_04575	0	BHU32_01385	0	EQU33_00645	BGL43_01070	EQT97_04210	EQT98_00110	EQU27_06220
	fructokinase (fk)	6	0	0	EQT93_01930	0	BGL47_02165	0	BGL44_01940	0	0	0	0	0
	fructose permease (fpe)	24	BGL41_01545	BGL42_02900	EQT93_03355	FG115_03370	BGL47_02680	BHU32_02420	BGL44_03540	EQU33_03115	BGL43_02465	EQT97_02555	EQT98_01870	EQU27_01530
	glucose-6-phosophate isomerase (g6pi)	24	BGL41_00130	BGL42_01500	EQT93_01935	FG115_04570	BGL47_02160	BHU32_01390	BGL44_01945	EQU33_00640	BGL43_01075	EQT97_04205	EQT98_00115	EQU27_06215

	mannitol dehydrogenase (Mandh)	21	BGL41_01550	0	EQT93_03350	FG115_03365	BGL47_02685	BHU32_02425	BGL44_03545	EQU33_03110	BGL43_02470	EQT97_02550	EQT98_01865	EQU27_01535
ribose metabolism	**ribose uptake protein (RbsU)**	6	0	0	EQT93_02810	0	BGL47_03220	0	BGL44_02820	EQU33_03525	0	0	0	0
	ribose transproter (RbsU)	24	BGL41_01510	BGL42_02865	EQT93_03390	FG115_03405	BGL47_02645	BHU32_02385	BGL44_03505	EQU33_03155	BGL43_02430	EQT97_02590	EQT98_01905	EQU27_01495
	ribokinase (rk)	21	BGL41_01555	0	EQT93_03345	FG115_03360	BGL47_02690	BHU32_02430	BGL44_03550	EQU33_03105	BGL43_02475	EQT97_02545	EQT98_01860	EQU27_01540
	ribose 5-phosphate isomerase A (rpiA)	24	BGL41_02915	BGL42_06130	EQT93_02660	FG115_04830	BGL47_03370	BHU32_03350	BGL44_02670	EQU33_03675	BGL43_02290	EQT97_01810	EQT98_02770	EQU27_03890
	ribose 5-phosphate isomerase A (rpiA)	3	0	BGL42_02905	0	0	0	0	0	0	0	0	0	0
	ribulose-phosphate 3-epimerase	24	BGL41_00580	BGL42_01955	EQT93_02390	FG115_04095	BGL47_01710	BHU32_01850	BGL44_02395	EQU33_00180	BGL43_01540	EQT97_01295	EQT98_00585	EQU27_02170
	ribose-phosphate pyrophosphokinase	24	BGL41_02295	BGL42_01015	EQT93_00270	FG115_00270	BGL47_00300	BHU32_00300	BGL44_00300	EQU33_04095	BGL43_00300	EQT97_00720	EQT98_00955	EQU27_00990
	ribose-phosphate pyrophosphokinase	24	BGL41_06255	BGL42_05665	EQT93_04945	FG115_06275	BGL47_04815	BHU32_05665	BGL44_04630	EQU33_05425	BGL43_05495	EQT97_03525	EQT98_04110	EQU27_04780
xylose metabolism	**sugar porter family MFS transporter (xpe)**	2	0	0	0	FG115_02900	0	0	0	0	0	0	0	0

						20								
	D-xylose proton-symporter (xylT)	11	BGL41_05965	0	0	0	0	BHU32_04500	0	0	BGL43_04710	0	EQT98_04720	EQU27_05210
	xylulokinase (xk)	12	BGL41_05915	0	0	FG115_02865	0	0	0	EQU33_05515	BGL43_04770	0	0	0
arabinose metabolism	**predicted Arabinose ABC transporter permease (ape)**	23	BGL41_06645	BGL42_06300	EQT93_05825	FG115_06475	BGL47_05615	BHU32_06025	BGL44_05490	EQU33_06525	BGL43_05835	0	EQT98_05590	EQU27_05815
gluconate metabolism	**gluconat permease (gpe)**	12	0	BGL42_02240	0	FG115_01800	0	BHU32_03515	0	EQU33_05065	0	EQT97_04490	EQT98_04950	EQU27_05310
	gluconokinase (gk)	24	BGL41_04870	BGL42_02270	EQT93_06310	FG115_01825	BGL47_06170	BHU32_03545	BGL44_06125	EQU33_05040	BGL43_06415	EQT97_04515	EQT98_06110	EQU27_06305
citrate metabolism	**citrate-sodium symporter**	24	BGL41_04280	BGL42_00285	EQT93_00975	FG115_00970	BGL47_01010	BHU32_01010	BGL44_01010	EQU33_03055	BGL43_04265	EQT97_00010	EQT98_03720	EQU27_00285
	citrate-sodium symporter	21	BGL41_06005	BGL42_05890	EQT93_05130	0	BGL47_04965	BHU32_04460	BGL44_04780	0	BGL43_04670	EQT97_04935	EQT98_04760	EQU27_05250
	citrate lyase subunit alpha (CitF)	22	BGL41_05980	BGL42_05915	EQT93_05105	0	BGL47_04990	BHU32_04485	BGL44_04805	EQU33_05585	BGL43_04695	EQT97_04910	EQT98_04735	EQU27_05225
	citrate (pro-3S)-lyase subunit beta (CitE)	21	BGL41_05985	BGL42_05910	EQT93_05110	0	BGL47_04985	BHU32_04480	BGL44_04800	0	BGL43_04690	EQT97_04915	EQT98_04740	EQU27_05230
	citrate lyase subunit	0	0	0	0	0	0	0	0	EQU3	0	0	0	0

	beta (CitE)								3_05590					
	citrate lyase subunit gamma (CitD)	21	BGL41_05990	BGL42_05905	EQT93_05115	0	BGL47_04980	BHU32_04475	BGL44_04795	0	BGL43_04685	EQT97_04920	EQT98_04745	EQU27_05235
	citrate (pro-3S)-lyase ligase (CitC)	21	BGL41_05995	BGL42_05900	EQT93_05120	0	BGL47_04975	BHU32_04470	BGL44_04790	0	BGL43_04680	EQT97_04925	EQT98_04750	EQU27_05240
	holo-ACP synthase (CitX)	22	BGL41_05975	BGL42_05920	EQT93_05100	0	BGL47_04995	BHU32_04490	BGL44_04810	EQU33_05580	BGL43_04700	EQT97_04905	EQT98_04730	EQU27_05220
	triphosphoribosyl-dephospho-CoA synthase (CitG)	22	BGL41_05970	BGL42_05925	EQT93_05095	0	BGL47_05000	BHU32_04495	BGL44_04815	EQU33_05575	BGL43_04705	EQT97_04900	EQT98_04725	EQU27_05215
malate metabolism	**malate permease**	17	BGL41_01270	BGL42_04435	EQT93_05670	FG115_03670	BGL47_02385	0	BGL44_06405	0	0	EQT97_05280	0	0
	malate permease	3	0	0	0	0	0	BHU32_06350	0	EQU33_05795	0	0	0	0
	malate dehydrogenase (maldh)	21	BGL41_06000	BGL42_05895	EQT93_05125	0	BGL47_04970	BHU32_04465	BGL44_04785	0	BGL43_04675	EQT97_04930	EQT98_04755	EQU27_05245
	NAD-dependent malic enzyme (me)	24	BGL41_01265	BGL42_04440	EQT93_05675	FG115_03675	BGL47_02380	BHU32_06210	BGL44_06410	EQU33_04650	BGL43_05645	EQT97_05275	EQT98_05870	EQU27_05890
	class II fumarate hydratase (fh)	24	BGL41_03870	BGL42_06430	EQT93_05430	FG115_02485	BGL47_05200	BHU32_04785	BGL44_05005	EQU33_01445	BGL43_05280	EQT97_05180	EQT98_05350	EQU27_04920

acetate metabolism	**acetate kinase (ak)**	24	BGL41_04160	BGL42_00450	EQT93_00835	FG115_00830	BGL47_00865	BHU32_00865	BGL44_00865	EQU33_01250	BGL43_00865	EQT97_00155	EQT98_02865	EQU27_00430
oxygen depletion	**NADH-Oxidase (NOX)**	24	BGL41_03455	BGL42_03865	EQT93_01840	FG115_05375	BGL47_02255	BHU32_05865	BGL44_01850	EQU33_02455	BGL43_01760	EQT97_03345	EQT98_04635	EQU27_05075
de novo purine biosynthesis	**amidophosphoribosyltransferase**	19	BGL41_00050	BGL42_01360	0	FG115_04695	0	BHU32_01315	0	EQU33_00715	BGL43_01020	EQT97_04345	EQT98_00040	EQU27_05615
	phosphoribosylamine-glycine ligase	19	BGL41_00070	BGL42_01380	0	FG115_04675	0	BHU32_01335	0	EQU33_00695	BGL43_01040	EQT97_04325	EQT98_00060	EQU27_05595
	phosphoribosylglycinamide formyltransferase	19	BGL41_00060	BGL42_01370	0	FG115_04685	0	BHU32_01325	0	EQU33_00705	BGL43_01030	EQT97_04335	EQT98_00050	EQU27_05605
	phosphoribosylformylglycinamidine synthase (purS)	19	BGL41_00035	BGL42_01345	0	FG115_04710	0	BHU32_01300	0	EQU33_00730	BGL43_01005	EQT97_04360	EQT98_00025	EQU27_05630
	phosphoribosylformylglycinamidine synthase (purQ)	19	BGL41_00040	BGL42_01350	0	FG115_04705	0	BHU32_01305	0	EQU33_00725	BGL43_01010	EQT97_04355	EQT98_00030	EQU27_05625
	phosphoribosylformylglycinamidine synthase (purL)	19	BGL41_00045	BGL42_01355	0	FG115_04700	0	BHU32_01310	0	EQU33_00720	BGL43_01015	EQT97_04350	EQT98_00035	EQU27_05620
	phosphoribosylformylglycinamidine cyclo-ligase	19	BGL41_00055	BGL42_01365	0	FG115_04690	0	BHU32_01320	0	EQU33_00710	BGL43_01025	EQT97_04340	EQT98_00045	EQU27_05610
	phosphoribosylaminoimidazolesuccinocarboxami	19	BGL41_000	BGL42_013	0	FG115_047	0	BHU32_012	0	EQU33_007	BGL43_010	EQT97_043	EQT98_000	EQU27_056

	de synthase		30	40		15		95		35	00	65	20	35
	adenylosuccinate lyase	24	BGL41_00080	BGL42_01390	EQT93_01895	FG115_04665	BGL47_02200	BHU32_01345	BGL44_01905	EQU33_00685	BGL43_01050	EQT97_04315	EQT98_00070	EQU27_05585
	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase	19	BGL41_00065	BGL42_01375	0	FG115_04680	0	BHU32_01330	0	EQU33_00700	BGL43_01035	EQT97_04330	EQT98_00055	EQU27_05600
depletion	**cytidine deaminase**	3	0	0	EQT93_04355	0	0	0	0	0	0	0	0	0
	guanine deaminase	19	BGL41_06050	BGL42_05850	0	FG115_02955	0	BHU32_04415	0	EQU33_05635	BGL43_04630	EQT97_04980	EQT98_04805	EQU27_05295
	guanine deaminase	5	0	0	EQT93_05175	0	BGL47_04925	0	BGL44_04740	0	0	0	0	0
	cytosine deaminase	5	0	0	0	0	0	0	BGL44_05405	EQU33_06360	0	EQT97_06240	0	0
	adenine deaminase	5	0	0	EQT93_06365	0	BGL47_05760	0	BGL44_05690	0	0	0	0	0
polyphosphate metabolism	**exopolyphosphatase**	24	BGL41_06185	BGL42_05735	EQT93_05015	FG115_06345	BGL47_04885	BHU32_05595	BGL44_04700	EQU33_05355	BGL43_05425	EQT97_03595	EQT98_04040	EQU27_04850
	exopolyphosphatase	3	0	0	0	0	0	BHU32_05445	0	0	0	0	0	0

	exopolyphosphatase	4	0	0	0	0	0	BHU32_05455	0	EQU33_05175	0	0	0	0
	polyphosphat kinase	4	0	0	0	0	0	BHU32_05450	0	EQU33_05180	0	0	0	0

Table A 4: Overview of genes involved in the stress metabolism of different *F. sanfranciscensis* strains. Orf ID of the first strain were the gene is detected is given. 1: gene present; 0: gene absent.

metabolism	**gene**	**Amount**	**Orf ID**	**TMW 1.1150**	**TMW 1.1152**	**TMW 1.1154**	**TMW 1.1221**	**TMW 1.1304**	**TMW 1.1470**	**TMW 1.1597**	**TMW 1.1730**	**TMW 1.2137**	**TMW 1.2138**	**TMW 1.2139**	**TMW 1.2140**	**TMW 1.2141**	**TMW 1.2142**	**TMW 1.2314**	**TMW 1.2323**	**TMW 1.392**	**TMW 1.53**	**TMW 1.54**	**TMW 1.640**	**TMW 1.726**	**TMW 1.897**	**TMW 1.907**	**TMW 1.936**
GSH and GSSG	**glutathione Peroxidase (gpo)**	24	TMW11150_BGL46_00365	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	glutathione dehydrogenase (gshR)	18	TMW11150_BGL46_06665	1	1	1	0	0	1	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1
	gluthation reductase	4	TMW11304_EQU35_04635	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
Cystin/Cystein	**cystine transport**	24	TMW11150_BGL46_02635	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	arginine ABC transporter ATP-	24	TMW11150_BGL46_0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	binding protein (artP)		1030																									
	ABC transporter permease	24	TMW11150_BGL46_03440	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	cystein transport permease (tcyB)	24	TMW11150_BGL46_01025	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Reverse Transsulfuration pathway (Methionine --> Cysteine)	**S-adenosylmethionine synthetase**	24	TMW11150_BGL46_01040	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DNA (cytosine-5-)-methyltransferase	5	TMW11304_EQU35_06135	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0
	DNA (cytosine-5-)-methyltransferase	1	TMW12139_BGL39_06765	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	DNA (cytosine-5-)-methyltransferase	1	TMW153_BHU32_06725	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	DNA (cytosine-5-)-methyltransferase	1	TMW1897_EQT97_04230	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	homocysteine S-methyltransfera	24	TMW11150_BGL46_0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	se		5285																								
Thioredoxin	**thioredoxin reductase**	24	TMW11150_BGL46_02460	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	thioredoxin reductase	24	TMW11150_BGL46_05000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	thioredoxin (trxA)	24	TMW11150_BGL46_00775	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	thioredoxin-like proteins	24	TMW11150_BGL46_02420	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	thioredoxin-like proteins (ytpP)	24	TMW11150_BGL46_01540	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Regulators and Sensors	**HTH- type transcriptional regulator (YodB)**	24	TMW11150_BGL46_01680	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	peroxide-responsive repressor (perR)	24	TMW11150_BGL46_02250	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	regulatory protein (spxA)	24	TMW11150_BGL46_02495	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	redox- sensing transcriptional repressor (rex)	24	TMW11150_BGL46_05425	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Proteases	**clpP**	24	TMW11150	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

against stress			_BGL46_04965																								
	clpX	24	TMW11150_BGL46_00430	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	ATP-dependent clp protease ATP-binding subunit clpE	24	TMW11150_BGL46_03465	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	ATP- dependent Clp protease ATP- binding subunit (clpC)	24	TMW11150_BGL46_03865	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	cell division protein (ftsH)	24	TMW11150_BGL46_06215	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
chaperons	**molecular chaperone GroES (groS)**	22	TMW11150_BGL46_05090	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
	molecular chaperone GroES (groS)	2	TMW11597_BGL45_03645	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	chaperonin (GroL)	24	TMW11150_BGL46_05085	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	molecular chaperone DnaJ	24	TMW11150_BGL46_01240	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	molecular	24	TMW11150	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	chaperone (danK)		_BGL46_01235																							
	nucleotide exchange factor (GrpE)	24	TMW11150_BGL46_01230	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Regulators against stress	**heat-inducible transcription repressor (hrcA)**	24	TMW11150_BGL46_01225	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	family transcriptional regulator (ctsR)	24	TMW11150_BGL46_02955	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
DN A repair proteins	**DNA replication/repair protein (RecF)**	24	TMW11150_BGL46_05595	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DNA repair protein (RecN)	24	TMW11150_BGL46_01815	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DNA repair protein (RecO)	24	TMW11150_BGL46_04050	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	recombinase RecA	24	TMW11150_BGL46_04395	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DNA repair protein (radA)	24	TMW11150_BGL46_05205	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
DN A mismatch repair protein	**DNA mismatch repair protein (mutL)**	24	TMW11150_BGL46_00710	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	DNA mismatch repair protein (mutS)	24	TMW11150_BGL46_00705	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Exinuclease proteins	**excinuclease ABC subunit A (UvrA)**	24	TMW11150_BGL46_04990	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	excinuclease ABC subunit A (UvrA)	12	TMW11150_BGL46_05965	1	1	1	1	0	1	1	0	0	1	0	1	1	0	0	1	0	0	0	1	1	0	0	0
	excinuclease ABC subunit B (uvrB)	24	TMW11150_BGL46_04995	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	excinuclease ABC subunit C (uvrC)	24	TMW11150_BGL46_00440	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Others	**DNA topoisomerase IV subunit A (parC)**	24	TMW11150_BGL46_04235	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DNA topoisomerase IV subunit B (parE)	24	TMW11150_BGL46_04230	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	helicase-exonuclease AddAB subunit AddA	23	TMW11150_BGL46_05750	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
	nuclease addA	1	TMW153_BHU32_04345	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

	SOS response regulator (lexA)	24	TMW11150_BGL46_01105	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	ATP- dependent DNA helicases (ruvA)	24	TMW11150_BGL46_00715	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Holliday junction DNA helicase (ruvA)	24	TMW11150_BGL46_00750	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Holliday junction DNA helicase (ruvB)	24	TMW11150_BGL46_00720	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	divalent metal cation transporter	2	TMW11150_BGL46_02120	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	divalent metal cation transporter	24	TMW11150_BGL46_02565	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	divalent metal cation transporter	2	TMW11152_EQU37_03285	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	divalent metal cation transporter (MnTH)	9	TMW11597_BGL45_04260	0	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0	1	0	0	1	0	0	1

12.3 Publication 4

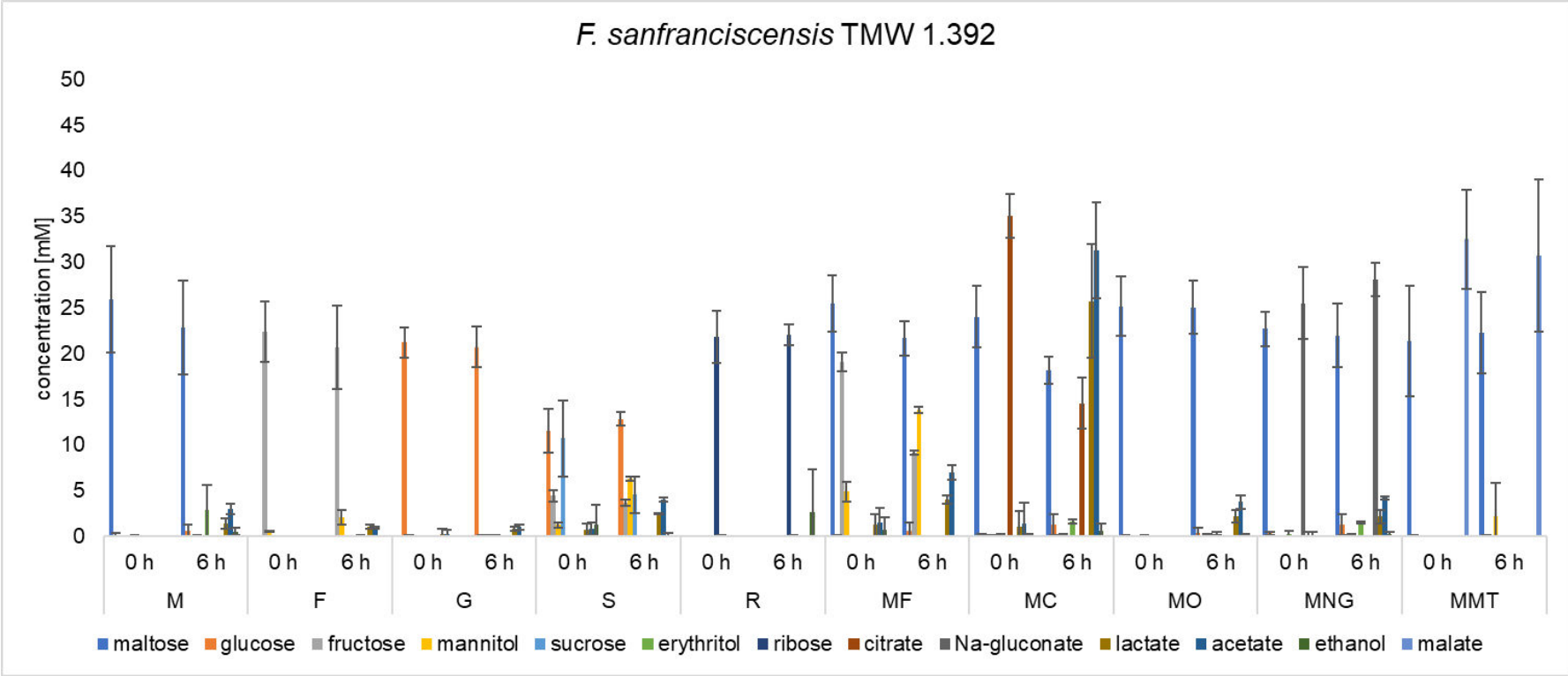

Figure A 3: HPLC analysis of *F. sanfranciscensis* TMW 1.392 metabolism. Concentration of carbohydrates and organic acids after 0h and 6h of incubation in ¼ Ringer's solution with 20 mM of Carbon-sources and putative external electron acceptors. Incubation in: M: maltose, F: fructose; G: glucose; S: sucrose; R: ribose; MF: maltose and fructose; MC: maltose and citrate; MO: maltose and oxygen (inoculation in Erlenmeyer's flask at 150 rpm); MNG: maltose and Na-gluconate; MNT: maltose and malate. The error bars show the standard deviation of the means of three independent replicates.

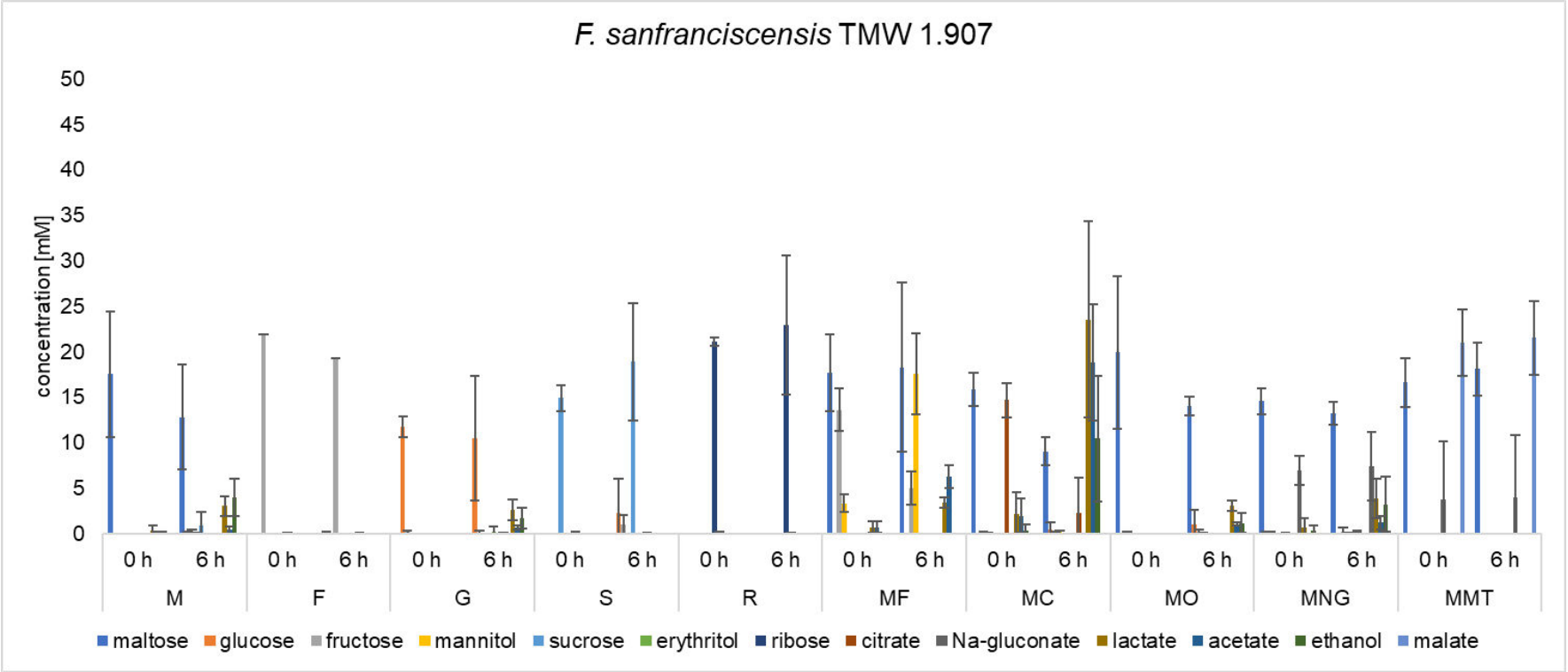

Figure A 4: HPLC analysis of *F. sanfranciscensis* TMW 1.907 metabolism. Concentration of carbohydrates and organic acids after 0h and 6h of incubation in ¼ Ringer's solution with 20 mM of Carbon-sources and putative external electron acceptors. Incubation in: M: maltose, F: fructose; G: glucose; S: sucrose; R: ribose; MF: maltose and fructose; MC: maltose and citrate; MO: maltose and oxygen (inoculation in Erlenmeyer's flask at 150 rpm); MNG: maltose and Na-gluconate; MNT: maltose and malate. The error bars show the standard deviation of the means of three independent replicates.

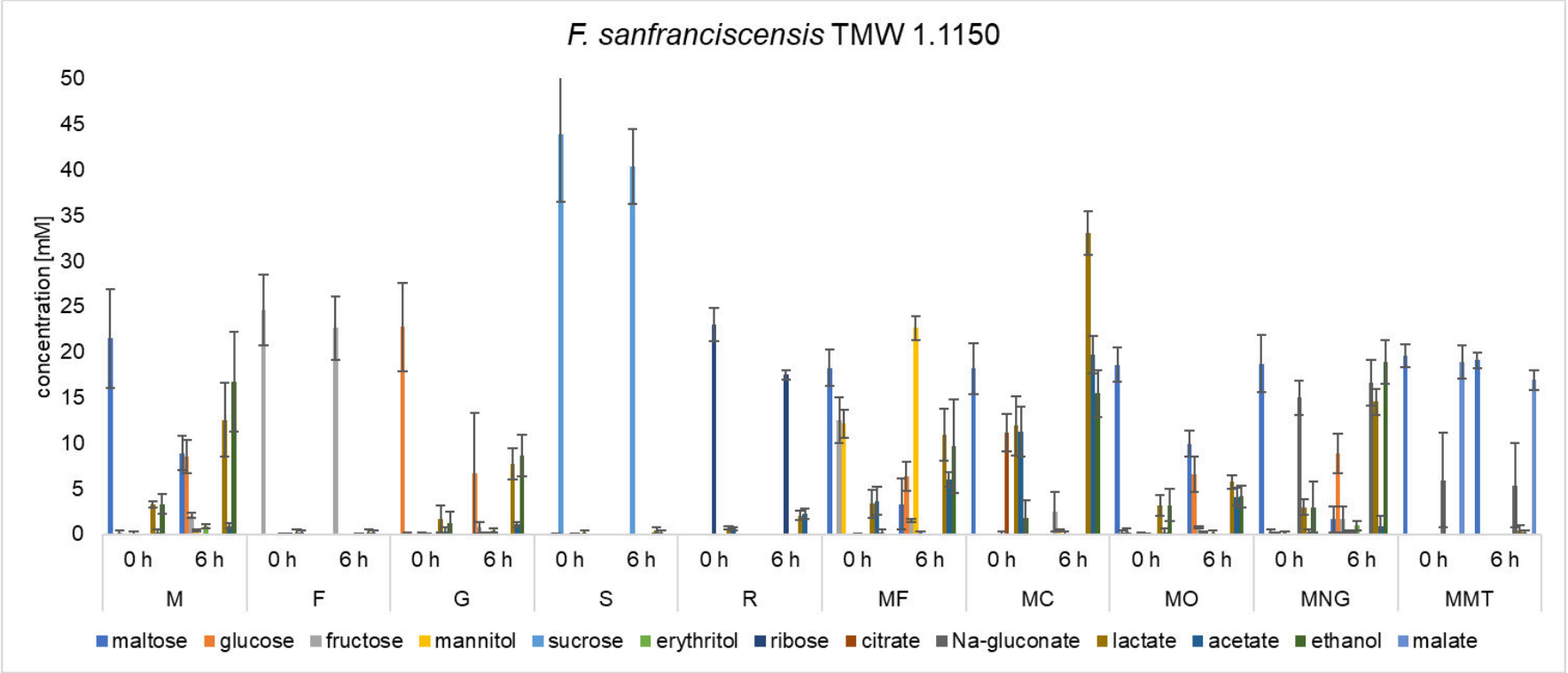

Figure A 5: HPLC analysis of *F. sanfranciscensis* TMW 1.1150 metabolism. Concentration of carbohydrates and organic acids after 0h and 6h of incubation in ¼ Ringer's solution with 20 mM of Carbon-sources and putative external electron acceptors. Incubation in: M: maltose, F: fructose; G: glucose; S: sucrose; R: ribose; MF: maltose and fructose; MC: maltose and citrate; MO: maltose and oxygen (inoculation in Erlenmeyer's flask at 150 rpm); MNG: maltose and Na-gluconate; MNT: maltose and malate. The error bars show the standard deviation of the means of three independent replicates.

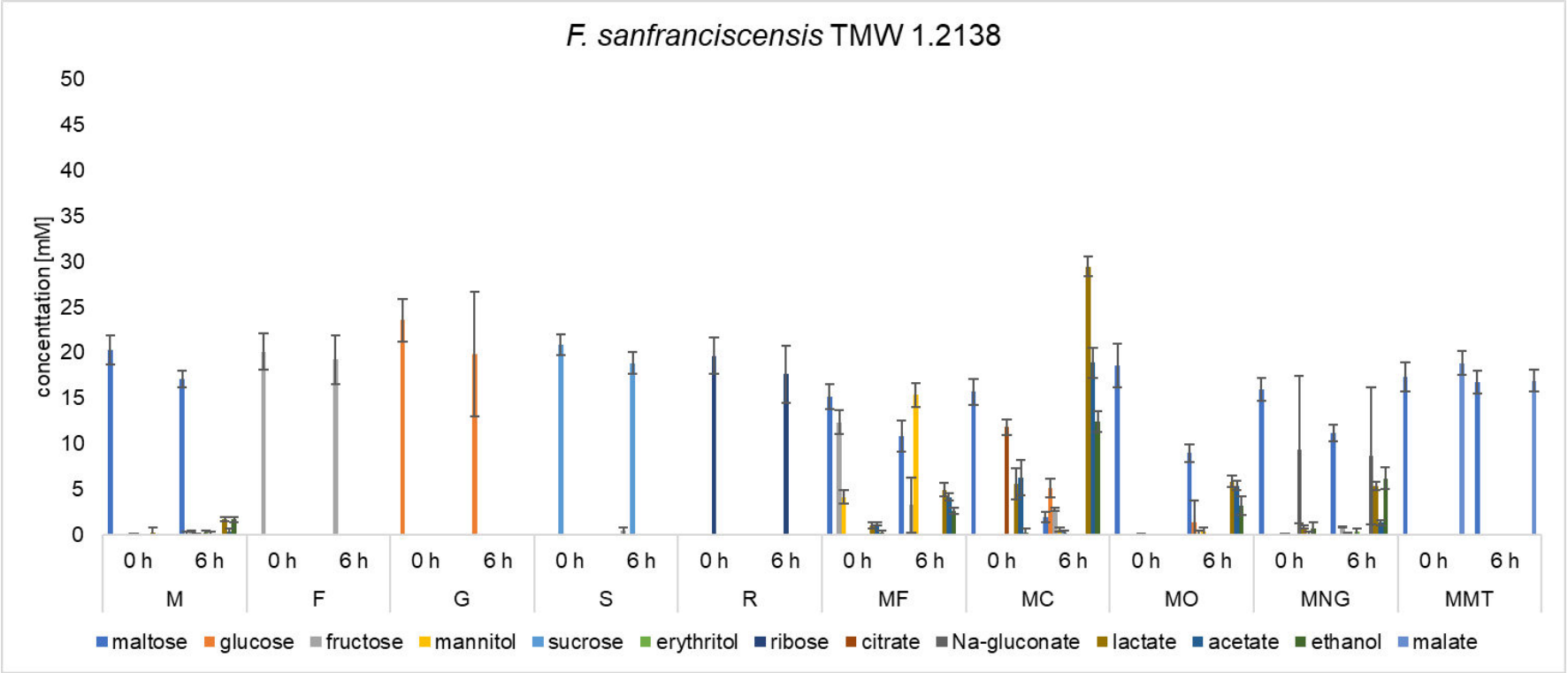

Figure A 6: HPLC analysis of *F. sanfranciscensis* TMW 1.2138 metabolism. Concentration of carbohydrates and organic acids after 0h and 6h of incubation in ¼ Ringer's solution with 20 mM of Carbon-sources and putative external electron acceptors. Incubation in: M: maltose, F: fructose; G: glucose; S: sucrose; R: ribose; MF: maltose and fructose; MC: maltose and citrate; MO: maltose and oxygen (inoculation in Erlenmeyer's flask at 150 rpm); MNG: maltose and Na-gluconate; MNT: maltose and malate. The error bars show the standard deviation of the means of three independent replicates.

13 Statutory declaration

I hereby declare that I wrote the present dissertation with the topic:

“Biodiversity and lifestyle of *Fructilactobacillus sanfranciscensis* and its interaction with yeasts in rye sourdough”

independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works. Other contributions to this work in terms of collaboration and supervised student theses are clearly indicated and acknowledged in the “publication and contribution” section.

Tuttlingen,