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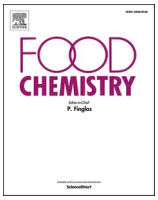

Fermentative *in situ* synthesis of isomalto/malto-polysaccharides in sourdough

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ABSTRACT

Some sourdough-derived lactic acid bacteria were shown to fermentatively synthesize potentially prebiotic isomalto/malto-polysaccharides (IMMPs) from maltodextrins. Therefore, the application of suitable starter cultures and adjusted fermentation conditions could be used to achieve a fermentative synthesis of IMMPs in sourdough, however, this has not been investigated yet. To monitor *in situ* IMMP synthesis, a new method based on *endo*-dextranase hydrolysis and quantification of released isomaltose was developed. Subsequently, it was applied to determine the amount of dextran equivalents (fermentatively formed α-1,6-linkages) in sourdoughs produced under different conditions. Of all seven investigated strains, *Limosilactobacillus reuteri* TMW 1.106 in combination with the addition of pregelatinized starch and starch-debranching enzymes produced by far the highest amount of IMMPs within 20 h of fermentation. Other changes in fermentation conditions did not significantly increase IMMP formation. However, applying adjusted conditions during sourdough fermentation may be used to increase the amount of potentially prebiotic carbohydrates in bread.

1. Introduction

The preparation of sourdough by fermentation of flour and water with lactic acid bacteria and yeast is a traditional method in the production of baked goods. For rye products, the use of sourdough is important to improve the quality of the resulting bread. Although the use of sourdough in wheat dough / bread is optional, it is often used to improve aroma, texture, shelf life, and nutritional quality (Gänzle, 2014; Gobbetti et al., 2019). The improvements are derived from different conversions of the ingredients during fermentation. Most importantly, organic acids, ethanol, and CO_2 are formed from maltose and glucose. These fermentable carbohydrates are derived from the degradation of starch by amylolytic enzymes such as α-amylase and β-amylase during fermentation (Gänzle, 2014). However, the molecular architecture of starch may also be modified during sourdough fermentation. We recently demonstrated that several lactic acid bacteria from sourdough or starch-rich environments form isomalto/malto-polysaccharides (IMMPs) from maltodextrins (Brand & Wefers, 2025). These compounds may also be formed in sourdough if these IMMP-forming lactic acid bacteria are used as starter cultures.

IMMPs consist of an α-1,4-linked chain on the reducing end and an α-1,6-linked chain at the non-reducing end and are synthesized by 4,6-α-glucanotransferases. These starch-converting enzymes belong to the GH70 family and are able to cleave α-1,4-glucosidic linkages and subsequently form α-1,6-linkages (Bai et al., 2017; Dobruchowska et al., 2012). The portion of α-1,6-linkages may reach up to 92 % and depends on the enzyme or the bacterial strain, the substrate, as well as the reaction conditions (Leemhuis et al., 2014). There are three subfamilies of the 4,6-α-glucanotransferases: GtfB, GtfC and GtfD (Gangoiti et al., 2018; Meng et al., 2016). Most of the already described enzymes belong to the GtfB subfamily which is further divided into type I and type II. GtfB type I enzymes synthesize IMMPs by forming linear α-1,6-linked chains, whereas type II enzymes form branched, reuteran-like products (Dong, Bai, Wang, et al., 2024). Most GtfB-like 4,6-α-glucanotransferases are found in lactic acid bacteria, including some strains which are commonly abundant in sourdough such as *Fructilactobacillus* (*Flb.*) *sanfranciscensis* or *Limosilactobacillus* (*Llb.*) *reuteri* (Bai et al., 2016; Brand & Wefers, 2025; Dong, Bai, Chen, et al., 2024; Dong, Bai, Wang,

Abbreviations: IMMPs, isomalto/malto-polysaccharides; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; *Llb.*, *Limosilactobacillus*; *Flb.*, *Fructilactobacillus*; *Lb.*, *Lactobacillus*; *Lpb.*, *Lactiplantibacillus*.

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et al., 2024; Dong, Wang, Li, et al., 2024; Gangoiti, van Leeuwen, Meng, et al., 2017; Ispirli et al., 2019; Kralj et al., 2011; Leemhuis et al., 2013).

The formation of IMMPs could improve bread quality in several ways. To begin with, it was shown that IMMPs result in higher dietary fiber contents depending on the portion of α-1,6-linkages (Leemhuis et al., 2014). Moreover, several studies indicated a prebiotic potential of IMMPs which may lead to different health benefits, for example a higher production of short chain fatty acids (SCFA) (Bai et al., 2016; Borewicz et al., 2024; Gu et al., 2018; Leemhuis et al., 2014; Mistry et al., 2020; Silva-Lagos et al., 2025; Wei et al., 2022). However, IMMP formation may also improve texture and shelf life of wheat bread. Nicin et al. (2024) used a recombinant 4,6-α-glucanotransferase (type I GtfB (Dong, Bai, Fan, et al., 2024)) from *Llb. reuteri* E81 in bread dough and observed a decreased digestibility of starch, improvements on hardness, stickiness, and elasticity of the bread crumb as well as positive effects on retrogradation properties. Other studies also reported a delayed retrogradation of starches that were modified by 4,6-α-glucanotransferases (Dong, Bai, Chen, et al., 2024; Li et al., 2018). Furthermore, some studies showed that the *in situ* synthesis of dextran in sourdoughs resulted in several improvements of texture and shelf life of wheat bread (Di Cagno et al., 2006; Katina et al., 2009). Due to the structural similarity of IMMPs and dextrans, these improvements on bread quality can also be expected for IMMPs.

However, previous studies applied recombinant 4,6-α-glucanotransferases in bread dough, but the fermentative *in situ* IMMP synthesis by common sourdough lactic acid bacteria has not been investigated yet. In addition, the amount of enzymatically synthesized IMMPs was not determined. Therefore, the aim of our study was to develop a method to analyze the amount of IMMPs in sourdough and bread samples, and to apply this method for the investigation of the fermentative *in situ* synthesis of IMMPs in wheat sourdough. To investigate the influence of different parameters on the IMMP formation, different starter cultures, fermentation temperatures, fermentation times, and ingredients were used.

2. Material and methods

2.1. Materials

Flb. sanfranciscensis DSM 20451, *Llb. panis* DSM 6035, *Llb. fermentum* DSM 20052, *Lactobacillus* (*Lb.*) *delbrueckii* subsp. *delbrueckii* DSM 20074, and *Lactiplantibacillus* (*Lpb.*) *argentoratensis* DSM 16365 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) GmbH, Braunschweig, Germany. *Llb. reuteri* TMW 1.106, *Flb. sanfranciscensis* TMW 1.1154, *Flb. sanfranciscensis* TMW 1.2138, and *Flb. sanfranciscensis* TMW 1.2139 were kindly provided by Prof. Rudi Vogel and Prof. Fabio Minervini. If not stated otherwise, all chemicals used were of "p.a." grade or better and were purchased from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, MA USA), VWR (Darmstadt, Germany) and Grüssing GmbH (Filsum, Germany). *Endo*-dextranase from *Chaetomium erraticum* was purchased from Merck (Darmstadt, Germany), and isoamylase from *Pseudomonas* sp. was purchased from Megazyme (Bray, Ireland). The following baking ingredients were kindly provided to us: Wheat flour type 550 by Bonback Halle (Saale) GmbH (Halle (Saale), Germany), dried yeast by VITAM Hefe-Produkt GmbH (Hameln, Germany), pullulanase (promozyme D2) by Novonesis (Bagsvaerd, Denmark), and pregelatinized flour by Kröner-Stärke GmbH (Ibbenbueren, Germany).

2.2. Sourdough preparation

The above mentioned strains were cultivated in modified Spicher medium (Capuani et al., 2012) (pH 5.4) which was composed of 10 g/L peptone from casein, 2 g/L meat extract, 7 g/L yeast extract, 2 g/L sodium gluconate, 5 g/L sodium acetate trihydrate, 5 g/L diammonium hydrogen citrate, 2.5 g/L potassium dihydrogen phosphate, 0.5 g/L cystein hydrochloride, 1 g/L tween 80, 0.2 mg/L of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin and pantothenic acid, 2 mL/L Spicher salt mix (5 g/L magnesium sulfate heptahydrate, 1.88 g/L manganese sulfate tetrahydrate, 1.25 g/L iron sulfate heptahydrate in water), 7 g/L fructose, 7 g/L glucose, and 7 g/L maltose. First, a preculture was grown statically at 30 °C or 37 °C for 1–7 days until an 1:10 dilution of the preculture reached an OD600 between 0.18 and 0.22. Subsequently, 50 mL of the preculture were centrifuged at 3100 rcf and 4 °C for 15 min to separate the cells from the medium. The cells were resuspended in 20 mL of 1 % (*w/w*) sodium chloride solution and mixed with 70 g of wheat flour type 550, 70 mL of tap water and 0.2 g of sodium chloride. To add gelatinized starch to the sourdough, 20 g of the flour were substituted with 20 g of pregelatinized flour or 20 g of dried bread (particle size: 315–630 μm) that was preswollen with 60 mL of boiling hot water. Starch debranching was carried out by adding 16.67 μL of pullulanase or 35 μL of isoamylase to the sourdough mixture. If not stated otherwise, incubation was carried out at 30 °C for 20 h. The growth of the starter culture was monitored through pH measurement. After incubation, the sourdough was frozen at −20 °C, lyophilized, and milled with a MM400 mixer mill (Retsch GmbH, Haan, Germany) with zirconia beads at 30 Hz for 30 s.

2.3. Bread preparation

To prepare bread with the produced sourdoughs, 140.2 g of sourdough were mixed with 330 g of wheat flour type 550, 210 mL of tap water, 5.3 g of sodium chloride and 4.4 g of dried yeast in a bread maker (Panasonic SD-YR 2550, Panasonic Europe B.V., Amsterdam, the Netherlands) for 20 min. After that, the bread dough was filled into a rectangular baking form and incubated at 30 °C for 2 h. The bread was baked in a household oven at 170 °C for 40 min using top and bottom heat. Subsequently, the bread was allowed to cool down to room temperature and was then cut into slices. Three slices were lyophilized and ground by using a food processor. Prior to analysis, the breadcrumbs were milled with a MM400 mixer mill (Retsch GmbH, Haan, Germany) with zirconia beads at 30 Hz for 30 s.

2.4. Analysis of α-1,6-linked glucose units in sourdough and bread samples

For each determination, 100 mg of lyophilized and milled sourdough or bread samples were suspended in 1.5 mL of deionized water. To enhance the enzymatic accessibility of the IMMPs, the samples were heated at 99 °C and 700 rpm for 1 h. Subsequently, 5 μL of *endo*-dextranase were added and enzymatic hydrolysis was carried out statically at 40 °C for 24 h. To inactivate the enzyme, the sample was heated at 95 °C for 15 min. After cooling down to room temperature, undissolved residues were removed by centrifugation at 15617 rcf for 10 min. To minimize losses of isomaltose, the residue was washed twice with 500 μL of deionized water. Subsequently, the supernatants were combined and 8 mL of 96 % (*v/v*) ethanol was added in 4 steps while shaking to precipitate polymeric starch (fragments). The precipitate was removed by centrifugation at 4468 rcf and 4 °C for 10 min, and residue was washed twice with 1 mL of ethanol/water 4:1 (*v/v*). Again, supernatants were combined and ethanol was evaporated in a vacuum concentrator (Eppendorf, Hamburg, Germany). The residual sample solution was diluted to a defined volume and analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-6000 system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Carbo-Pac PA20 column (150 mm × 3 mm inner diameter, 6.5 μm particle size, Thermo Fisher Scientific). The column temperature was 30 °C and the detector temperature 25 °C. The following gradient was used with a flow rate of 0.4 mL/min: column equilibration for 15 min with 12 mM NaOH, 0–20 min: isocratic 12 mM NaOH, 20–35 min: linear gradient from 12 mM NaOH

to 200 mM NaOH, 35–50 min: isocratic 200 mM NaOH +200 mM NaOAc, 50–80 min: isocratic 200 mM NaOH. Quantification of isomaltose was carried out by using an external calibration (1–150 μM). All samples were analyzed in triplicate. Dextran equivalents (= the amount of α-1,6-linked glucose units) were calculated from the molar concentration of isomaltose and glucose liberated from α-1,6-linked sections. The latter was included by calculating the molar concentration with a previously determined glucose-to-isomaltose ratio. Furthermore, the molar masses of anhydroglucose and anhydroisomaltose were used to convert the molar concentrations into the mass of dextran equivalents, accounting for the water added during hydrolysis.

To determine the glucose-to-isomaltose ratio, the molar concentrations of isomaltose and glucose which result from the *endo*-dextranase hydrolysis of linear α-1,6-linked glucopyranose chains were determined. For this analysis, the completely linear dextran from *Ligilactobacillus animalis* TMW 1.971 dextransucrase (Müller & Wefers, 2024; Pittrof et al., 2021) was hydrolyzed and analyzed by HPAEC-PAD as described above. The amounts of isomaltose and glucose were determined by using external calibrations (1–150 μM).

To verify the method, the linear dextran from *Ligilactobacillus animalis* TMW 1.971 dextransucrase was added to the reference sourdough bread (1 mg dextran to 100 mg bread) and the sample was analyzed as described above.

3. Results and discussion

3.1. Development of a method for the estimation of α-1,6-linked glucose units in sourdough and bread

To monitor IMMP synthesis in sourdough and bread, we developed a method based on specific partial enzymatic hydrolysis. Because the α-1,6-linked sections distinguish IMMPs from starch and because they primarily act as dietary fiber (Leemhuis et al., 2014), our method focused on the analysis of these structural elements. The cleavage of these dextran-like chains can be achieved by using *endo*-dextranase. The lactic acid bacteria used in this study form IMMPs through GtfB type I 4,6-α-glucanotransferases, thus, the α-1,6-linked sections are expected to be completely linear which was also suggested by the results of our previous study (Brand & Wefers, 2025). Consequently, an enzymatic hydrolysis of these dextran-like chains with *endo*-dextranase yields isomaltose and glucose as the sole products (Pittrof et al., 2021). Isomaltose should not be present in the raw material, thus, it can be used to estimate the portion of α-1,6-linked glucose units which are derived from IMMP synthesis. The absence of isomaltose in the raw material was confirmed by analyzing the flour used in this study. A representative HPAEC-PAD chromatogram of the enzymatic hydrolysate resulting from an IMMP-containing sourdough sample and the flour is shown in Fig. S1. However, glucose is ubiquitous in sourdough and bread samples, therefore, the portion of glucose derived from α-1,6-linked glucose units cannot be measured directly. Nevertheless, the relatively constant ratio of isomaltose and glucose after *endo*-dextranase hydrolysis can be used to estimate the glucose derived from dextran-like chains. To determine this glucose-to-isomaltose ratio, the linear dextran produced by *Ligilactobacillus animalis* TMW 1.971 dextransucrase (Müller & Wefers, 2024; Pittrof et al., 2021) was hydrolyzed under the same conditions. A molar glucose-to-isomaltose ratio of 0.320 ± 0.004 was determined from the amounts quantified in the hydrolysates (Table S1). This ratio was subsequently used to calculate the glucose derived from the hydrolysis of dextran-like structural elements from the amount of isomaltose (glucose-to-isomaltose ratio * molar amount of isomaltose = molar amount of glucose derived from *endo*-dextranase hydrolysis). The molar amounts of isomaltose and glucose were then used to calculate the dextran equivalents in mg (using the molar masses of anhydroisomaltose and anhydroglucose). To verify this approach, the linear dextran produced by *Ligilactobacillus animalis* TMW 1.971 dextransucrase was mixed with an IMMP-free reference bread. This sample was analyzed for the dextran equivalents by using the sample workup developed in this study, which included the removal of (un)dissolved, polymeric ingredients by ethanol precipitation and centrifugation as well as the removal of ethanol by evaporation. The quantification of isomaltose by HPAEC-PAD and the calculation of dextran equivalents as described above showed that 76.1 % of the dextran was found as dextran equivalents (Table S2). Therefore, losses during the sample workup were comparably low and isomaltose can be used for the estimation of α-1,6-linked glucose units in sourdough and bread samples. However, the content of the dextran-like structural elements may be slightly underestimated, which could be the result of incomplete enzymatic hydrolysis or some losses during the sample workup. Furthermore, the portion of isomaltose may show some slight variation depending on the structure and length of the substrate. We already demonstrated that the structure and length of the α-1,6-linked sections in IMMPs are quite different, depending on the bacterial strain, the substrate and the fermentation conditions (Brand & Wefers, 2025). Nevertheless, the determination of dextran equivalents provides a good semiquantitative estimation of the content of α-1,6-linked glucose units in complex food matrices.

3.2. Different starter cultures

In a first step, the 8 lactic acid bacteria (LAB) listed in section 2.1 were used as starter cultures in wheat sourdoughs and the *in situ* IMMP synthesis was analyzed. The strains originate from sourdough or other starch-rich environments (Brandt et al., 2020; Bringel et al., 2005; Rogalski et al., 2021; Rogosa & Hansen, 1971; Tieking et al., 2003; Wiese et al., 1996) and all of them were shown to synthesize IMMPs fermentatively (Brand & Wefers, 2025). In addition, a sourdough with *Flb. sanfranciscensis* TMW 1.2138 was prepared as control, because this strain is not able to synthesize IMMPs (Brand & Wefers, 2025). All sourdoughs were fermented at 30 °C for 20 h. To ensure the growth of the starter cultures, the pH of the sourdoughs was monitored. In each sourdough, the pH decreased significantly from approx. 5.6 to 3.4 (data not shown). In contrast, the pH of a flour water mixture which was incubated at 30 °C for 20 h only decreased to 5.4. Therefore, all strains showed significant growth in sourdough. To assess the IMMP formation during fermentation, the dextran equivalents were determined in all sourdoughs. The results are shown in Fig. 1.

In contrast to the non-fermented flour, the reference sourdough with *Flb. sanfranciscensis* TMW 1.2138 contained low amounts of α-1,6-linked glucose units (dextran equivalent of 7.1 mg / 100 g sourdough). This may be due to the growth of flour-derived, IMMP-forming lactic acid bacteria at low microbial density or other chemical or enzymatic transglycosylation reactions. However, all other sourdough samples showed clearly higher dextran equivalents. Between 45 and 72 mg dextran equivalents per 100 g of sourdough were found in the sourdoughs with *Flb. sanfranciscensis* TMW 1.2139, *Flb. sanfranciscensis* TMW 1.1154, *Flb. sanfranciscensis* DSM 20451, *Llb. panis* DSM 6035, *Lpb. argentoratensis* DSM 16365, *Llb. fermentum* DSM 20052, and *Lb. delbrueckii* subsp. *delbrueckii* DSM 20074. In contrast, the sourdough with *Llb. reuteri* TMW 1.106 contained even more α-1,6-linked glucose units (128 mg dextran equivalents per 100 g of sourdough). Therefore, it was confirmed that all strains which synthesized IMMPs from maltodextrin also formed IMMPs in sourdough. Although we observed varying portions of α-1,6-linkages after the fermentative synthesis from maltodextrin in our previous study (Brand & Wefers, 2025), all strains but *Llb. reuteri* TMW 1.106 led to quite similar portions of α-1,6-linked glucose units in sourdough. Because *Llb. reuteri* TMW 1.106 was able to produce by far the highest amount of α-1,6-linked glucose units, this strain was used as starter culture in the following experiments.

3.3. Fermentation temperature

Llb. reuteri strains are known to preferentially grow at higher fermentation temperatures up to 45 °C (Kandler et al., 1980), and it is

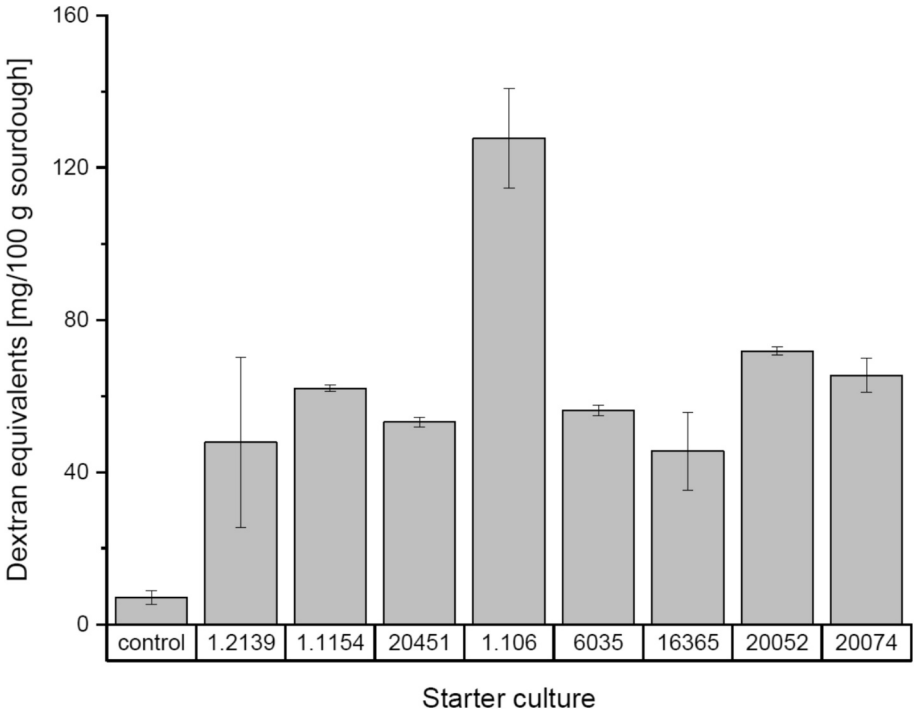

Fig. 1. Dextran equivalents (see sections 2.4 and 3.1) in sourdoughs which were produced by using different starter cultures. Control = *Fructilactobacillus* (*Flb.*) *sanfranciscensis* TMW 1.2138, 1.2139 = *Flb. sanfranciscensis* TMW 1.2139, 1.1154 = *Flb. sanfranciscensis* TMW 1.1154, 20451 = *Flb. sanfranciscensis* DSM 20451, 1.106 = *Limosilactobacillus* (*Llb.*) *reuteri* TMW 1.106, 6035 = *Llb. panis* DSM 6035, 16365 = *Lactiplantibacillus* (*Lpb.*) *argentoratensis* DSM 16365, 20052 = *Llb. fermentum* DSM 20052, 20074 = *Lactobacillus* (*Lb.*) *delbrueckii* subsp. *delbrueckii* DSM 20074.

unclear under which growth conditions 4,6-α-glucanotransferases are expressed. Therefore, it is possible that improved bacterial growth leads to an increased synthesis of IMMPs. Furthermore, the properties of the respective 4,6-α-glucanotransferases, for example the ratio between hydrolysis and transferase activity, may be influenced by the temperature (Bai et al., 2015). Thus, the effect of a higher fermentation temperature on the formation of IMMPs in sourdough was investigated. For this purpose, four sourdoughs were fermented at 30 °C, 37 °C, 40 °C, and 42 °C with *Llb. reuteri* TMW 1.106 as starter culture. Two samples of each sourdough were taken at two different times and analyzed for their dextran equivalents. The results are shown in Fig. 2.

Despite some minor variations between the dextran equivalents, which ranged from 128 mg to 174 mg dextran equivalents per 100 g of sourdough, the results did not allow for an unambiguous correlation between the fermentation temperature and the *in situ* synthesis of IMMPs in sourdough by *Llb. reuteri* TMW 1.106. The observed minor variations in the dextran equivalents (*e.g.* for the samples obtained from 30 °C and 37 °C after 20 h) could be caused by slight changes in other parameters. For example, slight differences in the handling of the starter cultures may impact IMMP synthesis, as this may influence the expression of 4,6-α-glucanotransferases.

3.4. Fermentation time

It has already been demonstrated that 4,6-α-glucanotransferases are cell-associated extracellular enzymes (Bai et al., 2016; Gangoiti, van Leeuwen, Gerwig, et al., 2017; Gangoiti, van Leeuwen, Meng, et al., 2017; Meng et al., 2016). Therefore, the course of the IMMP formation may be independent from the bacterial growth (even dead cells may contribute due to active enzymes). Thus, even longer fermentation times may result in higher portions of IMMPs. To analyze IMMP formation during prolonged fermentation, a wheat sourdough with *Llb. reuteri* TMW 1.106 was fermented at 37 °C for 192 h and several samples were taken at various fermentation times. The sourdough samples were analyzed for their dextran equivalents. The results are shown in Fig. 3.

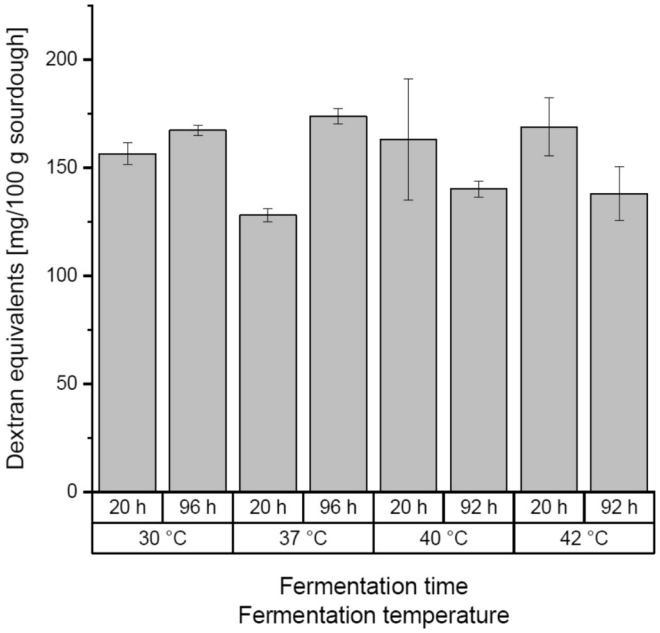

Fig. 2. Dextran equivalents (see sections 2.4 and 3.1) in sourdoughs prepared with *Limosilactobacillus (Llb.) reuteri* TMW 1.106 as starter culture, fermented at different temperatures and times.

At the beginning of the fermentation, α-1,6-glucosidic linkages were not detected. During the first 20 h of fermentation, the dextran equivalents increased significantly to 128 mg / 100 g of sourdough. After 26 h of fermentation, the amount of dextran equivalents was only slightly increased to 173 mg / 100 g of sourdough, but no further increase was detected for another 7 days. Consequently, a fermentation of 20–26 h is sufficient to obtain a high portion of α-1,6-glucosidic linkages. However,

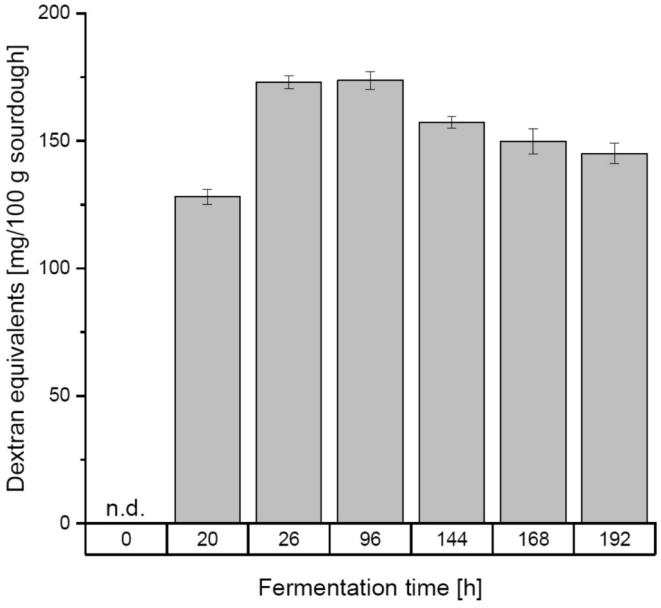

Fig. 3. Dextran equivalents (see sections 2.4 and 3.1) in a sourdough with the starter culture *Limosilactobacillus (Llb.) reuteri* TMW 1.106 after different fermentation times. Fermentation was carried out at 37 °C. n.d. = not detected.

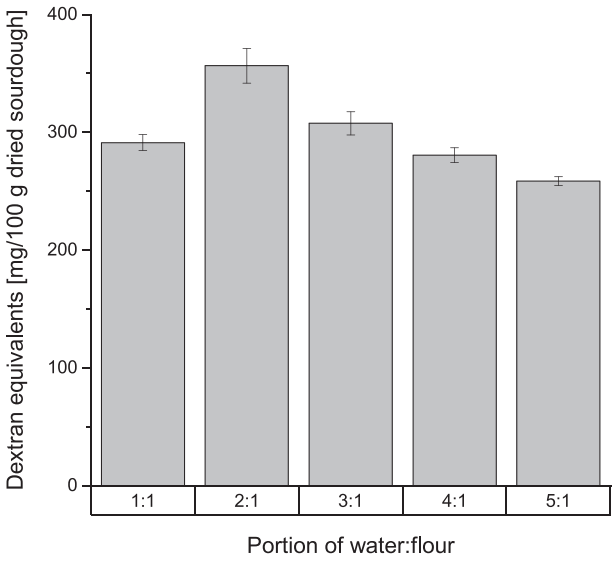

Fig. 4. Dextran equivalents (see sections 2.4 and 3.1) in different sourdoughs which were prepared with *Limosilactobacillus (Llb.) reuteri* TMW 1.106 as starter culture as well as varying portions of water and flour. Fermentation was carried out at 37 °C for 20 h.

further experiments would need to be conducted to analyze the relation between the growth of the starter culture and the formation of IMMPs.

3.5. Water content

The amount of water impacts the viscosity of the sourdough and water availability, which could in turn have an effect on the mobility and activity of the bacteria and the 4,6-α-glucanotransferases. A higher mobility could lead to an improved conversion of starch and therefore to a higher portion of α-1,6-linkages. In contrast, a higher water content results in lower substrate concentrations which could also influence the formation of α-1,6-linkages by 4,6-α-glucanotransferases. To investigate how the *in situ* synthesis of IMMPs is related to the water content in sourdough, five wheat sourdoughs with *Llb. reuteri* TMW 1.106 as starter culture and different portions of water and flour were fermented at 37 °C for 20 h. After lyophilization, the portion of α-1,6-linked glucose units was analyzed. In this case, the dextran equivalents were calculated on a dry matter basis (Fig. 4), because the dextran equivalents in the undried sourdough are strongly influenced by the significantly different water contents. The results are shown in Fig. 4.

Increasing the water to flour ratio from 1:1 to 2:1 led to a slight increase of the dextran equivalents from 291 mg / 100 g dried sourdough to 356 mg / 100 g dried sourdough. However, higher water contents did not lead to increasing portions of α-1,6-linkages and all samples showed dextran equivalents between 259 and 356 mg / 100 g dried sourdough. Because the differences between all samples are relatively small (within 97 mg / 100 g dried sourdough), it can be concluded that the water content has no significant effect on the *in situ* synthesis of IMMPs.

3.6. Addition of enzymes or pregelatinized ingredients

Previous research showed that the synthesis of IMMPs can be improved significantly by using solubilized amylose or by debranching soluble starch (Leemhuis et al., 2014). Therefore, isoamylase and pullulanase were added to the sourdough mix to enzymatically debranch amylopectin during fermentation and investigate the impact on the *in situ* synthesis of IMMPs. Furthermore, a sourdough in which a portion of the flour was replaced with pregelatinized flour was prepared, because most starch-active enzymes degrade gelatinized starch with a significantly higher efficiency than granular starch (Dong, Bai, Chen, et al., 2024; Zhong et al., 2022). Additionally, bread was added to one sourdough, because it contains high portions of gelatinized starch due to the baking process: Immonen et al. demonstrated that about 74 % of the starch in surplus bread can be enzymatically hydrolyzed (Immonen et al., 2021). For comparison, only 10 % of the starch in wheat flour is enzymatically degradable (Gómez & Martinez, 2023). The bread was prepared by using a sourdough with the IMMP-negative strain *Flb. sanfranciscensis* TMW 1.2138 as a starter culture. The enzymes as well as the pregelatinized ingredients were used isolated and in combination, and all sourdoughs were analyzed for their dextran equivalents. The results are shown in Fig. 5.

By adding the starch-debranching enzymes pullulanase or isoamylase, the dextran equivalents in sourdough were increased from 128 mg to 186 mg and 247 mg per 100 g of sourdough. Thus, debranching of starch improved the formation of IMMPs in sourdough. The observed variation between the two enzymes could result from differences in the amounts applied and their specific activity on starch. The addition of pregelatinized flour or bread led to significantly increased dextran equivalents (352 mg and 317 mg / 100 g sourdough, respectively). Therefore, the enzymatic accessibility of starch is also a major factor for the IMMP formation in sourdough. Based on these results, it was expected that the addition of enzymatically accessible, gelatinized starch also improves starch debranching and that the availability of soluble linear starch fragments results in an improved conversion of starch by 4,6-α-glucanotransferases. As expected, the combination of pullulanase and pregelatinized flour led to a significant increase in the amount of α-1,6-linked glucose units (712 mg dextran equivalents / 100 g sourdough). In contrast, the combination of bread and pullulanase only showed little differences to the sourdough without pullulanase after 20 h fermentation. However, a significantly higher content of α-1,6-linked glucose units was detected after another day of fermentation. Because our method only detects the α-1,6-linked sections of IMMPs, which may also be slightly underestimated, it can be expected that the actual IMMP contents are even higher. Consequently, the combination of an IMMP-producing starter culture, enzymatic starch debranching, and enzymatically accessible starch offers the possibility to reach IMMP contents close to 1 %.

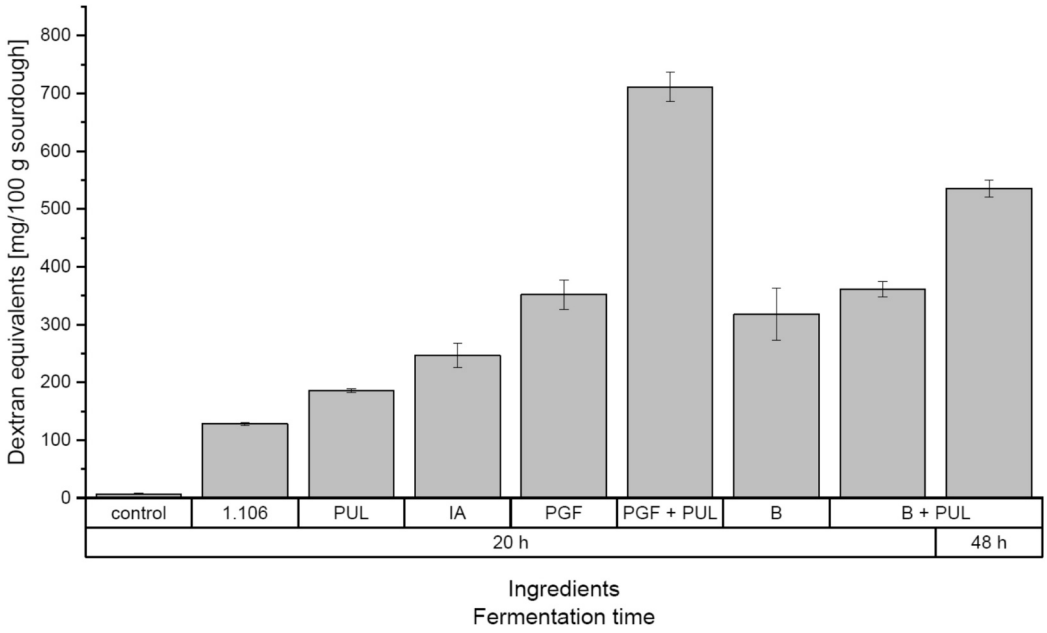

Fig. 5. Dextran equivalents in sourdoughs prepared with *Limosilactobacillus (Llb.) reuteri* TMW 1.106 as starter culture at 37 °C as well as different enzymes and/or ingredients. The IMMP-negative control (sourdough with *Fructilactobacillus (Flb.) sanfranciscensis* TMW 1.2138 as starter culture, fermented at 30 °C) and the starter culture in the standard recipe are shown for comparison. PUL = pullulanase, IA = isoamylase, PGF = a portion of the flour was substituted with pregelatinized flour, B = a portion of the flour was substituted with bread.

3.7. IMMP synthesis during breadmaking

To investigate if the *in situ* IMMP synthesis also occurs during breadmaking, a sourdough with *Llb. reuteri* TMW 1.106 as starter culture, with and without the addition of bread and pullulanase, was used to prepare sourdough breads with 20 % sourdough. Furthermore, a control bread was prepared by using the IMMP-negative strain *Flb. sanfranciscensis* TMW 1.2138 as starter culture. All sourdoughs as well as the respective breads were analyzed for the amount of α-1,6-linked glucose units. The results are shown in Fig. 6.

Our results clearly demonstrated that IMMPs are also formed during breadmaking, because clearly more α-1,6-linked glucose units were found than it would be expected from the amount of sourdough added. The control bread also contained small amounts of α-1,6-linked glucose units (22 mg dextran equivalents / 100 g sourdough) which may be derived from the growth of other lactic acid bacteria or other transglycosylation reactions. However, significantly higher amounts of α-1,6-linked glucose units were found in the other sourdough breads. In the bread prepared solely with *Llb. reuteri* TMW 1.106 as starter culture, the dextran equivalents formed during breadmaking clearly exceeded the ones brought in from sourdough. Nevertheless, the final amount of α-1,6-linked glucose units in the bread is comparably low (77 mg dextran equivalents / 100 g). In contrast, the dextran equivalents detected in the bread which was prepared from sourdough with bread and pullulanase was clearly higher (197 mg dextran equivalents / 100 g). Interestingly, a higher portion was formed in the sourdough than during breadmaking. Therefore, the extent of the IMMP synthesis during bread making could probably be increased by varying process parameters such as fermentation time and temperature of the final bread dough, or the baking temperatures. Further investigations, for example on the impact of fermentation temperature and duration as well as baking on the activity of the bacterial 4,6-α-glucanotransferases in bread dough, are needed to better understand and further improve the *in situ* synthesis of IMMPs.

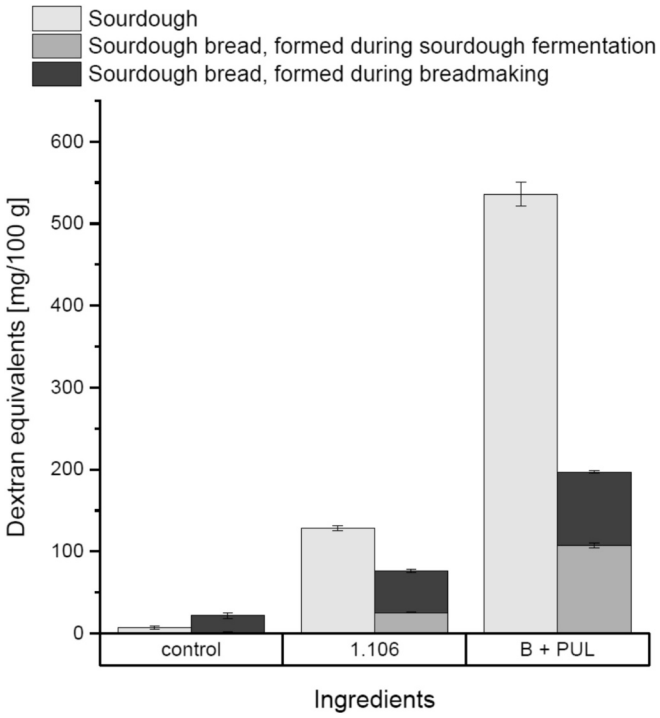

Fig. 6. Dextran equivalents in sourdoughs prepared with *Limosilactobacillus (Llb.) reuteri* TMW 1.106 as starter culture at 37 °C, with and without the addition of bread (B) and pullulanase (PUL), as well as in the breads made from the respective sourdoughs. The dextran equivalents in the sourdough prepared with the IMMP-negative control (*Fructilactobacillus (Flb.) sanfranciscensis* TMW 1.2138 as starter culture, fermented at 30 °C), as well as the respective bread are shown for comparison. The dextran equivalents in the breads are divided into those brought in from the sourdoughs (calculated *via* the absolute amount of dextran equivalents added to the bread dough) and those formed during the bread-making process (calculated by the total dextran equivalents minus the dextran equivalents from the sourdough).

4. Conclusion

Altogether, we demonstrated that it is possible to fermentatively synthesize significant amounts of IMMPs in sourdough and that the extent of IMMP formation can be measured by the developed enzymatic-chromatographic method. Several of the IMMP-forming starter cultures were able to synthesize IMMPs *in situ*, but *Llb. reuteri* TMW 1.106 was shown to form the highest portion of α-1,6-linked glucose units. In addition, the *in situ* synthesis of IMMPs in sourdough can be increased by adjusting fermentation conditions and by adding debranching enzymes or gelatinized starch. In our experiments, the portion of α-1,6-linked glucose units was up to 0.71 %, and it is likely that higher amounts can be achieved by further improving these parameters. Because the *in situ* IMMP synthesis is not limited to the sourdough fermentation, but can also occur during breadmaking, an adjustment of the corresponding conditions could further increase the IMMP amounts in bread. Consequently, sourdough fermentation with suitable starter cultures and conditions offers the possibility to form bioactive carbohydrates and to potentially improve the nutritional properties of the corresponding products. Furthermore, although additional research is required, the *in situ* synthesis of IMMPs by lactic acid bacteria in sourdough could also be used to improve the texture and shelf life of bread.

CRediT authorship contribution statement

Nele Brand: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Florian M. Stadler:** Investigation, Formal analysis. **Larissa Hahn:** Investigation. **Zoya Borisova:** Investigation. **Daniel Wefers:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

HPAEC-PAD chromatograms of the hydrolysates derived from an IMMP-containing sourdough as well as the flour used in this study, experimental data for the determination of the molar glucose-to-isomaltose ratio and the recovery of dextran equivalents in bread matrix.

Data availability

Data will be made available on request.

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