Effect of copy number of the *spoVA*^{2mob} operon, sourdough and reutericyclin on ropy bread spoilage caused by *Bacillus* spp.

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ABSTRACT

Bacillus spp. cause ropy bread spoilage of bread, which is characterized by a rotten fruity odor, followed by discoloration and degradation of the crumb. *Bacillus* spp. are wheat grain endophytes and form heat resistant endospores, therefore, process hygiene and heating during baking do not prevent ropy spoilage. This study used 8 strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* to determine whether the presence and the copy number of *spoVA*^{2mob} operon influences survival after baking; in addition, the spoilage phenotype was correlated with the presence of amylolytic enzymes in genomes of *Bacillus* spp.. The presence and copy number of the *spoVA*^{2mob} operon had only a minor effect on survival of *Bacillus* endospores. Strains of *B. amyloliquefaciens* caused ropy spoilage faster than strains of *B. subtilis*, this difference correlated to the number and type of extracellular amylases encoded in the genomes of the strains of *B. amyloliquefaciens* and *B. subtilis*. The inhibitory effect of sourdough on ropy spoilage was determined by addition of 3 – 24% sourdough fermented with *L. reuteri* TMW1.656. Addition of 12% and 24% sourdough, corresponding to a bread pH of 5.93 ± 0.041 and 5.53 ± 0.040, respectively, delayed ropy spoilage for 2 and more than 5 d, respectively. The comparison of addition of 12% sourdough fermented with the reutericyclin producing *L. reuteri* TMW1.656 and the isogenic reutericyclin-negative strain *L. reuteri* TMW1.656Δ*gtfA*Δ*rtcN* demonstrated that reutericyclin produced in sourdough inhibits growth of *Bacillus* in bread. In conclusion, sourdough inhibits germination of *Bacillus* spores in bread and the effect of sourdough is enhanced by reutericyclin.

KEY WORDS: *Bacillus* spp., Ropy spoilage, Extracellular amylase, Sourdough, Reutericyclin

1. INTRODUCTION

Bread or steamed bread is a staple of the daily diet in most regions of the world with a temperate climate (Kourkouta et al., 2017). Ropy spoilage of bread is caused by strains of *Bacillus* spp. including *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis* (Pepe et al., 2003; Sorokulova et al., 2003; Valerio et al., 2015, 2012). *Bacillus* endospores are present in flour and survive after baking. Their growth and production of amylases and proteases initially generate a typical fruity odor, subsequently result in a sticky and stringy crumb and slime formation, making the bread inedible (Corsetti et al., 2000; Pepe et al., 2003).

Bacillus endospores have been isolated from bakery environment and also from raw materials, such as wheat flour, yeast and bread improvers (Bailey and von Holy, 1993; Pepe et al., 2003). Contamination of wheat flour with *Bacillus* endospores relates to the stable occurrence of these organisms as part of commensal microbiota of plants including wheat (Chen et al., 2007; Fan et al., 2011; Jamal et al., 2019; Tilak and Reddy, 2006; Vessey and Buss, 2002), and to the presence of *Bacillus* as member of seed-borne endophytic microbial community (Robinson et al., 2016; Shahzad et al., 2016). *Bacillus* endospores remain viable throughout storage and processing of grains (Fangio et al., 2010; Needham et al., 2005) which makes the contamination of flour not accidental but unavoidable.

Endospores of *Bacillus* species are heat resistant (Setlow, 2006, 2003) and not inactivated during baking, where the crumb is heated to a maximum temperature of 100 °C for a few minutes. Strains of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* were reported to exhibit comparable heat resistance, amylase activity and spoilage ability (Setlow, 2006; Valerio et al., 2012). However, a large intra-species variation of heat resistance of *Bacillus* endospores was attributed to the presence the *spoVA*^{2mob}

operon (Berendsen et al., 2016). Strains of *B. amyloliquefaciens* and *B. subtilis* contain up to three copies of the *spoVA*^{2mob} operons and the heat resistance of spores increased with an increase of the copy number (Berendsen et al., 2016; Wang et al., 2018). A relationship between the *spoVA*^{2mob} operon and the potential to cause ropy spoilage is further suggested by the observation that all strains of *B. subtilis* and *B. amyloliquefaciens* that were isolated by Röcken and Spicher (1993) from ropy bread were later identified to harbor multiple copies of the *spoVA*^{2mob} operons (Li et al., 2019). A contribution of the *spoVA*^{2mob} operon to the ability of bacilli to survive baking, however, has not been determined experimentally.

Ropy spoilage of bread develops rapidly at warm and humid conditions (Vaičiulytė-Funk et al., 2015), and is prevented by chemical preservatives (Saranraj and Geetha, 2012) or the use of sourdough. In past years, the use of sourdough or sourdough products in baking has been re-established as the default method of bread production (Anonymous, 2019a, 2019b). Sourdough prevents ropy spoilage (Pepe et al., 2003; Sadeghi, 2008) through acidification and the inhibitory activity of undissociated organic acids (Rosenquist and Hansen, 1998).

Sourdough isolates of lactic acid bacteria were reported to produce bacteriocins with activity against rope-forming bacilli (Digaitiene et al., 2012; Pepe et al., 2003), however, their activity in bread is limited (Rosenquist and Hansen, 1998), likely because the bacteriocins are inactivated by proteases and thiol exchange reactions in sourdough (Gänzle, 2014). Some strains of *Limosilactobacillus reuteri*, previously named *Lactobacillus reuteri* (Zheng et al., 2020), are stable components of type II sourdoughs (Zheng et al., 2016) and produce reutericyclin, a heat stable and antimicrobial tetramic acid with activity against a wide range of gram-positive bacteria including rope-forming bacilli (Gänzle et al., 2000). Reutericyclin is produced in active concentration in

sourdough fermentation (Gänzle and Vogel, 2003), but a possible contribution to bread preservation has not been demonstrated. Therefore, the aim of this study was to determine whether ropy spoilage of bread is inhibited by the use of sourdough fermented with reutericyclin-producing *L. reuteri* TMW 1.656. To identify relevant spoilage organisms, strains of *Bacillus* spp. that were previously isolated from ropy bread or other cereals products were characterized with respect to the copy number of the spoVA^{2mob} operon, their heat resistance during the baking process, and with respect to the presence of extracellular amylases that may relate to the spoilage phenotype.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

The strains used in this study and their origin are listed in Table 1. Strains were maintained at −80 °C in 20% glycerol. Strains of *Limosilactobacillus reuteri* were incubated in modified de Man, Rogosa, and Sharpe (mMRS, Gänzle et al., 2000) medium at 37 °C for 24 h. The mutant strain of *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* was constructed by disruption of the reuteransucrase GtfA in *L. reuteri* TMW1.656Δ*rtcN* as described (Chen et al., 2016; Lin et al., 2015). Strains of *Bacillus* were grown aerobically on Luria-Bertani (LB) agar plates at 37 °C for 18 h.

2.2. Preparation of *Bacillus* endospore suspensions

Strains of *Bacillus* were surface plated on LB plates and incubated at 37 °C for 5 d to ensure the sporulation of more than 90% of cells (Li et al., 2019; Margosch et al., 2004). Sporulation was confirmed by staining with the Schaeffer-Fulton method (malachite green solution of 5 % and 0.1 % safranin) which results in green staining of spores and red staining of vegetative cells. Microscopic examination of spore suspensions confirmed that 90 to 99 % of the cells contained spores. Cells from 5 d old cultures on LB plates were collected by flooding the surface of the plates with 5 ml sterile distilled

water twice. The spore suspensions were washed with sterile water for four times by centrifugation at 3,000×g for 15 min at 5 °C and resuspended in sterile distilled water. Between the second and third wash cycles, the spore suspensions were treated at 80 °C for 10 min to kill vegetative cells. The spore suspensions were stored at −80 °C until further use (Li et al., 2019; Paidhungat et al., 2002).

2.3. Determination of survival of strains of *Bacillus* spp. with different copy number of the *spoVA*^{2mob} operons after baking.

To determine the contribution of the *spoVA*^{2mob} operon or multiple copies of the operons on survival after baking, we inoculated wheat dough with spores of 8 strains of *Bacillus* with a different copy number of the *spoVA*^{2mob} operon, and determined the survival after baking. The recipe of wheat bread is shown in Table 2. Ingredients were mixed with a KitchenAid 3 Qt mixer for 1 min (slow) and 14 min (fast), followed by a bulk proof at 25 °C for 60 min. The dough was divided into pieces of 150 g and each piece was rolled into a rectangular dough sheet, and inoculated by spreading 1.5 ml of spore suspensions of *Bacillus* spp. with a viable spore count of 10^8 spores/ml. Subsequently, the dough was rolled, shaped, proofed for 60 min at 32 °C, and baked at 180 °C for 20 min. The internal temperature of bread during baking was monitored by a thermocouple that was inserted to the bread during baking. The temperature profile during baking process is shown in Figure 1. Samples were taken from the dough before baking, after baking and cooling at room temperature for 1 h. Total aerobic plate counts of bread samples were determined by mixing 10 g of bread with 90 ml peptone in a stomacher. Serial dilutions in peptone water were surface plated on LB plates and were incubated at 37 °C for 18 h.

2.4. Determination of the spoilage phenotype of different strains of *Bacillus*

inoculated on dough

To monitor the typical characteristics of ropy spoilage including appearance, aroma and bacterial growth, two slices of each bread were placed in Petri dishes and stored at 30 °C for 7 days in a sealed container with 10 % NaCl solution to maintain the water activity (a_w) at 0.95 throughout storage. The slices were examined daily. Two pieces of bread of each sample were analyzed each day and the experiment was repeated 3 times. Representative images of slices of bread inoculated with *B. subtilis* FAD109 or *B. amyloliquefaciens* FUA2154 are shown in Figure 2.

2.5. Determination of the spoilage phenotype of different strains of *Bacillus* inoculated on bread

Uncontaminated bread was prepared following a similar procedure as indicated above. After baking and cooling at room temperature, the bread was sliced. Then 30 µl of spore suspensions from 5 strains of *Bacillus* were inoculated at 5 different spots on one bread slice, and 3 replicates of slices were prepared under the same procedure. Uninoculated slices that were incubated at the same conditions served as control.

The inoculated bread slices were placed in petri dishes in containers with 10 % NaCl solution (same as before) and incubated at 30 °C for 5 days. The bacterial growth and characteristics of ropy spoilage such as discoloration, visible lines, soft and sticky crumb, and slime were identified throughout five days of storage (Figure 2). The experiment was repeated 3 times.

2.6. Analysis of amylases encoded in the genomes of *Bacillus* spp.

Sequences of extracellular amylases and glucoamylases in *Bacillus* species were retrieved from the Universal Protein Resource (UniProt) (https://www.uniprot.org/) database. These protein sequences were used as query sequences for BLAST analysis

of whole genome sequences from the strains of *Bacillus* spp. (Li et al., 2019) used in this study. A different complement of amylases was found in *B. amyloliquefaciens* and *B. subtilis*. Therefore, we further blasted these sequences of amylases against genome sequences of additional *Bacillus* strains, including 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of *B. velezensis* in total (Li et al., 2019).

2.7. Preparation of sourdough

Anti-rope activity has been considered as an important characteristic of some lactic acid bacteria (Pepe et al., 2003). The reutericyclin positive *L. reuteri* TMW1.656 was used as reference strain and its anti-rope activity was compared to *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA*. After incubating the two strains in mMRS medium at 37 °C for overnight, 10 ml of culture were added into a 15 ml centrifuge tube, respectively. The culture was washed by centrifugation at 5000 rpm for 3 min and resuspended in 10 ml sterile tap water. The 10 ml of washed strain culture was mixed thoroughly with 10 g of reweighed white flour. The mixture was fermented at 30 °C for 24 h, corresponding to a sourdough pH of 4.60 ± 0.02 and 4.57 ± 0.03 for *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN*Δ*gtfA*, respectively. The fermented sourdough was placed at −20 °C overnight and then freeze-dried. After freeze drying, the dry sourdough was ground and stored at −20 °C until use.

2.8. Evaluation of the effect of sourdough on ropy spoilage

On the basis of slime production and heat resistance, *B. amyloliquefaciens* FAD 99 was selected as an indicator to determine the antagonistic activities of different sourdough dosage. Sourdough bread was prepared following the recipe in Table 2 by adding 0, 3, 6, 12 or 24 % freeze-dried sourdough made with *L. reuteri* TMW1.656, respectively (substitute white flour with freeze-dried sourdough). Each dough was inoculated with 1 ml spore suspension of *B. amyloliquefaciens* FAD 99 as described above. Sourdough

bread was prepared as described above after inoculation on dough with the spore suspension of *B. amyloliquefaciens* FAD 99. This strain was chosen because, among the strains of *Bacillus* investigated in this study, *B. amyloliquefaciens* FAD99 showed the greatest spoilage potential. The bacterial growth and ropy characteristics were observed over 7 d to determine the dosage of sourdough that inhibits growth of bacilli in bread. This experiment was carried out in three independent experiments.

2.9. Detection of the inhibition effect of reutericyclin on ropy bread spoilage caused by *Bacillus* species

The reutericyclin-positive *L. reuteri* TMW1.656 and the isogenic reutericyclin-negative derivative *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* were used to compare the inhibition effect of reutericyclin on ropy spoilage that caused by *B. amyloliquefaciens* FAD 99. The same procedure was applied to produce freeze-dried sourdoughs fermented with *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN*Δ*gtfA,* respectively. One ml spore suspensions from *B. amyloliquefaciens* FAD 99 was inoculated on the dough containing 12 % freeze-dried sourdough. The sourdough bread was baked as described before and bacterial growth and ropy spoilage characteristics were monitored daily. The experiment was performed in three independent replicates (independent baking trials).

2.10. Statistical analysis

Experiments were carried out at least in three replicates with different batches of spore suspensions and the same batch of freeze-dried sourdough. Statistical analysis of cell counts of *Bacillus* spp. after baking was carried out by ANOVA with Tukey's Honest Significant Difference test in RStudio. Significant differences were assessed with an

error probability of 5% (P<0.05).

3. RESULTS

3.1. Variations of heat resistance of spores from strains of *Bacillus* with different copy number of the *spoVA*^{2mob} operons in bread

Heat resistance of *Bacillus* endospores is dependent on the copy number of the *spoVA*^{2mob} operon (Berendsen et al., 2016; Li et al., 2019). Since ropy spoilage of bread is dependent on spore survival during the baking process, bread dough was inoculated with spores of strains of *Bacillus* with a different copy number of the *spoVA*^{2mob} operon and their survival after baking was determined (Figure 3). Overall, the lethality of the baking process, which exposed spores of *Bacillus* spp. to a temperature of more than 90 °C for about 10 min (Figure 1), was limited. Spore counts of *Bacillus* strains that did not encode for a *spoVA*^{2mob} operon were reduced by more than 1 log (CFU/g) after baking; spores of *Bacillus* strains that encoded three copies of the *spoVA*^{2mob} operons were reduced by less than 0.5 log (CFU/g); spores of *Bacillus* strains with two copies of the *spoVA*^{2mob} operons showed strain-specific survival (Figure 3). Surviving spores of *B. subtilis* and *B. amyloliquefaciens* caused visible bread spoilage after 1 – 2 days of incubation (Figure 2 and data not shown).

3.2. Appearance of ropy spoilage phenotype of different strains of *Bacillus* inoculated on dough

B. subtilis and *B. amyloliquefaciens* are recognized as the causative agents of ropy bread spoilage. To determine whether the spoilage phenotype of different strains of *B. subtilis* and *B. amyloliquefaciens* differs, spore suspensions of 5 different strains of *Bacillus* were inoculated at 5 different spots on one bread slice. The inoculation of bread rather than dough was chosen to eliminate the influence of spore survival after baking.

Spoilage characteristics were monitored for 5 d (Figure 4). The spores from 2 strains of *B. amyloliquefaciens* spoiled the bread more rapidly when compared to spores from 3 strains of *B. subtilis*. *B. amyloliquefaciens* FAD 99 visibly degraded the crumb after 1 day of incubation and formed slime after 3 – 4 d; crumb degradation and slime formation by spores from *B. subtilis* was observed only after 3 and 5 d, respectively (Figure 4). *B. amyloliquefaciens* FAD 99 was chosen as spoilage organism for the subsequent experiments.

3.3. Analysis of amylases encoded in genomes of *Bacillus* spp.

To identify putative genetic determinants for the different spoilage phenotypes of *B. amyloliquefaciens* and *B. subtilis* strains, genes coding for extracellular amylases and proteases were identified in the genomes of 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of *B. velezensis* (Li et al., 2019). Amylases were identified by BLAST analysis with protein sequences of extracellular amylases from *Bacillus* spp. that were available in the UniProt database as query sequences. Strains of *B. amyloliquefaciens* and *B. subtilis* differed with respect to the amylases that were identified in the genomes. Strains of *B. subtilis* and *B. velezensis* encode for AmyE as sole extracellular amylase while five extracellular amylases including a hyperthermostable α-amylase are encoded in genomes of each strain of *B. amyloliquefaciens* (Figure 5). All genomes encode for two glucoamylases, but β-amylases were not identified. The different complement of amylases likely relates to the different spoilage phenotype of strains of *B. amyloliquefaciens* and *B. subtilis*.

3.4. Effect of sourdough dosage on ropy spoilage

To determine the effect of sourdough on ropy spoilage, bread doughs were prepared with 0, 3, 6 12 and 24 % of freeze-dried sourdough fermented with the reutericyclin-

producing *L. reuteri* TMW1.656. Bread dough was inoculated with a spore suspension of *B. amyloliquefaciens* FAD 99 and the spoilage phenotype was monitored daily (Figure 6). The addition of 3 % sourdough did not affect spoilage, but spoilage was delayed by 2 – 3 days after the addition of 6 or 12 % sourdough corresponding to a bread pH of 6.2 and 5.9, respectively (Figure 6). The bread made with 24 % sourdough did not spoil during the 5 days of observation.

3.5. Detection of the inhibition effect of reutericyclin produced by *L. reuteri* on ropy bread spoilage caused by *Bacillus*

To compare the inhibitory effect of reutericyclin on ropy spoilage caused by FAD 99 *B. amyloliquefaciens*, sourdoughs were fermented with the reutericyclin positive *L. reuteri* TMW1.656 and its isogenic, reutericyclin negative *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* (Chen et al., 2016, this study). The GtfA – RtcN double mutant was used to avoid exopolysaccharide formation during sourdough fermentation, which may confound the assessment of slime formation during ropy spoilage. Bread was prepared with 12 % sourdough, a dosage that delays spoilage by 1 – 2 d (Figure 6), and bread dough was inoculated with spores of *B. amyloliquefaciens* FAD 99. Bread without reutericyclin showed visible line on the third day and became slimy on the fourth day (Figure 7). Bread with reutericyclin showed visible lines only on day five and slime was not observed until day seven (Figure 7).

4. DISCUSSION

Ropy spoilage of bread is frequent in warm and humid climate including Mediterranean countries, Africa as well as Australia (Voysey and Hammond, 1993). *Bacillus* spp., which produce extracellular slimy polysaccharides and possess proteolytic and amylolytic enzymes, are the major cause of ropiness (Corsetti et al., 2000; Pepe et al.,

2003). *Bacillus* endospores that are present in raw materials survive during baking (Pepe et al., 2003; Valerio et al., 2012) and lead to ropy spoilage if storage conditions support germination and growth (Vaičiulytė-Funk et al., 2015).

The *spoVA*^{2mob} operon plays an essential role in the heat resistance of *Bacillus* spores; its effect on heat resistance is dependent on the copy number of the operon in genomes of *Bacillus* spp. (Berendsen et al., 2016; Wang et al., 2018). This study used 8 strains of *Bacillus* isolated from ropy bread and daqu (Röcken and Spicher, 1993; Wang et al., 2018) to determine the impact of the copy number of the *spoVA*^{2mob} operon on heat resistance during baking process, where the temperature reaches up to 100 °C for several minutes. Viable counts of spores with two or three copies of *spoVA*^{2mob} operons were reduced by less than 1 log (CFU/g); however, the lethality of the baking process towards spores of strains that did not harbor a *spoVA*^{2mob} operon was also rather limited. Owing to this limited inactivation of endospores that do not harbour multiple copies of the *spoVA*^{2mob} operon, the presence of multiple copies of the operons did not appreciably improve survival during baking and is not a prerequisite for the ability of strains to spoil bread.

Strains of *B. subtilis* were most frequently isolated from ropy bread; however, several past studies misidentified *B. amyloliquefaciens* as *B. subtilis* (Pepe et al., 2003; Röcken and Spicher, 1993; Valerio et al., 2012) because the two species show a high level of similarity of their 16S rRNA gene sequence (Reginensi et al., 2013). This study compared the spoilage phenotype of 3 strains of *B. subtilis* and 2 strains of *B. amyloliquefaciens* and demonstrated that *B. amyloliquefaciens* caused spoilage of bread 2-3 days earlier than strains of *B. subtilis.* We further investigated the complement of extracellular amylases and proteases that are encoded in genomes of 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of the closely related species

B. velezensis (Li et al., 2019). Strains of *B. amyloliquefaciens* were differentiated from *B. subtilis* and *B. velezensis* by encoding for multiple extracellular α-amylases and gluco-amylases. Moreover, a hyperthermostable α-amylase was identified in each genome of *B. amyloliquefaciens* but not in *B. subtilis* and *B. velezensis*; this enzyme may remain active after baking and initiate starch degradation in bread. The comparison of the genotype of *B. subtilis* and *B. amyloliquefaciens* with the spoilage phenotype suggests that extracellular amylases and proteases contribute to ropy spoilage of bread, however, the contribution of individual enzymes remains subject to future studies.

Rope-forming bacilli are endophytes of plants including wheat, and their presence in seeds mediates vertical transmission of commensal plant microbiota to the offspring (Robinson et al., 2016; Shahzad et al., 2016). Therefore, contamination of wheat flour with *Bacillus* spores is unavoidable (Pepe et al., 2003; Saranraj and Geetha, 2012). The use of sourdough as a biopreservative has only limited effect against fungal bread spoilage (Axel et al., 2017; Quattrini et al., 2018) but inhibits growth of rope-forming bacilli (Röcken and Spicher, 1993; Rosenquist and Hansen, 1998; Valerio et al., 2008). The preservative effect of sourdough was mainly attributed to formation of organic acids and acidification of the bread crumb (Rosenquist and Hansen, 1998; Valerio et al., 2008). Acidification to a pH of 3.7 to 4.5 inhibits ropy spoilage (Pepe et al., 2003) and undissociated acetic acid enhances the preservative effect of sourdough (Rosenquist and Hansen, 1998).

Although sourdough has re-claimed its place as the standard process of bread-making, the use of sourdough or sourdough products may not always suffice to inhibit ropy spoilage. Dried sourdough products are standardized on the basis of the concentration of organic acids but typically much of the volatile acetic acid is lost during drying (Brandt, 2007). Moreover, not all sourdoughs are effective at inhibition of rope-forming

Bacillus spores due to their high pH and low concentration of organic acids (Katina et al., 2002). The concentration of organic acids in sourdoughs and bread dough fermented in bakeries is not as readily standardized as for stabilized sourdough products (Brandt, 2007). In addition, low pH and undissociated organic acids may alter the flavor and taste of bread (Hansen and Schieberle, 2005). In this study, addition of 24 % sourdough to a bread recipe effectively inhibited the growth of *B. amyloliquefaciens* but the combined use of organic acid with specific antimicrobial compounds may allow bread preservation without excessive acidity.

Bacteriocins of lactic acid bacteria were evaluated with respect to their activity against *Bacillus* spp. (Digaitiene et al., 2012; Pepe et al., 2003) even though nisin is the only bacteriocin which has a general inhibitory effect against strains of *Bacillus* (Rosenquist and Hansen, 1998). The use of nisin as additive or the use of nisin-producing starter cultures in sourdough, however, had no effect on growth of *Bacillus* in bread (Rosenquist and Hansen, 1998). This may relate to inactivation of nisin by proteases and thiol exchange reactions at the dough stage, or to their heat inactivation during baking (Gänzle, 2014). Reutericyclin is produced by *Streptococcus mutans* and few strains of *L. reuteri* (Lin et al., 2015; Tang et al., 2019). Reutericyclin is a tetramic acid derivative which resists proteolysis in dough and heat inactivation during baking (Gänzle et al., 2000). Reutericyclin exhibits a bactericidal mode of action against *Bacillus* spp., and inhibited spore germination (Gänzle et al., 2000). We used some of the same strains of *Bacillus* that were previously characterized with regards to their sensitivity to reutericyclin (Gänzle et al., 2000). Fermentation of sourdoughs with isogenic strains that produce comparable levels of organic acids but differ with respect to reutericyclin production (Lin et al., 2015) demonstrated that reutericyclin substantially delays growth of *Bacillus* spp. in bread. The use of reutericyclin-

producing strains thus allows the prevention of ropy spoilage with a reduced dosage of sourdough and corresponding reduced levels of acidity. The reutericyclin producing strain *L. reuteri* TMW1.656 was isolated from an industrial sourdough and also beneficially impacts bread texture through production of reuteran (Chen et al., 2016; Gänzle and Vogel, 2003).

In conclusion, multiple copies of the *spoVA*^{2mob} operon show only a limited effect on the heat resistance of *Bacillus* spores during baking. The different spoilage phenotype observed after growth of *B. amyloliquefaciens* and *B. subtilis* likely relates to the differential presence and expression of extracellular amylases and proteases in these species. Moreover, this study confirmed the inhibitory effect of sourdough and demonstrated that reutericyclin produced at the sourdough stage contributes to inhibition of growth of rope-forming bacilli in bread.

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FIGURES AND TABLES

Table 1 Origin of strains used in this study and copy number of the *spoVA*^{2mob} operon in each strain.

Microrganism	**Strain; origin**	**Copy # of *spoVA*^{2mob} operon/genome**	**Reference**
B. velezensis	[a]FUA 2155; daqu	0	(Wang et al., 2018)
B. subtilis	FUA 2114; malted oats	0	(Li et al., 2019)
B. subtilis	FAD 110; ropy bread	2	(Röcken and Spicher, 1993)
B. subtilis	FAD 109; ropy bread	2	(Röcken and Spicher, 1993)
B. amyloliquefaciens	FUA 2154; daqu	2	(Wang et al., 2018)
B. amyloliquefaciens	FUA 2153; daqu	2	(Wang et al., 2018)
B. amyloliquefaciens	FAD 99; ropy bread	3	(Röcken and Spicher, 1993)
B. amyloliquefaciens	FAD We; ropy bread	3	(Röcken and Spicher, 1993)
L. reuteri (reutericyclin positive)	TMW1.656; sourdough	n/a	(Gänzle and Vogel, 2003)
L. reuteri (reutericyclin negative)	TMW1.656Δ*rtcN*Δ*gtfA*	n/a	this study

[a] FUA number, Food Microbiology culture collection at the University of Alberta.

549 **Table 2** Wheat bread formula.

Ingredients[a]	Amount [g]
White wheat flour	100.0 minus weight of freeze-dried sourdough
Freeze-dried sourdough	0, 3.0, 6.0, 12.0, or 24.0
Sterile tap water	60.0
Yeast	2.0
Salt	2.0
Sucrose	2.0

550 [a] White wheat flour (Robin Hood) and baker's yeast (Fleischmann's Active Dry Yeast)

551 were obtained from a local supermarket.

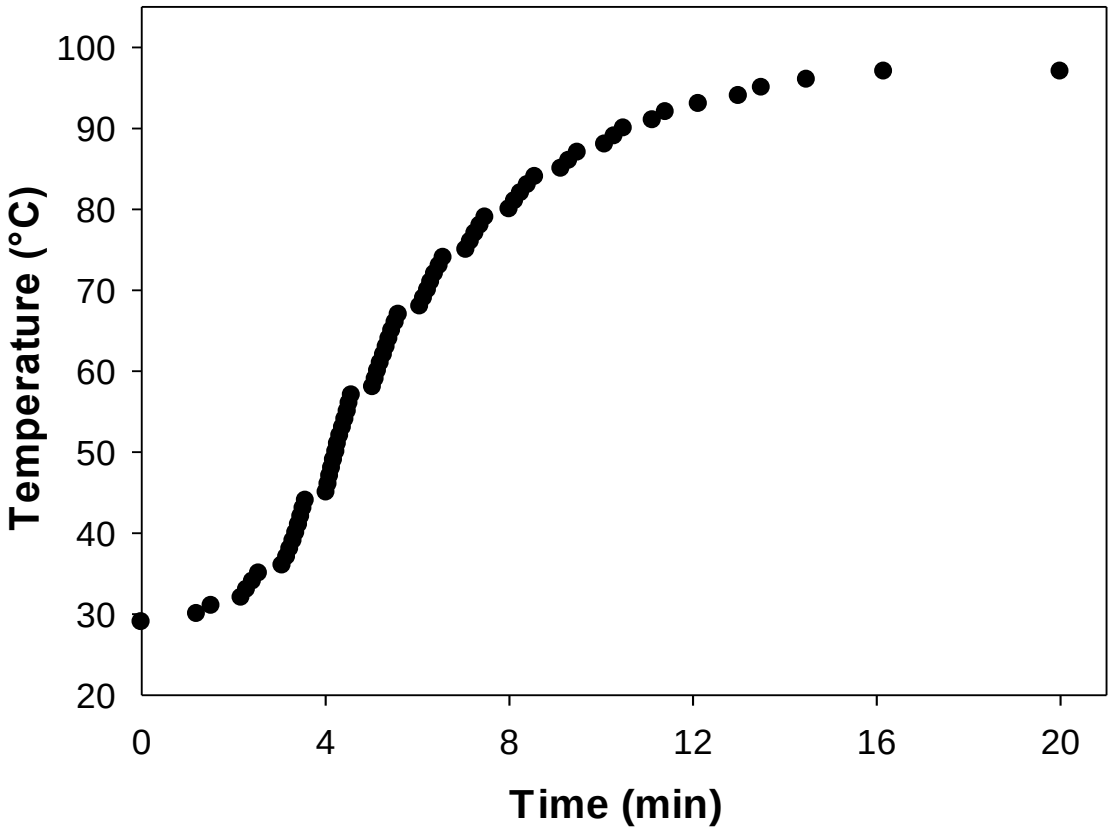

Figure 1. The temperature profile during baking process.

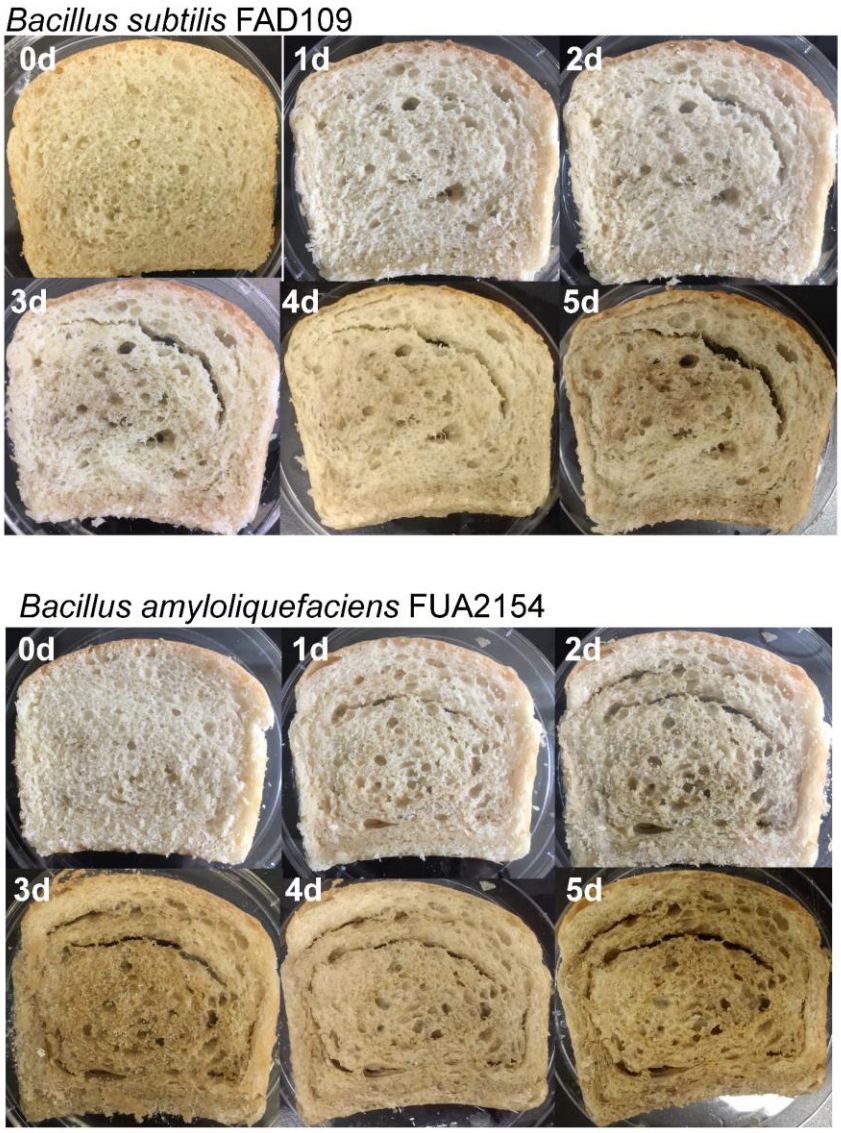

Figure 2. Images of bread slices that were inoculated with *B. subtilis* FAD109 (upper images) or *B. amyloliquefaciens* FUA2154 (lower images). Pictures of the same slice were taken at the day of baking (0d) and after 1 – 5 d of storage. Images are representative of three independent experiments.

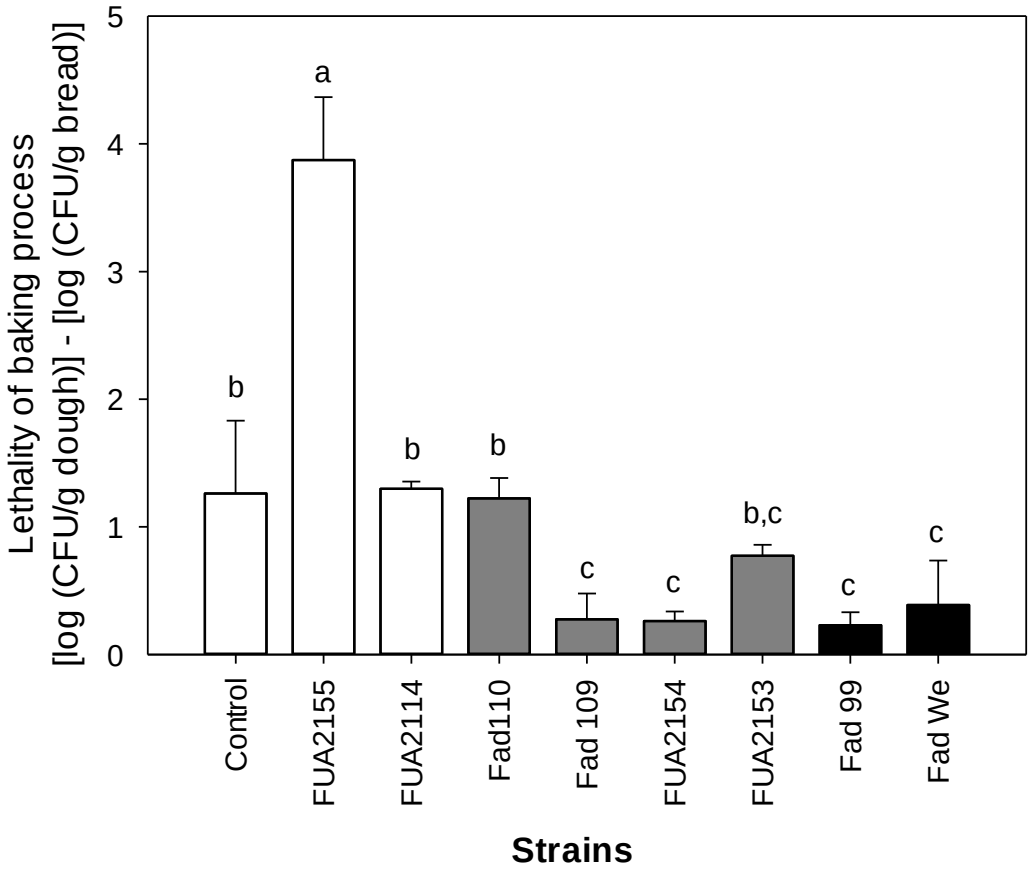

Figure 3. Lethality of the baking process [log (N_0/N)] towards spores of strains of *Bacillus* with different copy number of the *spoVA*^{2mob} operons. The color coding of the bars indicates the copy number of the *spoVA*^{2mob} operon as follows: white (0 copies), gray (two copies), and black (three copies). Strain number and species are indicated as follows: *B. velezensis* FUA 2155; *B. subtilis* FUA 2114; *B. subtilis* FAD 110; *B. subtilis* FAD 109; *B. amyloliquefaciens* FUA 2154; *B. amyloliquefaciens* FUA 2153; *B. amyloliquefaciens* FAD 99; *B. amyloliquefaciens* FAD We. Control refers to bread that was not inoculated with *Bacillus* endospores. The data are shown as means of three independent experiments, error bars indicate the standard deviation. Cell counts that do not share a common superscript differ significantly (P<0.05).

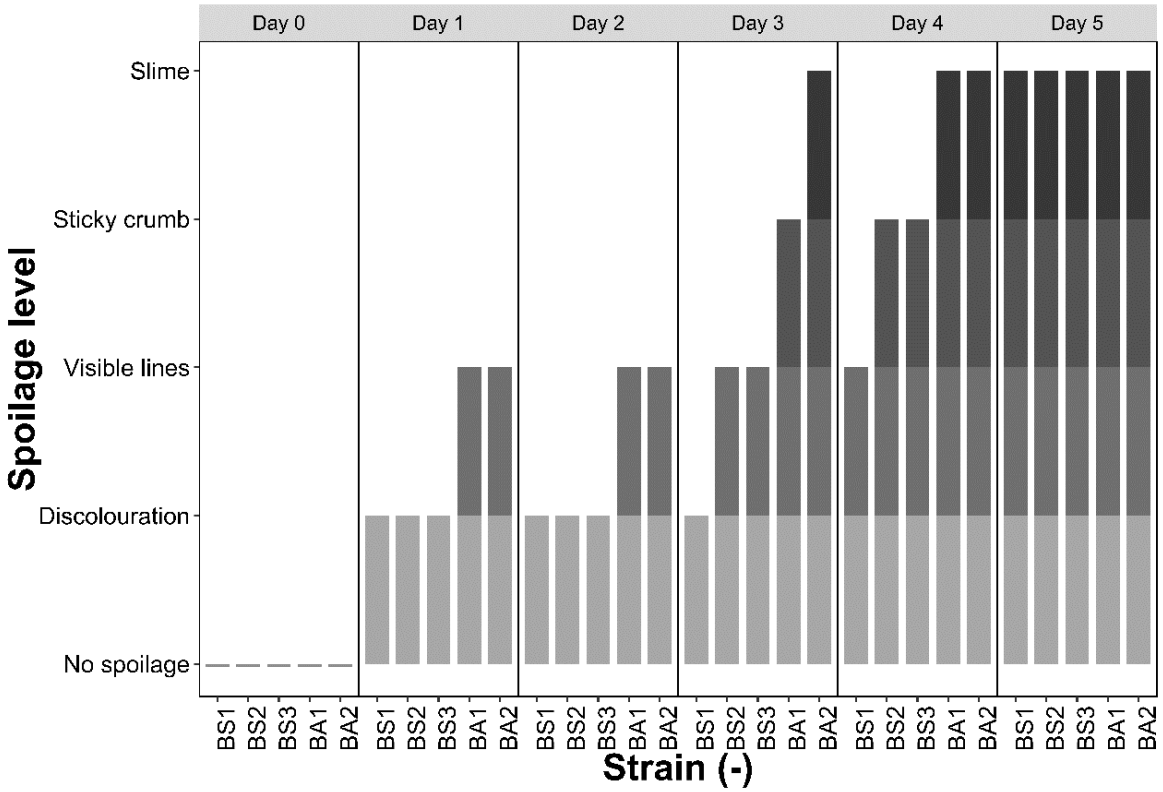

Figure 4. Kinetics of appearance of rope spoilage characteristics in breads contaminated with *B. subtilis* and *B. amyloliquefaciens* after baking. Contaminated slices were stored at 30 °C and a_w 0.95 for 5 days. Different colors of gray scale represent different levels of spoilage from low to high. The strain number is labeled as follows: BS1 (*B. subtilis* FUA 2114); BS2 (*B. subtilis* FAD 110); BS3 (*B. subtilis* FAD 109); BA1 (*B. amyloliquefaciens* FUA 2154) and BA2 (*B. amyloliquefaciens* FAD 99). The data represent results from three independent experiments.

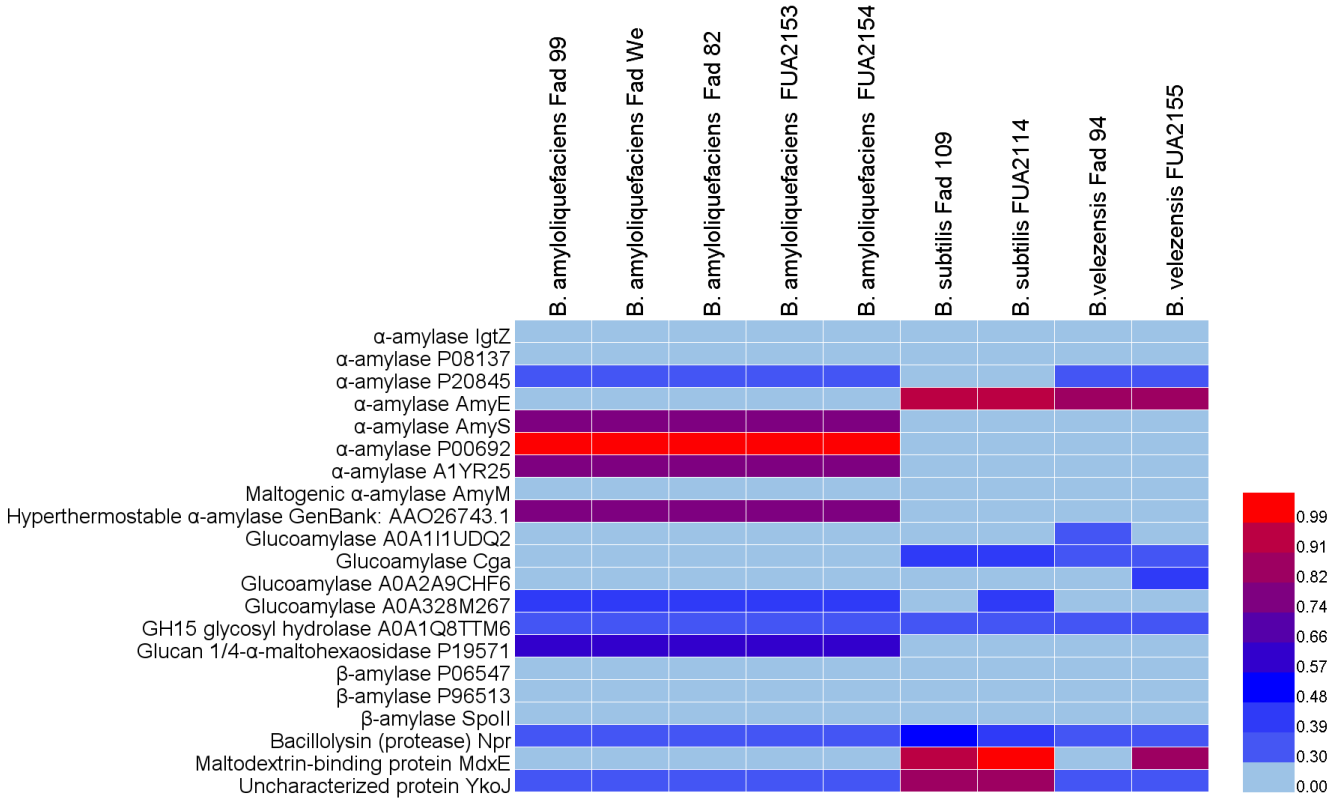

Figure 5. Identification of amylases, glucoamylases and other starch-active enzymes encoded in the genomes of 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of *B. velezensis*. With exception of the four glucoamylases, all proteins encode for a signal peptide that is predicted to mediate protein export. Uniprot protein accession numbers are indicated unless specified otherwise. **IgtZ** (A0P8X0) GH119 α-amylase; **P08137** GH13 α-amylase; **P20845** GH13 α-amylase; **AmyE** (P00691) GH13 α-amylase; **AmyS** (P06278 and P06279) GH13 α-amylase; **P00692** GH13 α-amylase;. **A1YR25** GH13 α-amylase; **AmyM** (P19531) GH13 maltogenic α-amylase; **GenBank: AAO26743.1** GH13 hyperthermostable α-amylase; **A0A1I1UDQ2** GH15 glucoamylase; **Cga** A0A3S4RT35 GH15 glucoamylase; **A0A2A9CHF6** GH15 glucoamylase; **A0A328M267** GH15 glucoamylase; **A0A1Q8TTM6** GH15 glycosyl hydrolase; **P19571** GH13 glucan 1/4-α-maltohexaosidase; **P06547** GH14 β-amylase; **P96513** GH14 β-amylase; **SpoII** P36924 β-amylase; **Npr** (P29148) M4 family peptidase bacillolysin (protease); **MdxE** (O06989) maltodextrin-binding protein of the bacterial solute-binding protein 1 family. **YkoJ** (O35012) uncharacterized protein (putative protease inhibitor).

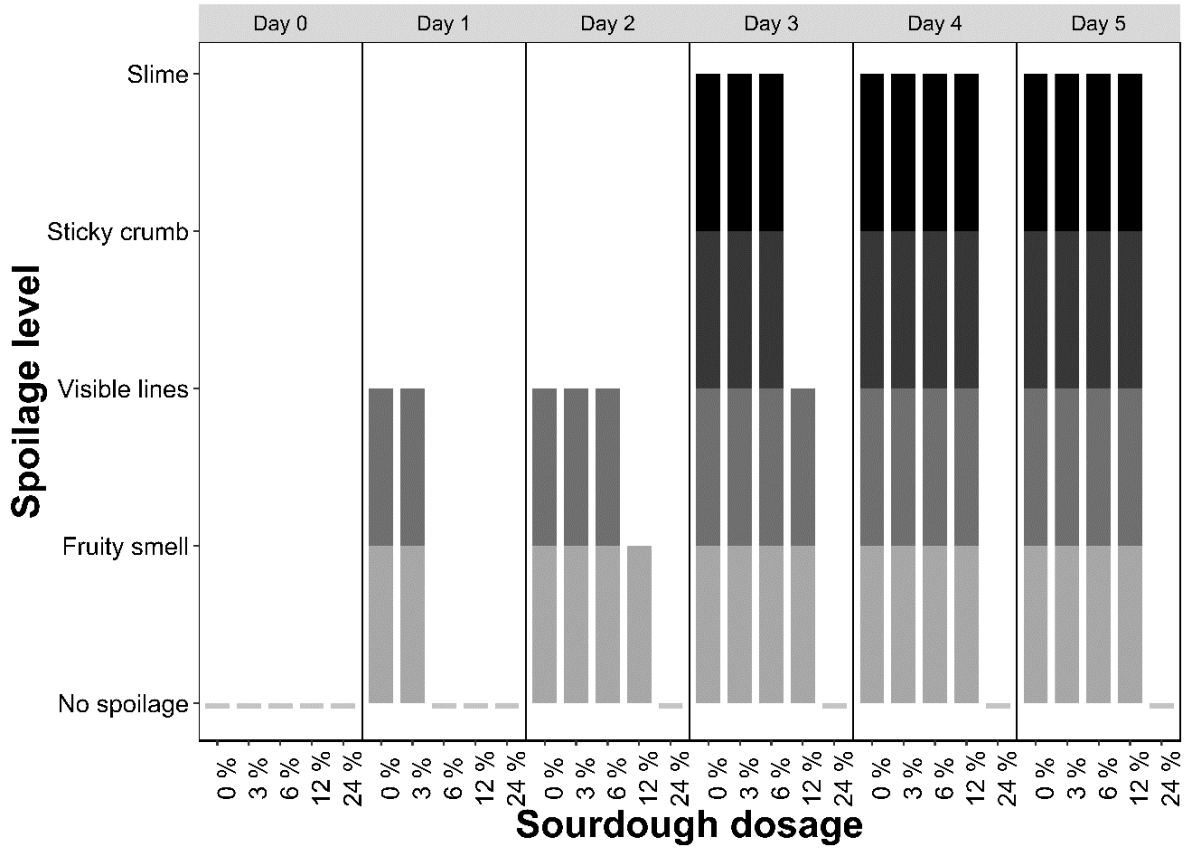

Figure 6. Kinetics of appearance of spoilage characteristics in breads baked from doughs contaminated with *B. amyloliquefaciens* (FAD 99) during storage at 30 °C and a_w 0.95 for 5 days. The doughs contained different dosage of sourdough fermented with reutericyclin-positive *L. reuteri* TMW1.656. Different colors of gray scale represent variations in levels of spoilage from low to high. Different sourdough dosage was labeled in the figure. The pH of bread produced with 0, 3, 6, 12 or 24 % sourdough was 6.40 ± 0.044; 6.30 ± 0.019; 6.17 ± 0.024; 5.93 ± 0.041; and 5.53 ± 0.040, respectively. Data represent results from three independent experiments.

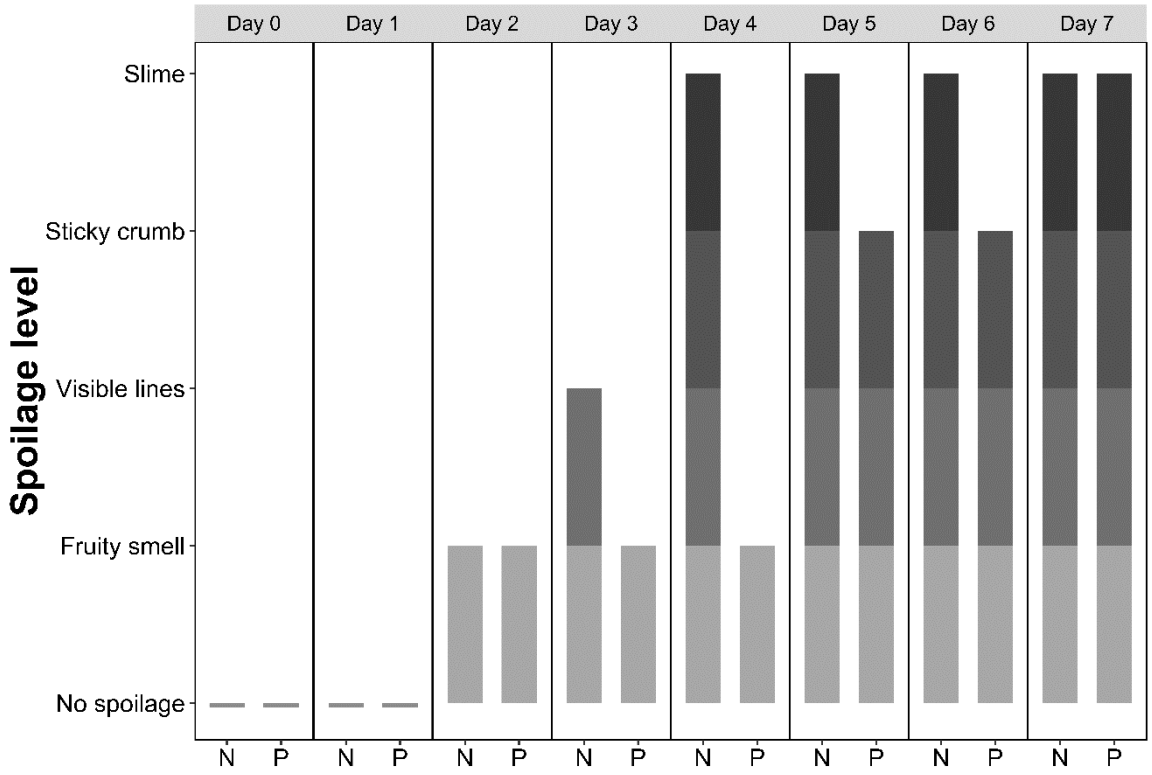

Figure 7. Effect of reutericyclin on the kinetics of appearance of ropy spoilage characteristics of breads baked from doughs contaminated with *B. amyloliquefaciens* FAD 99 during storage at 30 °C and a_W 0.95 for 7 days. Sourdough was added at a dosage of 12 %. Different colors of gray scale represent different levels of spoilage from low to high. "N" represented bread produced with sourdough fermented with the reutericyclin-negative *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA*; "P" represented bread produced with sourdough fermented with the reutericyclin-producing *L. reuteri* TMW1.656. The pH of bread produced with sourdough fermented with *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* was 5.93 ± 0.041 and 5.93 ± 0.021, respectively. Data represent results from three independent experiments.