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Viability of *Lactobacillus plantarum* P8 in Bread during Baking and Storage

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Abstract: Lactic acid bacteria are usually applied in sourdough fermentation to make bread with improved quality. Some lactic acid bacteria are probiotics that confer health benefits on the consumer when administered in adequate amounts. Given the high consumption of bread worldwide, bread is a potential vehicle of probiotic bacteria. However, it is challenging to make bacteria survive the harsh baking conditions. This paper discusses survival of *Lactobacillus plantarum* P8 in bread during baking and storage. The reduction of probiotic bacteria during baking was about 4 logs, with an initial viable count in the dough of about 10^9 cfu/g. The oven temperatures were 175, 200 and 235°C, respectively. Inactivation of bacteria during baking was primarily determined by thermal rather than by dehydration inactivation. During storage, viable counts of *L. plantarum* P8 increased by 2 – 3 logs in bread, due to bacterial growth. Because of their different temperature and moisture content history, the bread was divided into two domains: crust and crumb, which enabled model development. Using this, the experimental data on survival could be described with a modified Weibull model of thermal inactivation including the coupled effect of temperature and moisture content.

Keywords: bread baking, storage, inactivation kinetics, modeling, *Lactobacillus plantarum*

INTRODUCTION

Lactic acid bacteria (LAB), including obligately (e.g. *L. brevis*) and facultatively heterofermentative species (e.g. *L. plantarum*), have been widely involved in sourdough fermentation, giving acidification and structure to the grains and crumbs. In addition, the use of specific strains in breadmaking may delay firmness and staling (Corsetti et al., 2008). Palacios, et al (2006) found that inoculation of *L. plantarum* produces dough similar to that fermented by yeast alone, but with a larger dough volume. Valerio, et al. (2008) demonstrated that a sub-strain of *L. plantarum* (*L. plantarum* ITM21B) isolated from sourdough and its active metabolites could be used to prolong the *Bacillus* free-shelf life of yeast-leavened bread to 7 days at 30 $^\circ$C.

Some of those lactic acid bacteria and *Bifidobacterium* are probiotics that are claimed to have a positive effect on the health of the consumers when applied in sufficient numbers (>10^6~10^7 cfu/g) and on a regular basis (Ross, et al., 2005). Most probiotic products available on the market now are dairy products, however, there is an increasing amount of probiotics applied in non-dairy products including chocolate, biscuits, desserts and juice, etc. (Aragon-Alegro, et al., 2007; do Espírito Santo, et al., 2011; Reid, et al., 2007). Bread is a staple food in large parts of the world and almost consumed every day. In addition, bread is also known as a nutritious product that contains minerals, vitamins and dietary fibers (Cerqueira, 2014). Therefore, bread is an interesting potential vehicle of viable probiotics to study.

However, application of probiotics in bread is challenging, since the high temperature during the baking process significantly affects the viability of probiotic bacteria (Altamirano-Fortoul, et al., 2012; Zhang, et al., 2014). Many factors, including the temperature, moisture content and structure of the matrix, etc., will affect the retention of probiotic bacteria during baking through their effects on thermal and dehydration inactivation kinetics. Therefore, the aim of this study is to gain insight in the viability of *Lactobacillus plantarum* P8 in different parts of bread (crust and crumb) during baking and storage.

MATERIALS AND METHODS

Probiotic bacteria culture

The probiotic strain *Lactobacillus plantarum* P8 was provided by Key Laboratory of the Education Ministry of China, Inner Mongolia Agricultural University. The *L. plantarum* P8 culture was routinely prepared using MRS broth (OXOID, United Kingdom) as growth medium, incubated at 37 °C for 12 h and sub-cultured for 24 h. The cell pellets were then harvested from the MRS broth by centrifugation at 8000 g, at 4 °C for 15 min (Thermo Fisher Scientific, USA), and were re-suspended in UHT skim milk for the baking procedure.

Baking procedure

Bread dough was made following a recipe by Wang and Zhou (2004) with slight modification: wheat flour (100 g), sugar (4 g), fine salt (1.5 g), instant yeast (1 g), butter (3 g), UHT skim milk (65 g) with *L. plantarum* added. Dough was first made in a mixer (Hauswirt®, Germany), and then divided into small pieces (5 g, 30 g and 60 g). After having been made into spheres by hand, dough pieces were leavened for 1 h at 40 °C, 85 % RH in a climate chamber (Yiheng®, China). The bread was baked in an electric oven (Changdi®, China). The temperature in the bread during baking was monitored during baking by inserting K-type thermocouples into the surface and core sections of the dough; the sampling time was 1 s in all cases. The moisture contents (% kg/kg wet base) of the crust and crumb during baking were determined by weighing the samples after baking and after dehydration at 105 °C for 24 h. The baked breads were sealed in polyethylene bags and stored at 25 °C, 55 % RH in the climate chamber for 1 week.

Enumeration of viable bacteria

Viable counts of *L. plantarum* in bread were determined during baking and storage. Samples of crust or crumb were aseptically homogenized with sterile 0.1 % peptone water in a stomacher (iMix®, France) for 1 min. Serial dilutions were made in sterile 0.1 % peptone water and 100 µL solution was plated onto modified MRS agar broth (OXOID, United Kingdom). Vancomycin (20 ppm) and natamycin (200 ppm) were sterilized by filtration through a 0.22 µm polyethylenesulfone filter (Millipore®, USA), and were added into the MRS agar broth to inhibit the growth of *Bacillus strains* and yeast, respectively. These additions do not affect the growth of *L. plantarum*. The plates were incubated at 37 °C for 48 h. *L.plantarum* colonies were counted and the results were expressed as colonies forming units per gram of sample (cfu/g).

Determination of pH and TTA

The pH of the bread was measured by blending 10 g bread dough/crumb with 100 mL acetone/water (5/95, v/v) using a pH meter (Thermoscientific, USA). To determine the total titratable acidity (TTA), this suspension was titrated against 0.1 N NaOH to a final pH value of 8.5. The pH and TTA were measured before and after baking, as well as during storage. The TTA was expressed as the amount (mL) of NaOH used for titrating 10 g of dough or bread.

Statistical analysis

All data were presented as mean ± standard deviation. ANOVA was used to evaluate the difference between two means. Calculations were performed with Excel 2010 (Microsoft®, USA).

RESULTS AND DISCUSSIONS

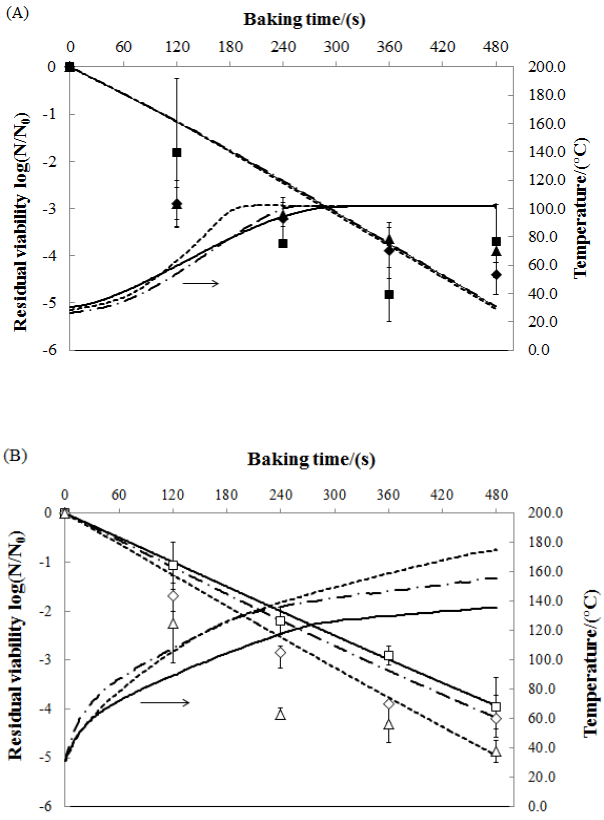
Viability of L. plantarum during bread baking

Thermal inactivation and dehydration inactivation of *L. plantarum* are expected to occur simultaneously because during bread baking both the temperature and the moisture content change significantly (Perdana et al., 2013; Zhang & Datta, 2006). During baking, the temperature and moisture content histories of bread crust and crumb are very different (Figure 1). In the crumb, the temperature increases slowly in the first 1 minutes of baking, followed by a fast rise to 100 °C, after which the temperature levels off, probably due to water evaporation. The moisture content of the crumb remains at the same level throughout the baking process (Purlis, 2011); Meanwhile, in the crust, water evaporates quickly and the moisture content decreases due to the high evaporation rate at the surface (Purlis & Salvadori, 2009). Therefore, different survival behaviour of *L. plantarum* may be expected in the crust and crumb.

Previous research shows that different inactivation mechanisms are induced at different temperature ranges: at high temperature thermal inactivation is more prominent while at lower temperature (<45 °C) dehydration inactivation becomes dominant (Fu & Chen, 2011; Jimmy Perdana et al., 2013). Thermal inactivation is affected by both temperature and moisture content (Perdana, et al, 2012). In addition, heat resistance of bacteria in an environment with lower moisture content is higher (Ansari & Datta, 2003). In this study, due to the high processing

temperature during baking, it is assumed that thermal inactivation is dominant.

The residual viable counts of *L. plantarum* in the crust and crumb of 5 g bread during baking at different oven temperatures are shown in Figure 1A and 1B (N_0, initial viable count, was $6.8 \cdot 10^8$ cfu/g). When the breads were baked at 175 °C, the viable counts of probiotics in the crust was higher than in the crumb ($P<0.05$). Although the temperature in the crust was much higher than in the crumb during baking, the heat-tolerance was also higher due to the lower moisture content (Figure 1). However, the opposite phenomenon was observed when the baking temperature was 235 °C ($P<0.05$), while no significant difference was found between the survival curves of probiotics in crust and crumb of bread baked at 200 °C ($P>0.05$) (Figure 1A and 1B). This might be because the inactivation rates increases especially when the temperature exceeds 100 °C, and the temperature in crust of bread baked at 235 °C increased more rapidly in the first minute of baking to 100 °C compared to the other baking temperatures, causing increased inactivation of *L. plantarum*. Overall, these results confirm that both temperature and moisture content have impact on the thermal inactivation kinetics.



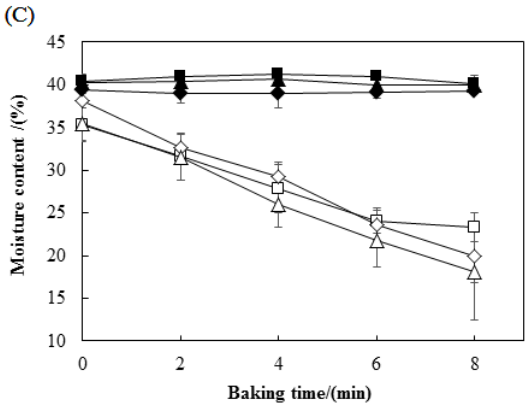


Fig. 1. A and B: Viability of *L. plantarum* (crumb: ■, 175 °C; ♦, 200 °C; ▲, 235 °C; crust: □, 175 °C; ◊, 200 °C; Δ, 235 °C) and temperature profiles in the crumb (A) and in the crust (B) (solid line, 175 °C; dot dash line, 200 °C; dotted line, 235 °C; predicted results of residual viability are also shown) C: Moisture contents of crumb and crust during baking at different temperature

Thermal inactivation model

A modified Weibull model for thermal inactivation (Equations 1 to 4) was used to fit the experimental data of both crust and crumb (Jimmy Perdana et al., 2013) by assuming that thermal inactivation is dominant and dehydration inactivation could be ignored. During baking, the moisture contents of bread crumb maintained at the same value (41 % w/w), and the moisture content of the crust at every time was obtained by linear interpolation (Figure 1C).

$$A_T = A_0 \exp\left(-\left(\frac{t}{\alpha}\right)^{\beta_T}\right) \quad (1)$$

$$\alpha = \alpha_{w,T} \exp\left[\ln\left(\frac{\alpha_{s,T}}{\alpha_{w,T}}\right)\exp\left(-p\frac{x_w}{(1-x_w)}\right)\right] \quad (2)$$

with

$$\log(\alpha_{w,T}) = \log(\alpha_{w,Tref}) - b_w(T - T_{ref}) \quad (3)$$

$$\log(\alpha_{s,T}) = \log(\alpha_{s,Tref}) - b_s(T - T_{ref}) \quad (4)$$

in which A_T is the cumulative residual viability after thermal inactivation, A_0 is the initial value, T the temperature, and α and β_T the Weibull distribution parameters of thermal inactivation. T_{ref} was set to the average temperature measured during baking.

The five parameters ($\alpha_{w,Tref}$, b_w, $\alpha_{s,Tref}$, b_s and p) were fitted using all the data obtained from the experiment on 5 g and 30 g bread (number of data point n=48; see Table 1). The optimized parameters were validated using data from 60 g breads (Figure 2A). The estimated residual viability of *L. plantarum* in the crumb and in the crust were shown in Figure 1A

and Figure 1B, respectively (solid line, 175 °C; dot dash line, 200 °C; dotted line, 235 °C). As can be seen, the calculated survival curves of probiotics in the *crumb* of bread baked at three different temperatures are overlapped, and statistical analysis on the experimental data also indicated that the difference between them was not significant. This is because the temperature and moisture content profiles in the crumb of 5 g bread were similar at different baking temperature (Figure 1A and 1C). In contrast, the influence of baking temperature on the thermal inactivation of probiotics in the *crust* was quite obvious (Figure 1B), both in the experiments and in the model descriptions.

Table 1. Optimized parameters of thermal and dehydration inactivation model .

Parameter	Unit	Value	95% CI
$\alpha_{w,Tref}$ [a]	Minute	0.0026	0.00004
b_w [a]	1/°C	0.0010	-
$\alpha_{s,Tref}$ [a]	Minute	84.64	0.50
b_s [a]	1/°C	0.0010	-
p [a]	-	0.11	0.00096
β_T [b]	-	1	-
T_{ref} [b]	°C	90	-
n [c]	-	48	-
RMSE[d]	-	0.92	-

[a] Thermal inactivation model parameters

[b] Not optimized

[c] Each data point is the mean value of results obtained from all the repeated experiment.

[d] Root of mean square error between measured and predicted results

It is mentioned above that the moisture content plays an important role in thermal inactivation. The results of the model fitting support this statement because the Weibull distribution parameter α increases with the decrease of moisture content when the temperature is the same, resulting in a higher residual viability (see Figure 2B and Equation 1).

In general, there is a reasonable agreement between the predicted and experimental results (Figure 2A), although the coupled effects of temperature and moisture content on the thermal inactivation need to be further studied.

Nevertheless, this model did not involve the possible influence of microstructure/microenvironment on the inactivation kinetics, and previous study indicated that surface roughness properties may protect probiotics during heating (Altamirano-Fortoul, et al., 2012). Therefore, in the future a new model could be developed, considering bacterial inactivation at different stages of bread baking including the change in microstructure during baking.

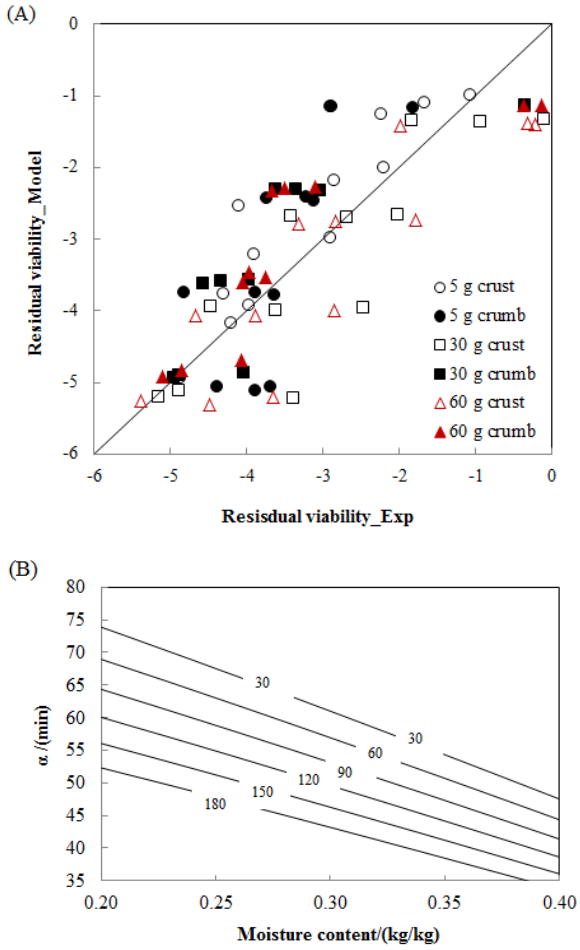


Fig. 2. A: Comparison between the measured and predicted residual viability of *L. plantarum* in different parts of bread baked at different temperatures; B: Contour plot of *T* as a function of x_w and Weibull distribution parameter α.

Viability of L. plantarum during bread storage

Even after baking, the bread matrix is a suitable substrates for growth of probiotic bacteria because nutrients for bacteria are abundantly present (Charalampopoulos, Pandiella, & Webb, 2002).

Figure 3 shows that the viable counts of *L. plantarum* increased during storage after baking from 5 log cfu/g to 8 log cfu/g in the crust and from 4 log cfu/g to 6 cfu/g in the crumb, and then levelled off at high viable counts (>10^6 cfu/g) retained for 3 days (a plateau was reached). These values may suffice for the bread to be a vehicle for the administration of probiotics at the time of consumption.

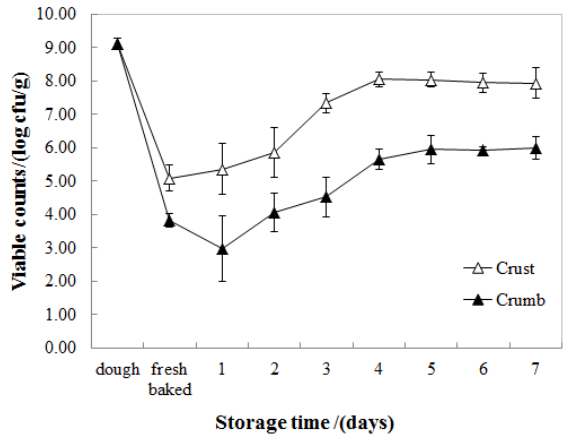


Fig. 3. Viable counts of *L. plantarum* in crust or crumb during storage (30 g bread baked at 175 °C for 8 min)

One can observe that both in the crust and the crumb, the bacteria need to recover from the baking process and adapt to the new environment (Corcoran, et al., 2004; Huang et al., 2014).

It is further noticeable in Figure 3 that the viable counts in the crust are higher than in the crumb. This is, firstly, due to a higher residual viability after baking (5 log cfu/g and 4 log cfu/g, respectively). During storage, moisture migrates from the crumb towards the crust until an equilibrium between crust, crumb and the environment is reached (Primo-Martín et al., 2006) (see Table 2). This moisture migration results in a higher moisture content (and water activity) in the crust bread, which is favorable for the bacteria to grow (Soukoulis et al., 2014).

Table 2. Variation of moisture content, pH and total titratable acidity of bread during storage (30 g bread baked at 175 °C for 8 minutes)

Days	Moisture content/(w/w)		pH value	TTA (mL 0.1 N NaOH)
	crust	crumb		
Dough	0.403± 0.005	0.403± 0.005	4.79± 0.05	7.38±0.11
0	0.296± 0.030	0.417± 0.010	5.19± 0.25	4.88±0.11
1	0.321± 0.006	0.403± 0.005	5.03± 0.11	5.28±0.39
2	0.327± 0.012	0.393± 0.006	5.22± 0.28	4.55±0.49
3	0.337± 0.012	0.392± 0.006	5.13± 0.29	4.50±0.71
4	0.339± 0.017	0.376± 0.020	5.04± 0.22	4.95±0.78
5	0.346± 0.007	0.384± 0.008	4.88± 0.13	5.85±0.92
6	0.360± 0.028	0.384± 0.005	4.73± 0.14	6.50±1.27
7	0.342± 0.031	0.380± 0.001	4.74± 0.04	6.80±0.99

It is not clear, however, why the final viable counts of *L. plantarum* in the crust are about two logs higher than in the crumb.

In addition, the pH of the bread decreased and the total titrable acid (TTA) increased during storage due to the production of lactic acid by *L. plantarum*, while the pH of bread from the control group (without probiotics addition) remained at around 6.00±0.09, TTA around 2.33±0.16 mL).

Fig. 4. Photos of bread after 5-day storage (left: control group; right: bread with *L. plantarum* addition; black arrow: mold).

The mold-free shelf life was prolonged by adding *L. plantarum* compared to bread without *L. plantarum* addition (see Figure 4). No mold was observed on the bread with probiotic addition after 5-day storage. This may be explained by production of anti-microbial compounds by lactic acid bacteria (including *L. plantarum* P8), which inhibit the growth of fungi (Moore, et al., 2008; W. Zhang, et al., 2015).

CONCLUSIONS

In this study, the residual viable counts of *L. plantarum* in bread during and after baking experiment were determined. Significant differences were observed between the probiotic viable counts in the crust and crumb of 5 g mini-breads baked at 175 °C and 235 °C, but not at 200 °C.

Thermal inactivation was dominant compared to dehydration inactivation during baking. A modified Weibull model reasonably described the residual viability of *L. plantarum* by considering thermal inactivation. The model may be further improved by considering different stages of bread baking and describing the influence of changes in microstructure on survival.

L. plantarum grows back in the bread matrix after baking. The probiotic bacteria grew by 2~3 Log cfu/g in bread matrix after 4-day storage and high viable counts (>10^6 cfu/g) were obtained and then stayed at the same level for 3 days . This growth was also evident in an extended mold-free storage time, compared to similar breads without *L. plantarum.*

NOMENCLATURE

A	Residual viability	-
b	Temperature dependency parameter of α for thermal inactivation	1/°C
n	Number of data points	-
N	Viable counts	cfu/g
p	Moisture content dependency of parameter α for thermal inactivation	-
T	Temperature	°C
t	Time	s
x	Moisture content	kg/kg

Greek letters

α	Weibull distribution parameter	Minute
β	Weibull distribution parameter	-

Subscripts

D	Dehydration inactivation
ref	Reference
s	Solid
T	Thermal inactivation
w	Water/dehydration
0	Initial
crit	Critical moisture content

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