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Abstract

The effect of encapsulation on the survival of *Lactobacillus plantarum* during isothermal heating and bread baking was investigated. Four encapsulating materials were evaluated, i.e., reconstituted skim milk (RSM), gum arabic (GA), maltodextrin (MD) and inulin. Freeze dried bacteria survived better in GA and RSM matrices during isothermal heating at 90 °C, which was explained by their high glass transition temperatures and physical entrapment of the bacterial cells in their dense microstructure. The survival of bacteria in bread during baking depended on the approach used to incorporate probiotics and physical properties of encapsulating materials, which was related to the exposure of the bacterial cells to moist-heat. Maximum survival of probiotic bacteria (>10^8 CFU/g bread) was achieved after 15 min baking at 100 °C when the RSM-probiotic powder was distributed on the dough surface. Furthermore, A Weibull model could describe the general trend of the inactivation kinetics of bacteria during isothermal heating (at 60, 75 and 90 °C) as influenced by the initial moisture content of the RSM-water mixtures (0.05, 0.60 and 0.90 kg/kg). Future development of bakery products with alive probiotic bacteria could benefit from this work.

Keywords: Freeze drying; survival; probiotic bread; baking; inactivation kinetics.

1. Introduction

Foods fortified with probiotics are increasingly introduced into the market (De Prisco & Mauriello, 2016; Rivera-Espinoza & Gallardo-Navarro, 2010). Bakery products are an emerging category within the probiotic food segment and have attracted increasing research interest (Pinto, Castro, Vicente, Bourbon, & Cerqueira, 2014; Reid, Champagne, Gardner, Fustier, & Vuillemard, 2007; Soukoulis et al., 2014; Vitaglione et al., 2015; Zhang, Huang, Ananingsih, Zhou, & Chen, 2014). To ensure that the addition of probiotic bacteria has the intended health benefit, a minimum number of living bacteria should be retained in the baked product at the time of consumption (> 6-7 log CFU/g) (Tripathi & Giri, 2014). This is however a challenge for baked products due to the high temperatures employed during baking, which may lead to a significant loss of viable bacteria (Zhang, Taal, Boom, Chen, & Schutyser, 2018). To facilitate the development of probiotic bakery products, it is important to study the survival of bacteria during the baking process.

A potential strategy to improve the survival of probiotic bacteria during baking is to encapsulate the bacterial cells in powder with protectants. Survival of probiotic bacteria in a solid matrix is influenced by the matrix composition when exposed to varying temperatures (Santivarangkna, Aschenbrenner, Kulozik, & Foerst, 2011). Ideally, probiotic bacteria are embedded in a dry glassy matrix to secure maximum survival (Broeckx, Vandenheuvel, Claes, Lebeer, & Kiekens, 2016; Krasaekoopt, 2017). It is crucial that the moisture content of the system is kept low, because the glass transition

temperature strongly decreases at increasing moisture content (Roos, 2010). Pitigraisorn, et al. (2017) encapsulated *Lactobacillus acidophilus* cells in alginate-based multi-layered microcapsules coated with an egg albumen-stearic acid composite. They found an increased survival of the encapsulated bacteria upon exposure to moist-heat (70 °C, 100 %RH, 30 min), which was explained by the hydrophobic properties of the encapsulation matrix that limited moisture transfer into the capsules. However, the heating temperature used in that study was relatively low (70 °C) compared to the actual temperature involved during baking. In another study, *Lactobacillus rhamnosus* R011 was entrapped in a whey protein gel, and the viability of the freeze dried cells were found higher during baking of biscuits (280 °C, 5 min) due to the limited rehydration of the incorporated whey protein (Reid et al., 2007). Improved survival of living bacteria during thermal processing has thus been achieved by encapsulation (Corona-Hernandez et al., 2013). However, more quantitative insight is needed, especially to explore the possibilities of encapsulation in relation to improved survival of probiotics during bread baking.

Therefore, the aim of this study was to investigate the protective effect of encapsulating materials on the survival of dried probiotics subjected to isothermal heating and bread baking. A model probiotic strain (*Lactobacillus plantarum* P8) was freeze-dried in four different matrices (reconstituted skim milk, gum arabic, maltodextrin and inulin) as protectants, respectively. The obtained powders were characterised on their physicochemical properties. Isothermal heating experiments with the dried powders

were conducted to investigate the heat resistance of the bacteria as influenced by the encapsulation matrix and its initial moisture content. Subsequently, the probiotic powders were incorporated into bread using three different approaches and the survival of bacteria in bread after baking was evaluated.

2. Materials and Methods

2.1 Bacterial culture

The probiotic strain of *Lactobacillus plantarum* P8 (ATCC-14917, hereafter termed LP) was provided by the Key Laboratory of the Education Ministry of China, Inner Mongolia Agricultural University. The bacteria were routinely cultured in MRS broth (OXOID®, United Kingdom). A single colony of LP was aseptically transferred from MRS agar plate to 10 mL sterile MRS broth, and pre-cultured at 37 °C for 12 h. Subsequently, 1 % v/v inoculum of LP was sub-cultured in 100 mL MRS broth at 37 °C for 24 h without agitation. The LP cell pellets were harvested by centrifugation (8000 g, 4 °C, 15 min), and were re-suspended in UHT skim milk or another solution as described in the next section.

2.2 Freeze drying of probiotic bacteria

The harvested LP cells were aseptically suspended in reconstituted skimmed milk (Devondale®, Australia), gum arabic from acacia tree (Sigma-Aldrich, Germany), maltodextrin (Dextrose Equivalent 13-17, Sigma-Aldrich, Germany), and inulin (Orafti GR®, Belgium) solutions with an initial solid content of 10 % w/w, respectively.

Reconstituted skim milk (RSM) was sterilized in an autoclave at 105 °C for 15 min (Zealway GR60DR, USA), while gum arabic (GA), maltodextrin (MD) and inulin solutions were sterilized at 75 °C for 10 min (Yonekura, Sun, Soukoulis, & Fisk, 2014). The LP cell suspensions in different solutions were transferred to sterile glass tubes and pre-frozen at – 20 °C for 12 h prior to the main vacuum-freeze-drying step in a freeze dryer (Sihuan Scientific Instruments Co., Ltd., China) for 50 h and the temperature was set at – 50 °C. Subsequently, the lyophilized matrices were fully grinded into fine powders in a mortar with a pestle. The powders were stored at 4 °C in sealed glass bottles in a desiccator.

2.3 Physicochemical analyses of the powders

2.3.1 Moisture content

To determine the moisture content of the freeze-dried powders (X_w, kg/kg), these were dried at 105 °C until a constant weight was reached. Subsequently, the moisture content was calculated as the weight of water removed during drying divided by the initial weight of the powder (AOAC, 2002).

2.3.2 Glass transition temperature

The glass transition temperature (T_g) of the freeze-dried powders was analysed by using differential scanning calorimetry (DSC, Mettler Toledo, USA) with a nitrogen-based cooling system (Behboudi-Jobbehdar, Soukoulis, Yonekura, & Fisk, 2013). A portion of each powder (5-10 mg) was weighed in a stainless steel DSC pan and hermetically

sealed. A sample was first scanned at the rate of 10 °C/min to 70 °C to erase the thermal history, and then cooled at 10 °C/min to 0 °C. A second scan was run up from 0 °C to 150 °C at a heating-rate of 10 °C/min. An empty pan was used as the reference. The onset and midpoint glass transition temperatures ($T_{g,onset}$ and $T_{g,mid}$) were analysed using Mettler Toledo Star (Columbus, OH, USA) software from the second heating scan thermographs.

2.3.3 Microstructure

Samples were fixed on an aluminium stub using a conducting carbon tape and coated with gold using a sputter to produce a conductive surface. Scanning electron microscopy (SEM) images were recorded using a Hitachi S4700 (Hitachi Ltd., Tokyo, Japan) to visualise the microstructure of the powders.

2.3.4 Hygroscopicity

The hygroscopicity of freeze-dried powders was determined according to a method modified from Fritzen-Freire et al. (2012). Samples of each powder were placed in aluminium weighing dishes, and stored at 75 % RH and 25 °C for 1 week. The hygroscopicity was expressed as grams of adsorbed water per 100 grams of dry solids (g/100 g).

2.4 Isothermal heat treatment

Isothermal heat treatment of powder (RSM, X_w = 0.05) or LP cell suspensions in RSM

146 solutions (X_w = 0.60 & 0.90) was conducted using a Thermomixer (Eppendorf,
147 Germany) at 60 °C, 75 °C and 90 °C for the designated time. For freeze-dried bacteria
148 (X_w = 0.05), 0.100 ± 0.001 g sample was weighed and transferred into a 2 mL sterile
149 centrifuge tube. To prepare cell suspension with a moisture content of 0.60, 150 μL
150 sterile Milli-Q water was added to the centrifuge tube to dissolve 0.100 g powder by
151 high-speed vortexing. To prepare suspension with a moisture content of 0.90, LP cells
152 were harvested from 100 μL MRS broth by centrifugation (8000 g, 4 °C, 15 min) and
153 then re-suspended into 100 μL 10 % w/w RSM. Samples in a 2 mL airtight centrifuge
154 tubes were heated in the Thermomixer with a rotation speed of 300 rpm. The heating-
155 up time was less than 60 s.
156
157 After heat treatment for the required time, the centrifuge tube was immediately
158 transferred to an ice-water bath to avoid further inactivation of the bacteria.
159 Subsequently, 900 μL cold peptone water (0.1 % w/w, 4 °C) was added to the sample
160 (for X_w = 0.60, 1350 μL peptone water was added). All of the bacteria-suspended
161 matrices were fully homogenized prior to making serial dilutions, and 100 μL diluted
162 solution was spread onto MRS agar broth (OXOID, United Kingdom). The plates were
163 statically incubated at 37 °C for 48 h, and the survival curves of LP during heat
164 treatment were obtained by plotting the log (N/N_0) versus the heating time, where N is
165 the viable count (CFU/g) at time t and N_0 is the initial viable count (CFU/g). In addition,
166 isothermal heat treatment of the other powders (i.e., GA, MD and inulin matrixes) at
167 90 °C for 30 min were conducted using the same method described above.

2.5 Preparation of bread supplemented with *L. plantarum*

Bread dough was prepared in a mixer (Hauswirt® HM730, China), according to the following recipe: wheat flour (100 g), sugar (4 g), fine salt (1.5 g), instant yeast (1 g), non-salted butter (3 g), and UHT skimmed milk (65 g) (Zhang et al., 2018). Three approaches were applied to incorporate LP cells into bread: i) Cell suspension: LP cell suspension in UHT skimmed milk was directly utilized to prepare the dough (control group); ii) Dry powder: freeze-dried bacterial powder (1 g) was thoroughly mixed into the dough as the last item; iii) Powder distribution: 0.03 g powder was evenly distributed on the surface of a dough ball (5 g), which was done before proofing to ensure good adhesion of the powder to the dough. The dough was then divided into balls of 5 g for the first two approaches, and the dough balls were proofed at 40 °C, 85 % RH in a climate chamber (Yiheng, Shanghai, China) for 40 min. Subsequently, bread samples were baked at 100 °C for 15 min and at 175 °C for 6 min in an electric oven (Changdi® CRTF30W, China), respectively. These temperature and baking time combinations were selected on the basis of 98 % estimated starch gelatinization as an indicator for proper baking (see Appendix, Fig. A1) (Zhang et al., 2018). Only the third approach was used to prepare bread with the GA, MD and inulin containing bacterial powders. Temperature profiles of the bread crust (surface) and crumb (core) during baking were recorded using K-type thermocouples (Omega®, USA). The moisture content of the bread after baking was determined according to the AOAC method 925.10 (AOAC, 2002).

2.6 Microbiological analysis

To determine the viable counts of LP in dough and baked bread, sample (5 g) was aseptically homogenized with 45 mL sterile peptone water (0.1 % w/w) in a stomacher (iMix®, Interlab, France). Serial dilutions of the suspensions (100 μL) were made in 900 μL sterile peptone water, and 100 μL solution was subsequently plated onto the MRS agar broth (OXOID®, United Kingdom) supplemented with 200 mg/L natamycin (Antai®, China). Natamycin was added to inhibit the growth of yeast on the MRS agar plate, which did not affect the growth of LP (Zhang et al., 2014). The plates were statically incubated at 37 °C for 48 h. After incubation, the viability of LP in bread was recorded as log CFU per gram of the sample (log CFU/g).

2.7 Weibull distribution model

The Weibull distribution function has been applied as a primary thermal inactivation model for vegetative bacteria (Pérez-Rodríguez & Valero, 2013; van Boekel, 2002). In this work, Weibull model is used to describe the survival of LP in RSM matrices with different initial moisture contents (X_w = 0.05, 0.60 and 0.90, see section 2.4). Weibull model is a statistical model with an empirical nature, which describes the distribution of inactivation times. The cumulative function of Weibull model for a survival curve is:

$$\log S(t) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \quad (1)$$

$$S(t) = \frac{N(t)}{N_0} \quad (2)$$

where *S(t)* is the survival rate of the bacteria after heat treatment for a certain time, α is

the scale parameter that represents here the average death time of the microbial population, and β is the dimensionless shape parameter (van Boekel, 2009). The scale parameter α can be described by the semi-empirical Bigelow model (Eqns. 3-5) (Perdana et al., 2013):

$$\alpha = \alpha_{w,T} \cdot exp\left[ln\left(\frac{\alpha_{s,T}}{\alpha_{w,T}}\right) \cdot exp\left(-p \cdot \left(\frac{X_w}{1 - X_w}\right)\right)\right] \quad (3)$$

with

$$\log(\alpha_{w,T}) = \log\left(a_{w,T_{ref}}\right) - b_w(T - T_{ref}) \quad (4)$$

$$\log(\alpha_{s,T}) = \log\left(a_{s,T_{ref}}\right) - b_s(T - T_{ref}) \quad (5)$$

in which T is the temperature (K), X_w is the moisture content (kg/kg), p is a dimensionless parameter that describes the dependency of α on the moisture content. The $\alpha_{w,T}$ and $\alpha_{s,T}$ are Weibull parameters at X_w = 1 (infinite dilution) and X_w = 0 (pure solid form), respectively, which are described with the empirical equations (Eqns. 4 & 5) with parameters of $\alpha_{T_{ref}}$ and b, where T_{ref} is set to 323.15 K (Mohácsi-Farkas, Farkas, Mészáros, Reichart, & Andrássy, 1999; van Boekel, 2009). The unknown parameters in the Weibull model, i.e., $a_{w,T_{ref}}$, $a_{s,T_{ref}}$, b_w, b_s, p, were estimated using the add-in Solver in Excel 2010 (Microsoft®, USA).

2.8 Statistical analysis

All the experiments were done independently in duplicate or more and all the data are presented as mean ± standard deviation (SD). One-way ANOVA and Student's t-test were used to evaluate the difference between two means, and a p-value smaller than 0.05 meant that the difference between two means was significant ($p \leq 0.05$).

3. Results and discussion

3.1 Effect of moisture content on the survival of bacteria in RSM powder

Fig. 1 shows the survival curves of *L. plantarum* in RSM matrices with different initial moisture contents (i.e., 0.05, 0.60 and 0.90) during isothermal heating at 60, 75 and 90 °C, respectively (see also Section 2.4). At the same heating temperature, the survival of LP strongly increased as the moisture content of the matrix decreased (Figs. 1A-1C). For example, the viability of LP in solutions (X_w = 0.60 and 0.90) decreased by 5 log after 300-s heating at 90 °C (Fig. 1B & 1C), whereas the bacterial viability in dried RSM powder (X_w = 0.05) decreased only by 0.75 log after the same treatment (Fig. 1A). This result is consistent with other studies, confirming that the heat resistance of bacteria increases at lower moisture content (Hansen & Riemann, 1963; Yesair, Bohrer, & Cameron, 1946). In a previous study, the heat resistance of *Lactobacillus plantarum* embedded in skim milk powder during heating at 150 and 200 °C was found highest when the initial water activity a_w of the powder was between 0.20 and 0.50 (Laroche, Fine, & Gervais, 2005). The water activity a_w of the dried RSM powder in our study (X_w=0.05 kg/kg) was approximately 0.30 according to the sorption isotherm of skim milk powder (Murrieta-Pazos et al., 2011). However, the water activity in the RSM-water mixtures with an initial moisture content of 0.60 and 0.90 is very high (a_w>0.9). This difference in water activity and moisture content and subsequent improved survival behavior upon heat treatment observed in this study is thus in agreement with the previous study (Laroche, Fine, & Gervais, 2005).

The general trend of the pronounced influence of moisture content on survival of LP in the RSM/water system could be described by Weibull model (Eqns. 1-2, see lines in Figs. 1A-1C), although discrepancy was found between the prediction and the actual inactivation data. This discrepancy may be attributed to the isothermal heating method, where time required to heat and cool samples was neglected, which may influence the results especially at elevated temperatures (see Fig. B1 in Appendix B). In this study, the shape parameter α of Weibull model was estimated for each survival curve by assuming that cells are equally susceptible to heat throughout the treatment at all conditions (i.e., β=1) (Pérez-Rodríguez & Valero, 2013) (see Table B1 in Appendix B). A contour plot of different isothermal temperature conditions (45-135 °C) was made as a function of moisture content and α according to Eqns. 3-5 (lines in Fig. 1D), and a high coefficient of determination was found (R^2=0.99). The parameters in the Bigelow model (Eqns. 3-5), $a_{w,T_{ref}}$, $a_{s,T_{ref}}$, b_w, b_s and p were estimated: 321 (s), 3810 (s), 0.031 (1/°C), 0.026 (1/°C) and 0.864 (-), respectively. An increase in the magnitude of α was observed at decreasing moisture contents and temperatures, indicating a higher survival of probiotics under these conditions (see Fig. 1D). However, at higher moisture contents (X_w >0.90), α was not sensitive to changes in moisture content anymore, and thus depended only on the heating temperature (Fig. 1D). A similar observation was reported for *L. plantarum* WCFS1 incorporated in maltodextrin solutions (Perdana et al., 2013).

278 **3.2 Physicochemical properties of freeze-dried probiotic powder**

279 Table 1 shows several physicochemical properties of the probiotic powders freeze-dried
280 in different matrices (i.e. RSM, gum arabic, maltodextrin and inulin). The moisture
281 content of the dried probiotic powder ranged from 0.028 kg/kg to 0.046 kg/kg and
282 varied little when different carrier matrices were used (t-test, p>0.05). Moreover, the
283 moisture content was similar to that of other freeze-dried probiotic powders (Chávez &
284 Ledeboer, 2007; Zayed & Roos, 2004). No significant difference in the final viability
285 of LP was found among groups (all above 10.5 log CFU/g, t-test, p>0.05), while the
286 bacterial viability before drying was about 11 log CFU/mL in the cell suspensions,
287 suggesting that the drying matrices used in this study had little influence on the viability
288 variation of LP during freeze drying (Broeckx et al., 2016).

289

290 The glass transition temperature (both onset and midpoint T_g) of the powder containing
291 10 wt. % gum arabic was the highest in comparison to that of other powders (Table 1).
292 It is assumed that the measured T_g values are not affected by the presence of the
293 bacterial cells (Fonseca, Obert, Béal, & Marin, 2001; Santivarangkna et al., 2011).
294 Because powders have similar water content, it is the anhydrous T_g of the drying matrix
295 that has the largest influence on the measured T_g of the probiotic powders. Therefore,
296 the high T_g of the GA bacterial powder is probably due to the high anhydrous T_g of gum
297 arabic. Unfortunately, only an approximated anhydrous T_g of gum arabic of 170 °C was
298 reported (Collares & Kieckbusch, 2004; Victória, Fernandes, & Vilela, 2014). This
299 anhydrous T_g of gum arabic was higher than that of RSM (92 °C), maltodextrin (DE13-

17, 153- 158 °C) and inulin (119 °C) reported in previous studies (Bhandari & Howes, 1999; Jouppila & Roos, 1994; Perdana et al., 2014). It is worthy to mention that the anhydrous T_g of maltodextrin (DE13-17) was also approximated based on a linear correlation between T_g and the 'Dextrose Equivalent (DE)' of maltodextrin (Bhandari & Howes, 1999).

All the four freeze-dried powders can be classified as hygroscopic because their hygroscopicity was higher than 10 g/100 g (Schuck, Anne, & Jeantet, 2012). In particular, the powders dried in gum arabic and maltodextrin appeared to be more hygroscopic than the other two, although no significant differences in hygroscopicity among groups was observed due to the large standard deviation ($p>0.05$) (see Table 1). Among the tested encapsulating materials, gum arabic and maltodextrin are hydrophilic compounds (Comunian & Favaro-Trindade, 2016). The hygroscopicity of RSM-probiotic powder was relatively low and close to the reported value for skim milk powder (10.2 g/100 g) (Schuck et al., 2012). The poor solubility of inulin in water can explain in the lower hygroscopicity of the corresponding probiotic powder (Mensink, Frijlink, Maarschalk, & Hinrichs, 2015).

Fig. 2 shows the morphology of the freeze-dried bacterial powders at the micrometre scale. Abundant intact LP cells were found fixed in the compact microstructure of RSM or GA matrices (Figs. 2A & 2B). Nevertheless, the bacteria cells seemed not so well embedded in the maltodextrin or inulin matrices (Figs. 2C & 2D): cells seemed to be included in the cavities of the continuous maltodextrin matrix, while the cells were

323 stacked on top of each other in inulin, resulting in a less obvious boundary between the
324 cells and the matrix. The distinct microstructure of the different bacterial powders is
325 difficult to explain, but is probably also related to the ice crystallization process during
326 freezing (Harnkarnsujarit, Charoenrein, & Roos, 2012).
327
328 **3.3 Effect of matrices on survival of bacteria during isothermal heating**
329 Fig. 3 shows that survival of LP during isothermal heating at 90 °C is influenced by the
330 drying matrices in which the cells are imbedded. The survival of LP cells was found the
331 highest in the GA matrix, followed by the RSM matrix. The protective effects of
332 maltodextrin and inulin on the LP cells were limited: the log reductions of bacteria in
333 GA, RSM, MD and inulin after 30-min heating at 90 °C were about 1.5, 2.75, 3.75 and
334 4.25, respectively (refer to Fig. 3).
335
336 The higher LP survival observed in GA may be due to the high T_g of this formulation
337 (Table 1), which is also suggested by Lodato, de Huergo, & Buera (1999) in a study on
338 the thermal stability of a yeast strain freeze dried in difference matrices. Although none
339 of the powders are in the glassy state at 90 °C, it may be expected that the mobility of
340 the molecules in the GA matrix is lowest compared to the other formulations, which
341 can explain the higher survival of the LP cells embedded in that matrix (Santivarangkna
342 et al., 2011). Moreover, the physical embedding of LP cells in the RSM matrix or the
343 compact GA matrix (Figs. 2A & 2B) seems better compared to the embedding in the
344 inulin and MD matrices (Figs. 2C & 2D), which may assist in protection of the bacteria

345 towards the harsh environmental conditions (Huang et al., 2014; Zheng et al., 2015).
346 Specifically a large number of bacteria were observed on the surface of the MD and
347 inulin powders, which suggests that bacteria in these matrices are less protected.
348
349 **3.4 Different approaches to incorporate probiotics in bread**
350 Different approaches may be applied to incorporate probiotic powders into bread, most
351 probably resulting in different survival during baking. In this study, the following three
352 approaches were used: i) addition of cell suspension in dough (control group); ii)
353 addition of dried probiotic powder to dough; and iii) application of dried probiotic
354 powder onto the surface of dough (De Prisco & Mauriello, 2016), as described in detail
355 in Section 2.5. The final viability of bacteria in bread prepared with dried probiotic
356 powders (using the second and third approaches) were compared to that of the control
357 group. Only RSM powder was used for these experiments and compared to cells
358 suspended in skim milk. As shown in Fig. 4A, the application of powder onto the dough
359 surface provided the highest viability of LP in baked bread, at the same baking
360 conditions (i.e., 6-min at 175 °C or 15-min at 100 °C). This can be explained by the
361 higher survival of LP at lower moisture content (see Section 3.1), even though the
362 temperature in the surface region of the bread is higher than in the core during baking
363 at 175 °C (Fig. 4B) (Zhang et al., 2018). The residual viabilities of the probiotics in
364 breads prepared with free cell suspension and powder mixed in the dough (the second
365 approach) were similar, i.e. 10^4 CFU/g after 6-min baking at 175 °C and 10^6 CFU/g
366 after 15-min at 100 °C, respectively (Fig. 4A). This suggests that the RSM matrix did

not protect the LP cells during baking even when supplied as a dry powder. The reason is probably the fast hydration of the powder, which exposed the bacterial cells to a more moist environment, and thus the cells became more susceptible to thermal inactivation (van Boekel, 2008).

Fig. 4A shows that the viability of LP in all three kinds of bread baked at 100 °C was 2 log higher than that of breads baked at 175 °C. The higher survival rate of LP can be attributed to the relatively low temperature reached (< 100 °C) inside the bread (Fig. 4B). The moisture contents of the bread crumb (0.34 kg/kg) was similar at the two baking temperatures (see Fig. 4B), as well as the crumb structure (data not shown). Remarkably, a high bacterial viability of 10^8 CFU/g was observed after baking at 100 °C when the third approach was used. This bacterial viability was even higher than viabilities reported in other studies in which a probiotic-containing edible film was applied onto the surface of partially-baked bread (Altamirano-Fortoul, Moreno-Terrazas, Quezada-Gallo, & Rosell, 2012; Soukoulis et al., 2014), or when a liquid sourdough was injected into baked bread (Lönner, 2008).

When the dried bacterial powder is applied onto the bread surface, the survival rate of LP after baking could be estimated with the earlier developed kinetic model in Section 3.1 (Eqns. 1-5 & Fig. 1D) and the measured temperature profiles of the bread surface during baking (Fig. 4B). We considered two extreme conditions: i) the powder maintained its low moisture content after proofing (X_w = 0.05); ii) the powder absorbed

water from the environment during proofing (X_w = 0.40, same as the dough). Based on these two more extreme situations, a linear semi-logarithmic survival curve is calculated (β = 1) using the temperature measurements retrieved each 10 s and using Eqns. 1 & 2, which are rewritten as:

$$\log\left(\frac{N_{i+1}}{N_i}\right) = -\frac{1}{2.303}\left(\frac{\Delta t}{\alpha}\right) \qquad (\mathrm{i} = 0,1,2\ldots \mathrm{n}) \qquad (6)$$

where Δt is the discrete time interval (Δt =10 s). The shape parameter α was changing along with the increasing temperature inside bread during baking (Figs. 1D & 4B), and was calculated based on Eqns. 3-5 at each time interval. Finally, the accumulated reduction of LP viability during baking can be estimated, i.e. $log(N/N_0)$. The log reduction of LP viability in bread was predicted between – 2.23 and – 8.16 after 6-min baking at 175 °C, and between – 0.31 and – 0.97 for baking at 100 °C for 15 min. The corresponding experimental results were – 2.46 and – 0.71, respectively, which fell within the range of the predicted values (Fig. 4A). Therefore, the kinetic model may be used to obtain a first approximation of the residual viability when the bacteria are applied as a powder on the dough surface.

3.5 Effect of matrices on survival of bacteria during bread baking

The influence of different drying matrices on the survival of LP during bread baking was investigated. The powder was added to bread by distributing it on the dough surface and a control group was made without adding probiotics (see Fig. 5). The RSM matrix showed the highest protective effect on LP cells during baking at either 100 °C or 175 °C ($p \leq 0.05$), followed by the inulin matrix (Table 2). However, no protective effect was

observed for gum arabic and maltodextrin during baking (Table 2), even though gum arabic performed the best during isothermal heating as discussed in Section 3.3 (Fig. 3). Fig. 5 shows that both GA and MD bacterial powders dissolved after proofing, while the RSM and inulin powders remained relatively dry, which is possibly due to the hydrophilic nature of GA and MD as compared to RSM and inulin (see Section 3.2). The hydration of powder is expected to negatively affect the survival of embedded LP cells, as the initially glassy powder will enter the rubbery state due to the 'plasticising effect' of water (Crowley, Kelly, Schuck, Jeantet, & O Mahony, 2016). Therefore, the dissolution of GA and MD powders after proofing is probably responsible for the low survival rate of LP during baking (Ansari & Datta, 2003). Furthermore, RSM led to higher viability compared to inulin, e.g. at 175 °C (log reduction was – 2.46 for RSM compared to – 4.01 for inulin), which may be related to the increased visual entrapment of bacteria into the matrix (Fig. 2).

It is important to note that in this study no browning of the surface of the breads occurred due to the relative low baking temperatures applied (Fig. 5). Although the surface temperature of bread baked at 175 °C exceeded 120 °C (the minimum temperature required for initiating color formation) in the late stage of baking (Fig. 4B), the baking time was too short to cause an obvious brown colour on the bread surface (Zanoni, Peri, & Bruno, 1995). In addition, although the extent of starch gelatinization was estimated to reach 100 % in the crumb after 15 min baking at 100 °C (see Appendix A, Fig. A1), the core temperature of the bread just reached 98 °C after baking (Fig. 4B). The short duration of the 98 °C baking plateau may have influence on the staling of the

bread (Besbes, Jury, Monteau, & Le Bail, 2014; Le-bail, Agrane, & Queveau, 2012).

4. Conclusions

The survival of encapsulated *L. plantarum* (LP) during subsequent isothermal heating and baking is indeed strongly influenced by the matrix composition and processing conditions. In particular the moisture content appeared to have large influence on the survival of bacteria upon exposure to heat. The Weibull model could describe the general trend of the bacterial inactivation kinetics during isothermal heating as influenced by the initial moisture content of the RSM matrix, which could be used to predict the survival rate of bacteria in baked bread. Application of the RSM-probiotic powder onto the surface of the bread could best delay the water migration from the dough into the dry powder, which was critical to maximally preserve the bacterial viability during baking. Incorporation of the dry powder in the bread crumb appeared not practical as the high moisture content in the crumb quickly rehydrates the powder and thus cancels out the protective effect of the encapsulation matrix. It is noted that application of powder on the dough surface slightly alters the appearance of the bread and baking time needs to be extended if browning of the crust is desired. Further evaluation of the organoleptic properties of the probiotic-fortified bread is therefore necessary.

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Conflict of Interest

All authors report no conflicts of interest.

Appendix. A. Starch gelatinization

The extent of starch gelatinization in dough was estimated using the method described in our previous study (Zhang et al., 2018). The starch gelatinization is described by a first-order kinetic model as a function of temperature (Fig. 4B) and time, and the extent of starch gelatinization in the crumb of 5 g dough was estimated to reach 98 % after 10-min baking at 100 °C and after 4.5-min baking at 175 °C, respectively (see Fig. A1).

Appendix. B. Supplementary results of Weibull model

Fig. B1 shows the parity plots of the logarithmic values of the residual viability of *Lactobacillus plantarum* obtained from experiments and calculated by Weibull model. The goodness-of-fit of Weibull model to the experimental inactivation data was acceptable in general, however some outliners were observed which was due to the

large standard deviation of the original data. The estimated parameter of Weibull model α, the corresponding root mean square error (*RMSE*) and the coefficient of determination (R^2) were shown in Table B1. A low RMSE value indicates a good fitting of the model to the data (Eqn. B1).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(Y_i - \hat{Y}_i)^2}{n}} \qquad (B1)$$

Where Y_i is the experimental result, and $\hat{Y}_i$ is the calculated value and n is the number of data points.

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Table 1. Physicochemical properties of bacterial powders freeze-dried in different matrices with the same 10 wt.% initial solid (RSM = reconstituted skim milk, GA = gum arabic, MD= maltodextrin DE13~17).

Property	RSM	GA	MD	Inulin
Moisture content (kg/kg)	0.046^{a} ±0.011	0.034^{a} ±0.019	0.028^{a} ±0.016	0.034^{a} ±0.016
Viable cell count (log CFU/g)	10.87^{a} ±0.22	10.76^{a} ±0.08	10.63^{a} ±0.21	10.54^{a} ±0.09
$T_{g,onset}$ (°C)	53.79^{b} ±2.51	60.64^{a} ±2.24	54.71^{b} ±2.49	48.80^{b} ±4.52
$T_{g,mid}$ (°C)	70.79^{c} ±1.00	80.28^{a} ±2.74	73.58^{b} ±0.18	68.80^{d} ±0.91
Hygroscopicity (g/100 g)	12.05^{a} ±5.28	20.05^{a} ±3.32	16.79^{a} ±2.49	13.35^{a} ±4.03

$^{a\text{-}d}$ Parameters with different superscript letters within the same row have significant differences ($p \leq 0.05$).

672 **Table 2**. Viability of *L. plantarum* in bread supplemented with different bacterial
673 formulations before and after baking at 175 °C for 6 min or at 100 °C for 15 min.

Property	RSM	GA	MD	Inulin
Initial viable count (log CFU/g)	8.77^{a} ±0.03	8.04^{b} ±0.06	8.13^{ab} ±0.18	8.17^{ab} ±0.24
Viable count at175 °C (log CFU/g)	6.31^{a} ±0.19	2.99^{c} ±0.12	2.95^{c} ±0.24	4.16^{b} ±0.16
Log reduction at 175 °C (-)	-2.46	-5.05	-5.18	-4.01
Viable count at 100 °C (log CFU/g)	8.03^{a} ±0.10	4.95^{c} ±1.23	6.57^{b} ±0.43	7.42^{b} ±0.11
Log reduction at 100 °C (-)	-0.74	-3.09	-1.56	-0.75

674 $^{a\text{-}d}$ Parameters with different superscript letters within the same row have significant
675 differences ($p \leq 0.05$).
676
677

678 Table B1. Estimated Weibull parameter α, the corresponding root mean square error
679 (*RMSE*) and the coefficient of determination (R^2) for each experimental condition as
680 described in Section 2.4 for isothermal heating of RSM-water mixtures with different
681 initial moisture contents.

Moisture content X_w (kg/kg)	T (°C)	α (s)	R^2 values	*RMSE* values
0.05	60	1900	0.84	0.19
	75	760	0.78	0.38
	90	280	0.76	0.61
0.60	60	282	0.94	0.26
	75	108	0.55	1.19
	90	46	0.69	1.40
0.90	65	184	0.72	0.75
	75	46	0.62	1.46
	90	17	0.63	1.27

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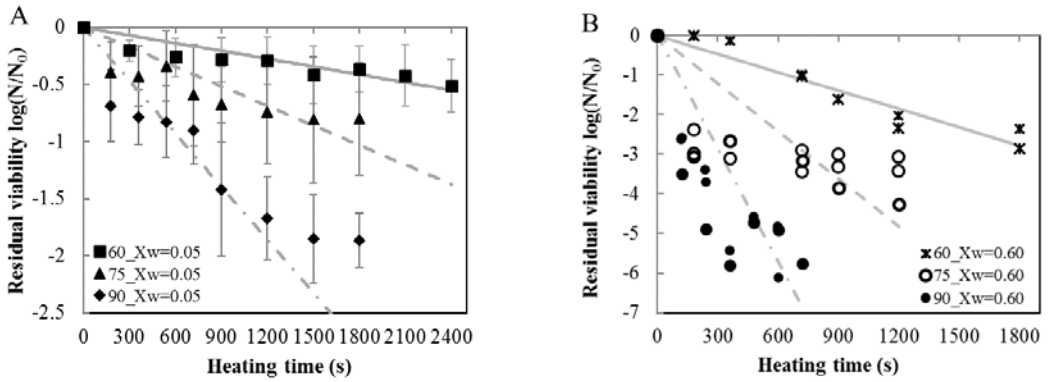

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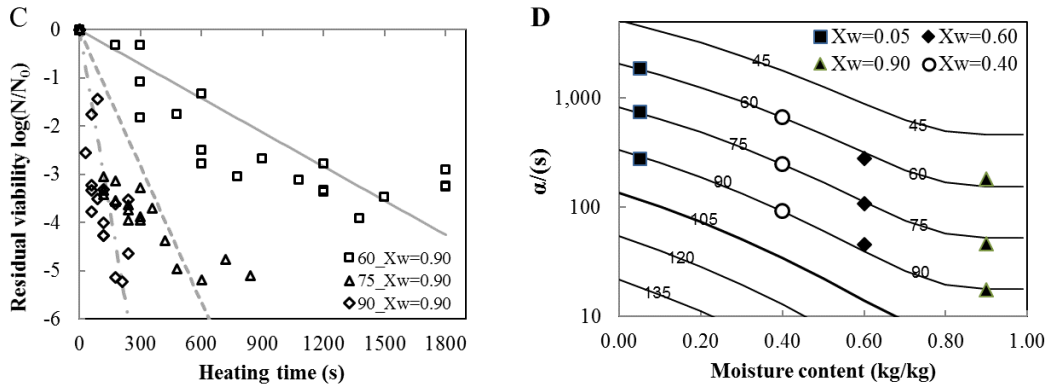

687 **Fig. 1**. Survival curves of *L. plantarum* in RSM matrixes with different initial moisture
688 contents (A: X_w=0.05; B: X_w=0.60; C: X_w=0.90) during isothermal heat treatment at 60
689 °C, 75 °C and 90 °C; solid lines and dashed lines represent fitted results of Weibull
690 model, and error bars represent standard deviation (n=4). D: The scale parameter α
691 estimated based on experimental data for each *T*-X_w combination (■, X_w=0.05; ♦,
692 X_w=0.60; ▲, X_w=0.90) and the predicted α (○, X_w=0.40); lines represent the contour plot
693 of temperature as a function of α and moisture content based on Eqns. 3~5 (R^2=0.99).
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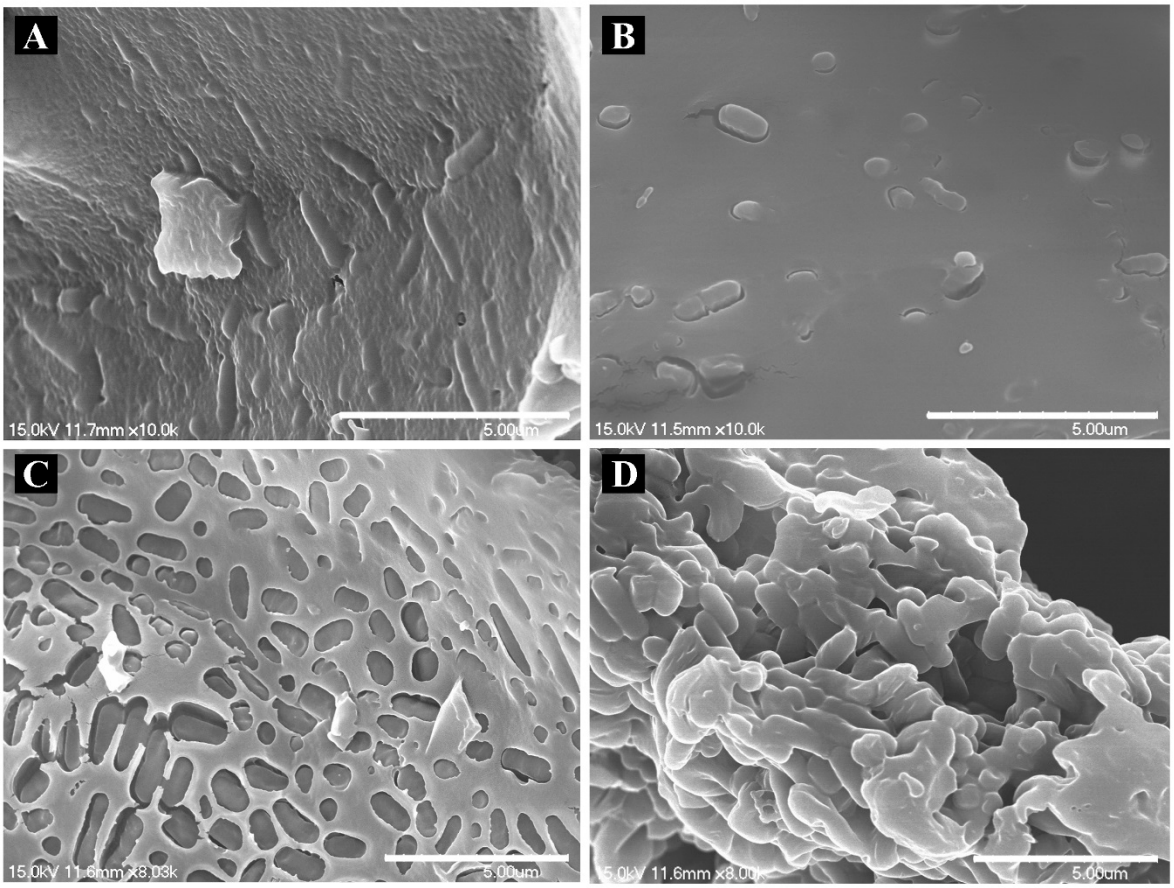
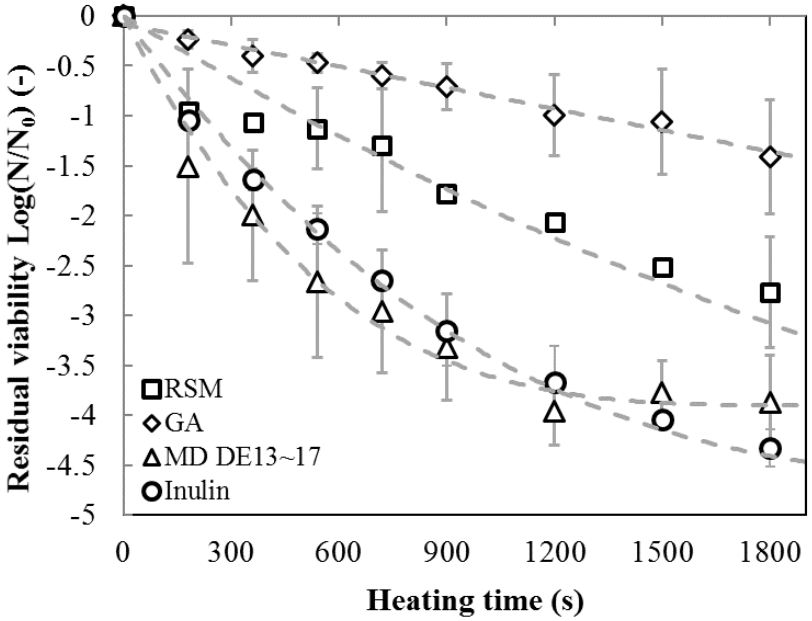


Fig. 2. SEM images of *Lactobacillus plantarum* freeze dried in different matrices (A: RSM; B: gum arabic; C: maltodextrin DE13~17; D: Inulin), scale bars represent 5.00 μm.



703

704 **Fig. 3**. Semi-logarithmic survival curves of *L. plantarum* freeze-dried in different

705 matrices during isothermal heat treatment at 90 °C for 1800 s (□, RSM; ◊, GA; Δ, MD

706 DE13~17; ○, inulin). Dashed lines are drawn to guide the eye and the error bars indicate

707 the standard deviation.

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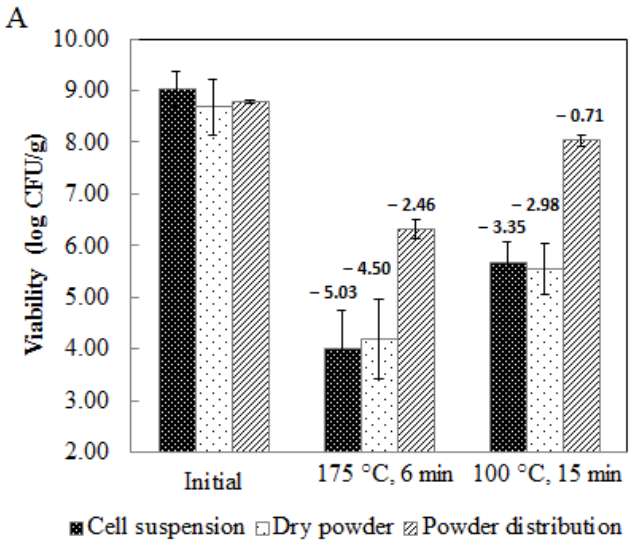

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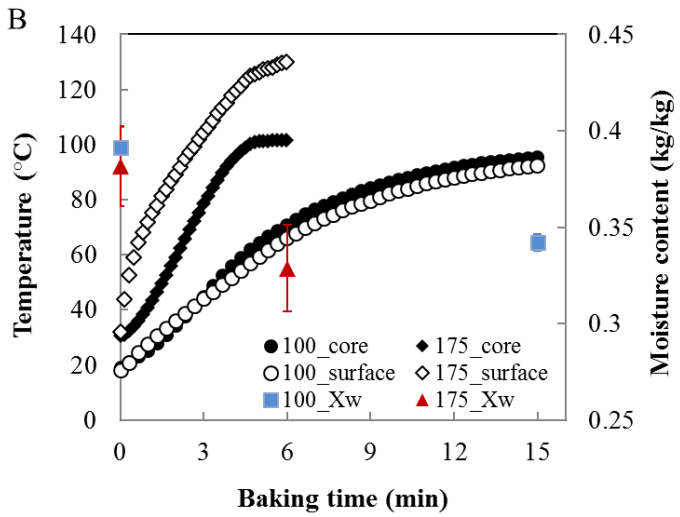
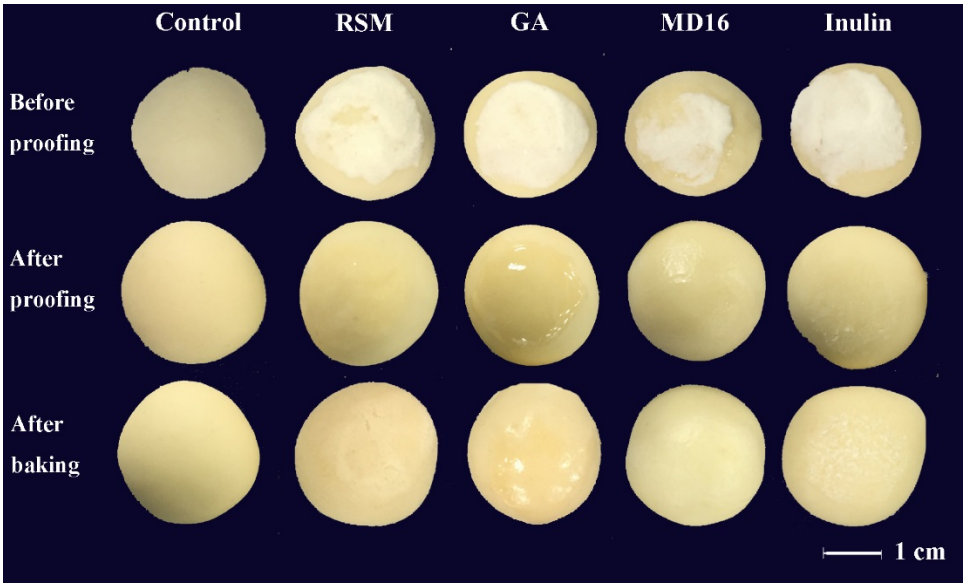


711

712 **Fig. 4**. A: Viable counts of *L. plantarum* in bread before and after baking at 175 °C for
713 6 min or at 100 °C for 15 min with three different approaches to incorporate probiotics
714 into bread (i.e., cell suspension, dry powder and powder distribution); the
715 corresponding log reduction of the LP viability was marked on top of each bar; B:
716 Temperature profiles of the core and the surface of bread during baking at 175 °C (6
717 min) and 100 °C (15 min), and the average moisture contents (kg/kg) of the dough and
718 the baked bread (▲, 175 °C; ■, 100 °C).

719



720

721 **Fig. 5**. Digital images of the dough or the bread supplemented with different bacterial

722 powders that were evenly distributed on the surface of the dough before proofing (bread

723 was baked at 175 °C for 6 min or at 100 °C for 15 min, and the appearance of bread

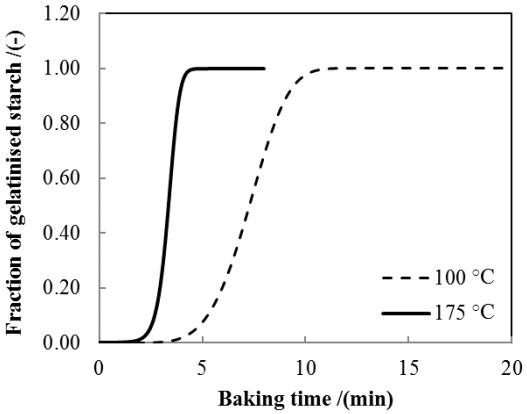
724 samples baked at these two conditions was similar, so only the images of one group

725 were shown).

726

727

728



729

730 **Fig. A1**. Estimated extent of starch gelatinization in the crumb during baking of 5 g

731 bread at 100 °C (dashed line) and 175 °C (black line).

732

733

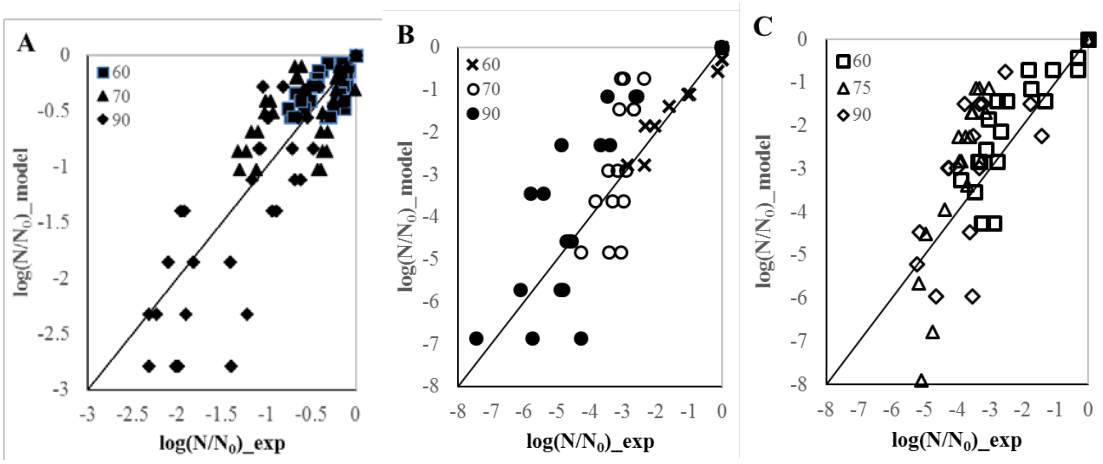


Fig. B1. Parity plots of the residual viability of *Lactobacillus plantarum* in RSM matrices during isothermal heating (at 60, 75 and 90 °C, respectively) obtained from experimental data and calculated by Weibull model. The symbols represent the results from all the replicates for each experimental condition, i.e., different initial moisture contents (kg/kg): (A) X_w=0.05; (B) X_w=0.60; (C) X_w=0.90.