LWT - Food Science and Technology 131 (2020) 109705

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Impact of carrier agents and temperature during storage of dry inocula of *Salmonella enterica*: A contribution to the validation of low water activity foods processing interventions

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ARTICLE INFO

Keywords:
Low moisture foods
Challenge tests
Food safety modernization act
Thermal processing
Dried foods

ABSTRACT

In this study, dry inocula were developed using four different carriers [talc, fine sand (0.05–0.2 mm), regular sand (0.2–0.6 mm), and calcium carbonate] for validation of inactivation steps of low moisture foods against *Salmonella enterica*. Besides, the changes in a_w and the survival of *S. enterica* in the dry inocula made with the four carriers were evaluated throughout the storage period of up to 150–180 days at 4 and 25 °C. Among the carriers evaluated fine and regular sand seem to constitute the best carrier agents for longer preservation of *S. enterica* cells to be employed in dry inocula. Besides, the storage of inoculated carrier agents under refrigeration (4 °C) enhanced the stability of the dry inocula prepared, maintaining *S. enterica* counts unaltered for ~ six months ($p < 0.05$). Overall, based on the results, it has been found that the a_w variation during storage did not directly influence *S. enterica* survival in the dry inocula. However, considering only the a_w of the dry inocula obtained using the different carrier agents, it is possible to propose combinations between carrier agents and a_w of low moisture foods (LMF). The findings of this study will be highly relevant for the development of stable dry inocula for use in the validation of the inactivation steps of LMF.

1. Introduction

Low moisture foods (LMF) industries are required to validate the ability of a lethal step to deliver a specified number of decimal reductions of a target microorganism (Food safety modernization act FSMA, 2011). Because of the occurrence of salmonellosis outbreaks, *Salmonella* is considered the target microorganism for killing steps of LMFs (FDA, 2009; Industry handbook for safe processing of nuts, 2016). Then, for lethal step validation, an LMF needs to be inoculated with *Salmonella* or a surrogate microorganism. However, the inoculation procedures must not modify food properties such as a_w and structure to avoid changes in microbial inactivation kinetics, which would affect the number of decimal reductions caused by the inactivation step. Therefore, to avoid these changes, a dry inoculum is preferable.

Additionally, two further requirements when inoculating microorganisms in LMF for validation studies comprise the need for the inoculum to be as homogeneous as possible ("Microbiological Challenge Testing," 2003; "Parameters for determining inoculated pack/challenge study protocols," 2010) and for the population of target microorganism to remain stable as long as possible during dry inoculum storage. In this sense, several *Salmonella* dry inoculation methods for use in validation tests have been reported in the literature (Enache et al., 2015; Liu, Xu, Xie, Zhu, & Tang, 2019; Trinetta, McDaniel, Magossi, Yucel, & Jones, 2019; Xu et al., 2018). Numerous carrier agents have been tested, such as talc, sand, calcium carbonate, and silica. Nonetheless, limitations described in the studies include complicated procedures for inoculum preparation and food inoculation, as well as, difficulties in inoculum homogenization, achievement of high-concentration inoculum and changes in food properties due to the inoculum used (Beuchat & Mann, 2015; Blessington, Theofel, & Harris, 2013; Bowman, Waterman, Williams, & Ponder, 2015; Enache et al., 2015; Shrestha & Nummer, 2016).

One aspect barely studied comprises the evaluation of the effect of medium-to long-term storage on *Salmonella* titer in the carriers used to prepare the dry inocula. The effect of storage of a dry inoculum on *Salmonella* concentration was only studied in talc (Enache et al., 2015) and chalk (Hoffmans & Fung, 1992), for up to 30 and 180 days, respectively. Assessing the impact of medium-to long-term storage on

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https://doi.org/10.1016/j.lwt.2020.109705
Received 1 April 2020; Received in revised form 28 May 2020; Accepted 1 June 2020
Available online 10 June 2020
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Salmonella survival in carriers is a primary concern for the inoculation of LMF with a dry inoculum for thermal processes validations. Therefore, in this study, dry inocula were developed using four different carriers for validation of inactivation steps of LMF against *Salmonella enterica*. Also, the stability of the dry inocula made with the four carriers during storage for up to 180 days at two temperature conditions was evaluated.

2. Material and methods

2.1. Carrier agents

Four carrier agents were chosen (talc, two types of sand, and calcium carbonate) for the preparation of the dry inocula. These carriers were chosen considering their previous use for *Salmonella* dry inocula preparation (Beuchat & Mann, 2015; Blessington et al., 2013; Bowman et al., 2015; Enache et al., 2015; Shrestha & Nummer, 2016). The talc was from Dinâmica, São Paulo, Brazil ($3MgO{\cdot}4SiO_2{\cdot}H_2O$). Two types of sand with different grain sizes were used: fine (0.05–0.2 mm) and regular (0.2–0.6 mm) (Campinas/SP, Brazil). The calcium carbonate employed was from Dinâmica, São Paulo, Brazil ($CaCO_3$, PM: 100,09).

2.2. Salmonella strains

Five strains of *S. enterica* were selected for the preparation of the dry inocula. The strains belonged to the following serotypes: Senftenberg 775W, Havana IOC 2310, Infantis IOC 2327, Mbandaka IOC 2317, and Typhimurium IOC 2328. The last four serotypes were isolated from final product samples of soybean bran produced in different processing plants located in Brazil and deposited at Institute Oswaldo Cruz Culture Collection of Enterobacteriaceae. The Senftenberg serotype was employed as previous studies demonstrated its unique heat resistance (Ng, Bayne, & Garibaldi, 1969).

2.3. Liquid inocula preparation

In order to obtain the inoculum, each of the five *S. enterica* strains was separately inoculated in Tryptic Soy Broth (TSB; Kasvi, Italy), following incubation at 37 °C for 24 h. This procedure was repeated two consecutive times, and the same incubation conditions were employed. Then, the *Salmonella* cells of each strain were centrifuged, washed and further resuspended with saline solution (NaCl 0.85%; Dinâmica, São Paulo, Brazil), following the adjustment of cell concentration through optical density measurement in spectrophotometer at 630 nm wavelength as previously described (DU6408, Beckman, California, USA) (Jesus et al., 2016; Sant'Ana, Landgraf, Destro, & Franco, 2013). The inoculum for each *S. enterica* strain was standardized at a concentration of approximately 10^8 CFU/mL, and these were further combined in equal proportions to generate a pool made with the five *S. enterica* strains. After that, the verification of *S. enterica* concentration (in CFU/mL) was performed through pour plate count using Xylose Lysine Deoxycholate Agar (XLD, Acumedia, Michigan, USA) and incubation at 37 °C per 24 h.

2.4. Dry inocula preparation

The dry inocula were prepared based on adaptations from previous studies published in the literature (Blessington et al., 2013; Enache et al., 2015; Shrestha & Nummer, 2016). The liquid inoculum was added in the proportions of 1:1 (w/w) in the calcium carbonate and 1:2 (w/w) in the talc and two types of sand. The proportions were determined in preliminary tests conducted (data not shown). The liquid inoculum was added to each carrier and further gently homogenized and placed in sterile trays. The trays were covered with sterile aluminum foil and then disposed in an oven at 35 °C/24 h for drying. Then, the trays were kept at room temperature for further 24 h for complete drying. The time and temperature combination for drying was chosen based on preliminary experiments (data not shown). After drying, the dry inocula were sifted, homogenized in a mortar, and placed in sterile, tightly closed plastic bags (polypropylene - PE). The bags with the dry inocula were stored at room temperature (25 ± 2 °C) and under refrigeration (4 ± 2 °C), to determine in which conditions *S. enterica* would be more stable through storage.

2.5. Survival of S. enterica during storage of dry inocula prepared with four carriers

The survival of *S. enterica* in the dry inocula was determined after drying and during the storage period at 25 ± 2 °C and 4 ± 2 °C for up to 180 days. For the enumeration of *S. enterica* in each carrier, an aliquot of 5 g was suspended in 45 mL of 0.1% peptone water (Acumedia, Michigan, USA), following decimal dilution and drop plating in XLD agar (Acumedia, Michigan, USA), which were incubated at 37 °C/24 h. The counts of *S. enterica* in each carrier stored at different temperatures were expressed as CFU/g. Besides, the water activity (a_w) of each carrier was measured after drying and throughout the storage period using an a_w measurement equipment (Aqualab CX2T, Decagon Devices, Washington, USA).

2.6. S. enterica survival kinetics in dry inocula prepared with four different carriers

The Weibull model (Mafart, Couvert, Gaillard, & Leguerinel, 2002) was fitted to the survival data of *S. enterica* in the four carriers throughout the storage period. This model was used as the data did not follow first-order kinetics. The model is described by equation (1):

$$\log S(t) = -(t/\delta)^p \quad (1)$$

where S is the survivors' fraction at a particular time (t), δ is the required time for the first decimal reduction to take place, and p is the model curvature parameter. The estimation if survival kinetics was done using Microsoft Excel add-in, GinaFiT (Geeraerd, Valdramidis, & Van Impe, 2005).

2.7. Statistical analyses

The statistical differences in the survival parameters of *S. enterica* in the dry inocula were assessed by ANOVA and two-sample *t*-test using XLSTAT 2019.2.2. (Addinsoft, Boston, USA). The correlation between the a_w in different carriers during the storage of the dry inocula was verified through the Pearson correlation ($p = 0.05$) using the *ggpubr* package of R (version 3.3.1) (The R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

In this study, four carrier agents were used to prepare dry inocula of *S. enterica*, and the survival of this pathogen inoculated in the carriers was assessed throughout storage of up to 180 days at 4 and 25 °C. As shown in Table 1 (and Supplementary Figs. S1 and S2), the four carrier agents tested resulted in dry inocula with different initial a_w values even after the standardization and preparation procedures employed. For instance, at 4 °C the inoculum prepared with calcium carbonate presented the highest initial a_w after drying (0.946 ± 0.011) ($p < 0.05$), while inocula prepared with talc, fine sand, and regular sand did not present significantly different initial a_w values, i.e., 0.489 ± 0.042, 0.635 ± 0.069 and 0.601 ± 0.089, respectively ($p > 0.05$) (Table 1). Similar initial a_w values were also obtained for the four carrier agents when the storage was 25 °C (Table 1), and again the dry inoculum with calcium carbonate presented the highest a_w ($p < 0.05$). Even though the inoculum prepared with calcium

Table 1
Initial and final a_w of dry inocula of *S. enterica* prepared with different carriers and stored at 4 and 25 °C for up to 180 days[a,b].

Carrier	Temperature (°C)	Initial a_w	Final a_w
Fine Sand (0.05–0.2 mm)	4	0.648 ± 0.211[a,B]	0.724 ± 0.088[b,B]
	25	0.635 ± 0.069 [a,B]	0.645 ± 0.059 [a,A]
Regular sand (0.2–0.6 mm)	4	0.608 ± 0.114 [a,B]	0.670 ± 0.000 [a,B]
	25	0.601 ± 0.089 [a,B]	0.671 ± 0.005 [a,A]
Calcium carbonate	4	0.946 ± 0.011 [a,A]	0.968 ± 0.000 [a,A]
	25	0.927 ± 0.033[a,A]	0.711 ± 0.000[b,A]
Talc	4	0.489 ± 0.042 [a,B]	0.656 ± 0.062 [a,Bc]
	25	0.542 ± 0.061 [a,B]	0.669 ± 0.014 [a,A]

[a] Different lowercase letters in the same column indicate significant difference ($p < 0.05$) according to Tukey test amongst the a_w of dry inocula made with different carriers and stored at the same temperatures.
[b] Different uppercase letters in the same line indicate significant difference ($p < 0.05$) according to *t*-test, between initial and final a_w of a dry inoculum stored at 4 or 25 °C.
[c] After 150 days of storage.

carbonate was dried during extra 24 h at 37 °C in an attempt to achieve an initial a_w of ~0.6, this approach was not adequate as after this period (total drying time 72 h), Salmonella was not detected (10^2 CFU/g). The high a_w values observed in the inoculum prepared with calcium carbonate may also be linked to the formation of clusters that hampered its pulverization and further drying till lower a_w values. This behavior has also been reported in a previous study (Blessington et al., 2013). Despite this, these data indicate that it is not feasible/practical to expect to obtain dry inocula with similar initial a_w values when different carriers are used. These differences in a_w values of the prepared dry inocula are related, among others, to the inherent properties, such as initial moisture, size, shape, and arrangement of each carrier studied that difficult the drying process (Qiu, Zhang, Tang, Adhikari, & Cao, 2019; Ratti, 2001). Despite this, these properties can be exciting and useful for the preparation of dry inocula with a_w values that fall in the ranges of different LMF that need to be submitted to validation tests.

Another critical aspect regarding the development and further use of dry inocula is that the materials do not experience significant a_w changes throughout the storage period. This aspect is essential because changes in a_w during the storage period may impact on the titer of the inoculated microorganism, may allow the growth of contaminating microorganisms (fungi, for instance) or may result in physical changes of the material (agglutination, for instance). Therefore, the a_w of the dry inocula prepared with the four carriers was also assessed at the end of the storage period (up to 180 days). The data indicated that a_w of the dry inocula remained constant through the storage period for almost all the materials ($p > 0.05$). The only exceptions were an increase of a_w observed in the dry inoculum prepared with fine sand stored at 4 °C and a decrease of a_w detected in the dry inoculum prepared with calcium carbonate stored at 25 °C ($p < 0.05$) (Table 1). As a result, non-significant differences were observed in the final a_w of the dry inocula prepared with the four carriers when the storage was done at 25 °C ($p > 0.05$). However, when the storage was done at 4 °C, calcium carbonate still presented the highest a_w ($p < 0.05$). These changes in a_w indicate that maximum storage periods will vary with the type of carriers used to prepare the dry inoculum. For instance, for storage of the dry inoculum at 25 °C made using calcium carbonate as the carrier, a maximum of 90 days of storage should be used. On the other hand, 120–150 days would be the maximum time for storage of the dry inoculum made with fine sand and maintenance of a_w close to initial values (~0.62–0.64) (Table 1). Knowing how a_w changes during the storage of dry inocula are essential for validation of inactivation steps of LMF because modifications in the water content of foods will impact on inactivation kinetics of target microorganisms. Increases in a_w are known to drastically decrease the heat resistance of foodborne microorganisms, such as Salmonella (Liu, Rojas, Gray, Zhu, & Tang, 2018; Taylor, Tsai, Rasco, Tang, & Zhu, 2018; Tsai et al., 2019). Therefore, if the a_w of the inoculum used is not within the range of the a_w of the food, the whole validation procedure will likely be flawed because the target microorganism will be inactivated quickly, resulting in more number of decimal reductions.

In addition to a_w, other critical aspects related to dry inocula preparation comprise the reproducibility of the protocols employed and the maintenance of the titer of the target microorganism unaltered during storage. The reproducibility in the preparation procedure relates to the achievement of dry inocula with similar counts of the target microorganism, from the inoculation of the carrier agent with the liquid inocula until their drying. The initial concentration of the target microorganism in an inoculum is vital as it is used in the establishment of the end-point of the killing step (Pflug, 2010). In this study, a decline in the counts of *S. enterica* of 1–2 log CFU per g has been observed between the liquid inocula and the dry inocula (data not shown). This decline in the counts of *S. enterica* of up to 2 log CFU per g was observed even after four replications of inocula preparation (data not shown). Similar decreases in *S. enterica* counts during preparation were also reported (Shrestha & Nummer, 2016), while a higher decrease in the counts (of up to 2.5 log CFU per g) have also been observed (Blessington et al., 2013; Bowman et al., 2015). The stability in the decline of *S. enterica* counts between the liquid inocula preparation until their drying indicates that the procedures employed for the preparation of the dry inocula, no matter the carrier agents, were adequate and reproducible.

The other critical aspect of dry inocula preparation (i.e., keeping the concentration of the target microorganism stable through the storage period). The stability of microbial target during storage of the dry inoculum is of utmost relevance for assessing the reproducibility of delivered lethality by the inactivation step of LMF because it allows the use of inoculum with the same resistance. Validating inactivation steps of LMF with different batches of dry inoculum may result in non-reproducible trials because the inactivation kinetics of microorganisms may change from batch to batch depending on the inoculum's cultivation conditions (den Besten, Wells-Bennik, & Zwietering, 2018; Págan, Mañas, Alvarez, & Sala, 1998). Therefore, the counts of *S. enterica* in two types of sands, calcium carbonate, and talc, were assessed through the storage of the dry inocula at 4 and 25 °C (Fig. 1). A variance analysis was carried out (ANOVA) to evaluate the significance of the factors of variables present in the inocula prepared considering the carrier agent, the a_w, and the storage time. For the dry inocula stored at 4 °C the carrier agent ($p = 0.0006$) and storage time ($p < 0.0001$) presented significant differences besides the carrier agent × time ($p = 0.0097$) combination. For the dry inocula stored at 25 °C, the same behavior was observed, with p-values of 0.0007 and lower than 0.0001 being obtained, respectively. The a_w did not present significant differences in any of the evaluated conditions.

The decline in *S. enterica* counts through the maximum storage period (180 days) of the dry inocula was characterized by a non-log linear pattern (Fig. 1). As such, the Weibull model was fitted to the *S. enterica* counts (Fig. 1). The R^2 were > 0.81, showing a very good fit of the model to the data, especially for the survival curves, which

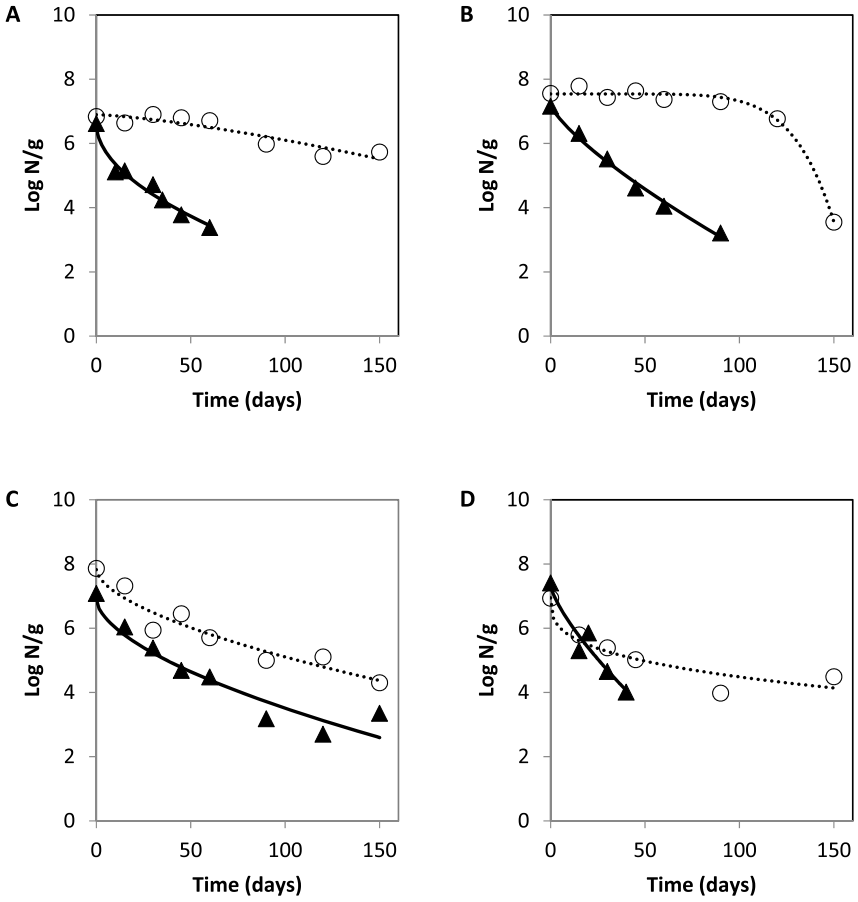

Fig. 1. *Salmonella enterica* survival in fine sand (a), regular sand (b), calcium carbonate (c), and talc (d), stored at 4 °C (○) and stored at 25 °C (▲). The dotted and straight lines represent the Weibull model fitted to the survival data at 4 °C and at 25 °C, respectively.

$R^2 > 0.90$. The RMSE values indicate the ability of the model to predict the data accurately.

As shown in Table 2, the survival kinetics parameters of *S. enterica* were affected by the carrier agent used to prepare the dry inocula and by the storage temperature (Table 2). The times for the first decimal reduction (δ) of *S. enterica* at 4 °C were ~2 and 6 times higher when the carrier agents used for dry inocula preparation were fine and regular sand compared with calcium carbonate and talc, respectively ($p < 0.05$). With these carrier agents, a time of approx. 120 days were needed for the first decimal of *S. enterica* to be observed when storage was carried out at 4 °C (Table 2). On the other hand, when calcium carbonate and talc were used to prepare the dry inocula, the δ were ~50.4 and 19.4 days at 4 °C, respectively ($p > 0.05$). Studies published have also demonstrated that Salmonella is capable of surviving in the sand, talc or chalk (calcium carbonate) (Beuchat & Mann, 2015; Blessington et al., 2013; Bowman et al., 2015; Enache et al., 2015; Shrestha & Nummer, 2016). Sand is the carrier agent that most frequently enables better Salmonella survival, from 170 (Blessington et al., 2013) up to 480 days, when storage was done at 4 °C.

In this study, the highest δ values were observed when storage was done at 4 °C instead of 25 °C ($p > 0.05$), highlighting that low temperature seems to be crucial for higher stability in the concentration of *S. enterica* in the dry inocula (Fig. 1). Analysis of *S. enterica* was possible in all carrier agents stored at 4 °C for up to 180 days (quantification

Table 2
Survival kinetic parameters dry inocula of *S. enterica* prepared with different carriers and stored at 4 and 25 °C for up to 180 days[a,b].

Carrier	Temperature (°C)	δ (days)	p	R^2	RMSE
Fine Sand (0.05–0.2 mm)	4	$118.4 \pm 18.8^{A,a}$	1.29 ± 0.06	0.81	0.0402
	25	$8.9 \pm 2.9^{B,ab}$	0.62 ± 0.15	0.97	0.0492
Regular sand (0.2–0.6 mm)	4	$124.5 \pm 3.8^{A,a}$	7.01 ± 0.52	0.97	0.0162
	25	$14.3 \pm 3.3^{B,a}$	0.76 ± 0.04	0.93	0.0482
Calcium carbonate	4	$50.4 \pm 30.2^{A,ab}$	1.06 ± 0.34	0.94	0.1014
	25	$15.9 \pm 9.8^{A,a}$	0.86 ± 0.18	0.88	0.3536
Talc	4	$19.4 \pm 16.4^{A,b}$	0.45 ± 0.24	0.95	0.5073
	25	$2.2 \pm 5.9^{A,b}$	0.29 ± 0.08	0.94	0.4416

[a] Different uppercase letters in the same column indicate significant difference ($p < 0.05$) in δ values according to *t*-test for the dry inocula made with the same carrier and stored at 4 and 25 °C.

[b] Different lowercase letters in the same column indicate significant difference ($p < 0.05$) in δ values according to Duncan test for the dry inocula made with different carriers and stored at the same temperature (4 or 25 °C).

limit of 10^2 CFU/g). However, at 25 °C, *S. enterica* survived longer in calcium carbonate (150 days), even though the δ was equal to 15.9 days. Fourteen days was also the δ obtained when the dry inoculum was made with regular sand; however, *S. enterica* was counted only during 90 days (quantification limit of 10^2 CFU/g). At 25 °C, *S. enterica* was counted for up to 60 days of storage in fine sand and 40 days in talc (Table 2, Fig. 1).

The shape parameter (p) values of the fitted Weibull model to the data varied significantly as a result of the different patterns of *S. enterica* inactivation curves (concave or convex) observed during dry inocula storage (Fig. 1). The inactivation curves did not approach a log-linear behavior, no matter the storage temperature and carrier agent used to prepare the dry inocula. Deviations of log-linear inactivation curves can be common when microorganisms are exposed to mild inactivation or stressful conditions (Bermúdez-Aguirre & Corradini, 2012; Ozturk, Kong, & Singh, 2020; Portela et al., 2019; Wang et al., 2015). These deviations likely occur because, under these conditions, sub-lethal injuries take place, i.e., cells and cellular components are not equally sensitive to heat (Smelt & Brul, 2014).

Overall, based on the results, it has been found that the a_w variation during storage did not directly influence *S. enterica* survival in the dry inocula. However, considering only the a_w of the dry inocula obtained using the different carrier agents, it is possible to propose the following combinations between carrier agents and a_w of LMF: i) calcium carbonate would be suitable to prepare dry inoculum of foods with a_w 0.70–0.95, ii) fine sand could be used for the preparation of dry inoculum of foods with a_w of 0.60–0.70, iii) regular sand could be applied for making dry inoculum of foods with a_w 0.50–0.60 and iv) talc could be used for the preparation of dry inoculum of foods with a_w 0.40–0.50.

CRediT authorship contribution statement

Marianna M. Furtado: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Beatriz S. Silva:** Data curation, Formal analysis, Investigation, Visualization, Writing - original draft. **César Faviero:** Data curation, Formal analysis, Investigation, Visualization. **Verônica O. Alvarenga:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Anderson S. Sant'Ana:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

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

Acknowledgments

A.S. Sant'Ana thanks to CNPq (Grants #302763/2014–7 and #305804/2017–0). This study was financed, in part, by CAPES (Finance Code 001).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109705.

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