Short Communication

Survival Kinetics of *Salmonella* Typhimurium in Oat and Pea Protein Flours

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ABSTRACT

Oat and pea protein flours have been used as ingredients for plant-based food products. These low-moisture ingredients have been implicated in *Salmonella* recalls, most likely due to post-processing contamination. Oat and pea protein flour do not undergo pathogen kill step, especially for minimally cooked or ready-to-eat food. This study aimed to quantify the survival kinetics of *Salmonella* Typhimurium in oat and pea protein flours at storage temperatures of 4°C and 25°C. Powders were inoculated at ~8 log CFU/g via seed inoculation method and tested for homogenous *Salmonella* distribution. The inoculated samples were enumerated on days 0, 3, 5, 7, 21, 35, 49, and 63. This study showed the impact of storage temperatures on S. Typhimurium survival and proved that refrigerated storage did not control microbial growth. The findings suggest that flour treatment should be based on its composition because composition affects *Salmonella* survival.

Keywords: low-moisture food; oat flour; pea protein flour; predictive modelling; *Salmonella* Typhimurium

1. Introduction

Cereals and legumes are excellent sources of nutrients for the human diet. Cereals like wheat, corn, rice, and oats are typically rich in carbohydrates and soluble fibers, whereas legumes like lentils, chickpea, and peas are protein-rich ingredients. Oat (*Avena sativa* L.) is a healthy ingredient for many food applications due to its high content of ß-glucan (Jokinen *et al.*, 2023). Oat carbohydrates comprise starch, a great candidate as a naturally sourced food stabiliser (Rawala et al., 2023). Oat also contains protein ranging from 45-60% (Kaleda *et al.,* 2021). On the other hand, pea (*Pisum sativum* L.) is a seed legume mainly used to make soup in Western countries or roasted to be consumed as a snack in Asian countries

(Kronenberg, 2022). Despite their differences in nutritional profiles and food purposes, oats and peas are suitable as gluten-free and non-allergenic ingredients for meat-substitute products (Onkowijoyo and Peterson, 2023; Kaleda *et al.*, 2021). For instance, Kaleda *et al.* (2021) formulated an oat-pea blend to be used as an alternative to soy or wheat as ingredients to produce a fibrous meat-like structure.

Upon harvest, oat and pea are determined for further processing to make them palatable. They undergo milling to be turned into flour. Flour exhibits a long shelf-life as low water activity can hinder microbial growth. In a household condition, flour may be kept at room temperature, or consumers may seal the leftovers and store them refrigerated. The presence of foodborne pathogens in flour is mostly unnoticeable as pathogens typically reside in a low level and do not cause apparent adverse attributes. Given that flour is often used as raw ingredients to make other food products, the presence of foodborne pathogens poses a great concern as flour may serve as a vehicle for pathogen proliferation in foods that are high in moisture and nutrients (Sánchez-Maldonado *et al.,* 2018).

Salmonella is Gram-negative, non-spore former, flagellated, and facultative anaerobes. *Salmonella* causes gastroenteritis, which could lead to septicemia, particularly in immunocompromised individuals (Centers for Disease Control and Prevention, 2022). *Salmonella* has persisted in long-term storage of low-moisture commodities (Sánchez-Maldonado et al., 2018). *Salmonella* was found to acquire enhanced thermal resistance to subsequent thermal treatment when *Salmonella* was pre-exposed to a dry environment (Syamaladevi *et al.,* 2016).

Salmonella has been repeatedly implicated in major recalls and foodborne outbreaks linked to various cereal products. In 2018, 135 cases with 34 hospitalisations were reported when Kellogg's Honey Smack wheat breakfast cereals were recalled due to *Salmonella* contamination (Centers for Disease Control and Prevention, 2018). Most recently, General Mills recalled *Salmonella*-tainted wheat flour, which caused 14 illnesses and 3 hospitalisations (Centers for Disease Control and Prevention, 2023), and Quacker Oats Company recalled their granola bars and cereal products due to possible *Salmonella* contamination (U.S. Food and Drug Administration, 2023). These outbreaks demonstrated that *Salmonella* ended up in finished products if raw ingredients were contaminated. Determining factors related to *Salmonella* survival in flours is critical to establish adequate *Salmonella* controls in finished products. Therefore, this study aimed to assess the effect of flour composition, represented by oat and pea protein flours, on the survival of *Salmonella* Typhimurium during two-month storage at 4°C and 25°C.

2. Materials and Methods

2.1 Background microbial counts

Organic oat flour (batch no. 188Z883, Helsinki, Finland) and organic pea protein isolate (batch no. OPO222 0216, Batu caves, Malaysia) were purchased online from a local vendor in Malaysia. Background microflora was performed by randomly taking 25 g of powder from the original package, diluted in 225 mL of 0.1% buffered peptone water [BPW] (Oxoid Ltd, Basingstoke, United Kingdom) in a sterile Whirl-Pak bag (Nasco, Madison, WI) and homogenized for 3 min by using a stomacher (Bagmixer® 400-p, Interscience, Puycapel, France). Afterward, 0.1 mL was plated on plate count agar [PCA] (Oxoid Ltd) for total plate count (TPC) and on potato dextrose agar [PDA] (Oxoid Ltd) for yeast and mold count (Y/M). PCA was incubated at 37ºC for 24 h while PDA at 25ºC for 3 to 5 days. The presence of *Salmonella* was detected using tryptic soy agar [TSA] (Oxoid Ltd) supplemented with 0.05% of ammonium ferric citrate (Sigma-Aldrich, St. Louis, MO), 0.03% of sodium thiosulfate (R&M Chemicals, Semenyih, Malaysia) and 0.6% yeast extract (Oxoid Ltd), which then referred as MTSA. Black colonies on MTSA indicates presumptive *Salmonella* counts. Background microflora counts were conducted in duplicate.

2.2 Inoculum preparation

Salmonella Typhimurium (ATCC 14028, Thermo Fisher Scientific, Waltham, MA) was chosen for its association with *Salmonella* outbreaks in flour (McCallum *et al.,* 2013; Zhang *et al.,* 2007;). Inoculum was prepared using the agar-lawn method as described by Ahmad *et al.* (2022). Once revived from frozen

culture, a single colony of *Salmonella* on the tryptic soy agar plate supplemented with 0.6% yeast extract [TSAYE] (Oxoid Ltd, Basingstoke, United Kingdom) was transferred into 10 mL of tryptic soy broth supplemented with 0.6% yeast extract [TSBYE] (Oxoid Ltd) and incubated for 24 h at 37ºC. To prepare the bacterial lawn, 1 mL of aliquot was spread onto the TSAYE plate and incubated for 24 h at 37ºC. To harvest, 1 mL of sterile 0.1% buffered peptone water [BPW] (Oxoid Ltd) was added onto TSAYE and gently scraped on the surface using an L-shaped spreader. The inoculum was transferred into a sterile test tube and immediately used for flour inoculation. The inoculum concentration was 11.25±0.1 log CFU/g.

2.3 Sample inoculation

A 100 g of oat and pea protein flour containing 1 mL of inoculum was prepared through seed inoculation method following Ahmad *et al.* (2022). Briefly, 10 g oat flour and 1 mL of inoculum was transferred into a Whirl-Pak bag (118 mL, Nasco, Madison, WI). The inoculum was homogenized evenly by hand massaged and stomached (Bagmixer® 400-p, Interscience, Puyacapel, France) for 3 min. Next, in a new Whirl-Pak bag (710 mL, Nasco), the prepared seed inoculum was homogenized with remaining 90 g of uninoculated oat flour. The inoculum sample was then hand massaged and stomached for 3 min. Then, homogeneity test was conducted by taking three 1-g samples from three random spots and plating on modified tryptic soy agar [MTSA] (Oxoid Ltd) supplemented with 0.05% of ammonium ferric citrate (Sigma-Aldrich, St. Louis, MO), 0.03% of sodium thiosulphate (R&M Chemicals, Semenyih, Malaysia) and 0.6% yeast extract (Oxoid Ltd). If *Salmonella* population reached a standard deviation <0.3 log CFU/g in flour, it was considered homogeneous (FAO/WHO, 2016; Ahmad *et al.*, 2022).

2.4 Storage conditions

A total of 100 g inoculated flours were individually weighed (Model Setra, Johnson Scale Co., Pine Brook, NJ) and packed in 1 g of Whirl-Pak bag (118 mL, Nasco) for enumeration purposes, while 2 g packed for water activity analysis. The samples were sealed in a zip lock bag and stored in a plastic container separately for each temperature storage (4 and 25ºC). A total of 6 bags (3 bags containing 1 g and 3 bags containing 2 g of inoculated flour) were pulled for *Salmonella* enumeration and water activity (AquaLab, METER Group, Inc, Pullman, United States) measurement on each analysis day. Two biologically independent batches of flours were conducted.

2.5 Salmonella enumeration

Enumeration was conducted on day 0, 3, 5, 7, 35, 49, and 63. The enumeration was conducted by sampling 1 g of inoculated oat flour for each storage temperature. Each sample were diluted with 9 mL of 0.1% BPW, stomached for 3 min, and further diluted in 1:10 and Then, 100 µL of appropriate dilution was plated on duplicate MTSA for enumeration of S. Typhimurium survivors where black colonies were counted after 24 h incubation at 37ºC. The acceptable colony was in the range of 25 to 250 CFU/g (U.S. Food and Drug Administration, 2001). Each *Salmonella* population count in CFU/g was converted to log. The mean log CFU/g of triplicate subsamples at each time point were then subtracted from those at time 0, to obtain log reduction. Duplicates batches were used for curve-fitting.

2.6 Data analysis

IPMP 2013, developed by USDA, is a curve-fitting tool for assessing the kinetic of pathogen growth or survival (Huang, 2014). Log linear model is described in Eq. 1. The survival of bacteria is a function of time (y(t)). t, was chosen to fit *Salmonella* survival curves. D is the time (day) required to reduce bacterial population by 1 log. D-value is an estimated parameter in log linear model to compare *Salmonella* resistance during different storage temperatures. y0 corresponds to the intercept. Root-mean square error (RMSE) (Eq. 2) indicates the goodness of fit. Confidence level (CL) of 95% was also determined. Student's t-test (t-test) from Microsoft Excel (Version 16.16.22) was used to determine significant differences for log

reduction and water activity changes between 4 and 25ºC.

$$\log N = \log N_0 - \frac{t}{D} \quad (1)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N-P}} \quad (2)$$

3. Results and Discussion

3.1 Background microbial counts

Both uninoculated oat and pea protein flours showed < 25 CFU/g for *Salmonella*, aerobic bacteria, yeast, and mould. A low level of background microflora is desirable as the growth of microflora during storage would have less impact on quantifying *Salmonella* Typhimurium used in this study. On the other hand, if the uninoculated flours posed a high microbial background, it implies that they should not be used in this study as the accuracy and reliability of *Salmonella* survival results may be compromised. *Salmonella* Typhimurium may not grow well during enumeration, leading to underestimation of *Salmonella* survival.

3.2 Inoculum homogeneity

Oat flour exhibits an initial *Salmonella* concentration of 7.10 - 8.66 log CFU/g, with a standard deviation ranging from 0.04 - 0.21 log CFU/g (n=4). For pea protein flour, initial *Salmonella* concentration ranged from 8.58 - 9.14 log CFU/g, with a standard deviation of 0.01 - 0.16 log CFU/g (n=4). In this study, homogeneous microbial distribution in inoculated flours was achieved because the standard deviation of the initial *Salmonella* population for each biologically independent batch was less than 0.30 log CFU/g. According to the World Health Organization (2016), the variability of microbial distribution in food is categorized into three food groups in which a standard deviation of 0.20 log CFU/g for liquid food, 0.40 log CFU/g for reasonably mixed food, and 0.80 log CFU/g for less mixed foods. In this study, 0.30 log CFU/g was selected as a conservative value for reasonably mixed food like flour.

3.3 Salmonella survival during storage

Salmonella Typhimurium survival kinetics in both oat and pea protein flours during storage at 4ºC and 25ºC are depicted in Figure 1. *Salmonella* population exhibits a linear reduction trend in both oat and pea protein flours at all storage temperatures. It was also observed that *Salmonella* reduction for both flours was less than 1 log reduction across the storage period, except for *Salmonella* in oat flour stored at 25ºC. In oat flour, *Salmonella* declined below the limit of quantification after 35 days of storage at 25ºC. Shi *et al.* (2022) presented a similar pattern of *Salmonella* survival in other low-moisture food stored at similar temperatures and duration storage time for different teas. *Salmonella* survival at 4ºC storage was shown to be more stable than at 25ºC storage.

The estimated D-values for *Salmonella* Typhimurium in oat flour were 9.4±0.9 days at 25ºC and 216.2±95.4 days at 4ºC. For pea protein flour, estimated D-values of 163±4 days at 25ºC and 1955±4059 days at 4ºC were observed in pea protein flour (Table 1). Compared to the D-value, a high standard error for *Salmonella* in pea protein flour stored at 4ºC could be due to stable *Salmonella* population level across the storage period, which also yields to a negative value in 95% of the lower confidence limit. Nevertheless, low RMSE values, ranging from 0.09 to 0.48, imply log linear model is an appropriate model fitting for *Salmonella* survival data in this study.

3.4 Water activity of flours during storage

No significant difference in the water activity (a_w) of oat flour for both temperatures was reported on the initial toward the end of the analysis day ($p>0.05$). The a_w analysis is important to ensure that no storage abuse occurs during the storage of oat flours, which could impede the reliability of this study. According to Syamaladevi et al. (2016) and Finn *et al.* (2013), one of the significant contributing factors to *Salmonella* activity in LMF is a_w, widely applied in many food industries to indicate the shelf stability of food products. Based on Brett *et al.* (2009), oat flour may typically have a_w below 0.75. Therefore, as the a_w of oat flour in this study was less than 0.75 for both storage temperatures, it can be affirmed that the oat flour was not negatively affected during the storage period by other external factors such as moisture migration.

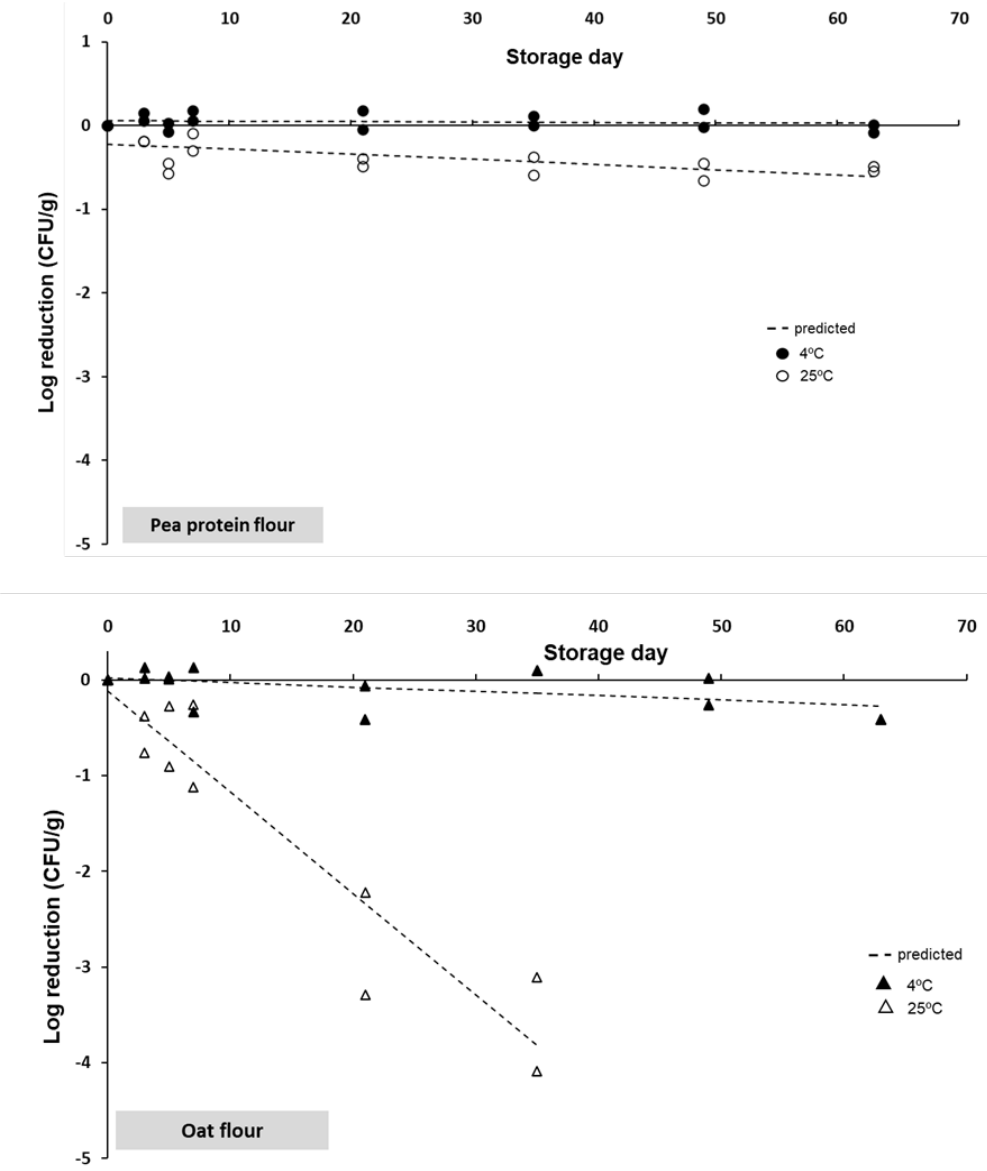

Figure 1. Survival kinetic of *Salmonella* Typhimurium in oat and pea protein powders stored at 4ºC and 25ºC, with predicted line obtained using log-linear model. Each symbol represents triplicate samples per time point. Two biologically independent batches were used for each temperature. *Salmonella* was below the limit of quantification (< 25 CFU/g) after day 35 at 25ºC

Table 1. Parameter estimation of *Salmonella* Typhimurium in oat and pea protein powders during storage at 4ºC and 25ºC via log-linear model obtained from IPMP 2013

Sample	Storage temperature (ºC)	D-value (day)	Standard error (day)	RMSE (log CFU/g)	95% CL*
Oat	4	216.2	95.4	0.18	[11.5, 420.9]
	25	9.4	0.90	0.48	[7.2, 11.7]
Pea protein	4	1954.7	4054	0.09	[-6740,10649]
	25	162.7	48.7	0.16	[58.3, 267.1]

*CL = confidence interval [lower limit, upper limit]

Overall, *Salmonella* survival at 25ºC was greatly affected by flour composition as the decline of *Salmonella* population was lower in pea protein ($P<0.05$) as compared to oat flour. It is most likely that a higher protein content in pea protein contribute to persistence of *Salmonella* when stored at 25ºC. Previous studies revealed that *Salmonella* inactivation was greatly influenced by food composition where *Salmonella* was more thermally resistant in fat-rich matrices as opposed to carbohydrate-rich or protein-rich matrices (Ahmad *et al.*, 2022). Water vibrations theory was proposed to justify differences in *Salmonella* inactivation during heat treatment (Syamaladevi *et al.,* 2016). However, no heat treatment was introduced in this study, yet difference in *Salmonella* survival was observed between flours.

Salmonella survival in low moisture food was understood to be temperature dependent, with higher temperature storage leading to higher destruction in *Salmonella* survival across the storage period (Usegi *et al.* 2006). These findings corroborate those of Beuchats and Mann (2011) and Beuchats and Mann (2014), who found that *Salmonella* exhibit better survival behavior at 4°C than at 25°C storage environment. *Salmonella* posed a higher survival rate at low refrigeration temperatures as a reaction to various environmental stressors, which cause a dormant state and slow down the bacteria metabolism (Humphrey, 2004; Shi *et al.,* 2022). This study revealed that the lower the storage temperature, the greater the *Salmonella* stability, and thus the greater the *Salmonella* survival in oat flour. As low temperatures have been shown to support *Salmonella* survival mechanisms, it is assumed implicitly that post-heating and physical processing of oat flour has a positive influence on *Salmonella* survival in low storage temperatures of oat flour. *Salmonella* might experience a lethal injury which activates survival mechanism by transitioning into a viable but non-culturable (VBNC) state while retaining the pathogenicity properties in the food matrix (Webster, 2011). The VBNC state of *Salmonella* survival response is concerning in oat flour as this poses a threat to consumer safety if the oat flour was contaminated during any stage before or after the oat flour was used as raw material in the processing of a product that is oat based.

Conclusion

As a conclusion from this study, the storage of oat and pea protein flours at room or refrigeration temperature would not lower *Salmonella* Typhimurium survival. Despite popular belief that storing opened bags of food products like powders in the refrigerator ensures that it lasts longer, this study shows that storage in refrigerated conditions keeps *Salmonella* more stable in such products compared to room temperature storage. The consumption of contaminated products, especially if it is not exposed to heat at

all, or inadequate heat treatment application leads to serious issues and life-threatening outbreaks. Manufacturers, food handlers and consumers must take necessary measures by ensuring proper sanitation and hygiene practices, as well as applying correct handling techniques, storage, and cooking temperatures.

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