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Metabolomics elucidating the effect of water activity on the thermal resistance of *Salmonella* in wheat flour

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ABSTRACT

With mounting evidence indicating an enhanced thermal resistance of *Salmonella* at lower a_w, the effectiveness of thermal treatment in wheat flour decontamination is challenged. Therefore, this study was carried out to evaluate the thermal resistance of three *Salmonella* strains, including Enteritidis (ATCC 13076), Typhimurium (ATCC 14028) and Newport (ATCC 6962), at 65 °C in wheat flour at three a_w levels (0.33, 0.53 and 0.69), and to explore the mechanisms of the difference in thermal resistance via nuclear magnetic resonance (NMR)-based metabolomics. The results showed that except for the insignificant difference between the reductions of *S.* Newport at 0.53 and 0.69 flour a_w ($P > 0.05$), a remarkable decreasing trend in *Salmonella* cell reduction with decreasing flour a_w was observed after the 20-min thermal treatment. By comparing the metabolic profiles of each strain recovered from the lower-a_w (0.33 or 0.53) flour with that from the a_w-0.69 flour, the metabolic differences implying more efficient misfolded protein degradation, higher availability of amino acids as osmoprotectants, larger throughput of energy production by ATP synthase as well as wiser glucose allocation in the metabolic network were suspected to contribute to the strains' enhanced thermal resistance. Overall, the study adds to the evidence for the effect of lower a_w in increasing the thermal resistance of *Salmonella* in wheat flour. Meanwhile, the identified discriminative metabolic pathways may be artificially modified in the future to help ease *Salmonella* inhibition during cooking or any types of thermal treatments.

1. Introduction

Salmonella contamination has long been a thorny issue for the flour industry. Ever since the first documented outbreak occurred in Australia in 1952 (Eglezos, 2010), *Salmonella* spp. have been implicated in multiple outbreaks of wheat flour and its related products worldwide (McCallum et al., 2013; U.S. Food & Drug Administration, 2015; Zhang et al., 2007). The long-term survival of *Salmonella* under desiccation is to blame for the high outbreak incidence. According to previous research, in the low-moisture condition, although the growth and proliferation of *Salmonella* may not be supported, the pathogen can survive for several months or even years (Beuchat et al., 2013; Canakapalli, Sheng, & Wang, 2022; Hildebrandt, Marks, Anderson, & Grasso-Kelly, 2020; Jarvis et al., 2016). As a result, it leaves a "time bomb" for consumers if the flour has already been contaminated. Considering this, the Food and Drug Administration has declared that flour and its related products could not be considered as ready-to-eat foods and should be eaten only after cooking (U.S. Department of Agriculture, 2022).

To respond to the government's call, and simultaneously to meet the demand for a wider variety of foods, multifarious cooking means (e.g., baking, boiling, frying and grilling) have been utilised to prepare wheat flour-based foods. Nevertheless, the cooking processes cannot necessarily guarantee the safety of these foods, the reason is the cross-protection of low-water activity (a_w) exposure to heat stress in *Salmonella* (Finn, Condell, McClure, Amézquita, & Fanning, 2013; Fong & Wang, 2016). In fact, a positive correlation between *Salmonella*'s desiccation resistance and thermal resistance has been well-documented (Beuchat et al., 2013; Liu et al., 2018; Smith, Hildebrandt, Casulli, Dolan, & Marks, 2016). Since McDonough and Hargrove initially proposed a significantly prolonged cooking time in eliminating *Salmonella* when the moisture content of dried milk powder was reduced from 25% to 7% in the 1960s (McDonough & Hargrove, 1968), the increased heat tolerance of *Salmonella* due to the decrease in a_w in other low-moisture foods (LMFs), including wheat flour, has also been revealed successively by researchers (Laroche, Fine, & Gervais, 2005; Liu, Sheng, Canakapalli, & Wang, 2022; Smith & Marks, 2015; Villa-Rojas et al., 2013). However,

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the causes of such cross-protection phenomenon remained unclarified yet. To unravel this mystery so as to develop countermeasures to the increased thermal resistance, understanding the differences in *Salmonella*'s thermal resistance mechanisms at different flour a_w levels may be useful.

Metabolomics, the analytical profiling technique which provides an instantaneous snapshot of the metabolic status in a cell, has found its increasing application in mechanism study (Manchester & Anand, 2017). Nuclear magnetic resonance (NMR) spectroscopy, with non-invasive, non-destructive and high-reproducible properties, is one of the most used metabolomics tools (Emwas, 2015). For instance, it has been applied to study the mechanisms of inhibiting *Listeria monocytogenes* on salmon by the combined treatment of electrolysed water and moderate heat (Wu, Zhao, Lai, & Yang, 2021). It has also been used to investigate the metabolic variations in *Salmonella* and Shiga toxin-producing *Escherichia coli* on fresh vegetables under organic acid treatments (Guo, He, Wang, & Yang, 2022; Wang, Gao, & Yang, 2022). Better still, the bottleneck of isolating microbial cells from flour has been broken through by our recent work (Wang, Zhou, & Yang, 2022), which freed metabolomics studies from the constraints of food matrices. Taking advantage of this, NMR could be used in the current work to enlighten us on the mechanisms of the increased thermal resistance of *Salmonella* caused by the decrease in the a_w of wheat flour.

Overall, in this study, we aimed to verify the effect of flour a_w on *Salmonella*'s thermal resistance in our experimental setting. Besides, by using NMR spectroscopy, this would be the first study venturing into the underlying mechanisms via metabolomics. The results of the study would manifest the differences in heat-induced metabolic responses of *Salmonella* at different flour a_w, from which hints would be obtained for the development of targeted measures to reduce the thermal resistance of the *Salmonella* hazard before the wheat flour is put on the market.

2. Materials and methods

2.1. Bacterial strains and inocula preparation

Three *Salmonella* strains from the top human salmonellosis-causing serotypes, including Enteritidis (ATCC 13076), Newport (ATCC 6962) and Typhimurium (ATCC 14028) (Bugarel, Tudor, Loneragan, & Nightingale, 2017; Centers for Disease Control and Prevention, 2018; Petsong, Benjakul, Chaturongakul, Switt, & Vongkamjan, 2019), were utilised in this study. Before use, the strains were resuscitated from their glycerol stocks (stored at – 80 °C) by two consecutive transfers (24 h at 37 °C) in tryptone soya broth (TSB; Sigma-Aldrich, St. Louis, MO, USA) (Forghani et al., 2019). Additional consecutive transfers with a stepwise increase in nalidixic acid (Sigma-Aldrich, St. Louis, MO, USA) concentration were then applied to gradually adapt the strains to 100 mg/L of nalidixic acid (Wang et al., 2022). To rule out any effects that might be exerted by the naturally existing microbes in wheat flour, media used in the study were all supplemented with 100 mg/L of nalidixic acid (Kharel, Adhikari, Graham, Prinyawiwatkul, & Yemmireddy, 2018; Parnell, Harris, & Suslow, 2005).

Upon adaptation, the working culture of each strain was individually prepared by inoculating the strain into 100 mL of fresh TSB (1:100, v/v) and incubating at 37 °C for 24 h. The suspension was centrifuged at 4500g for 10 min (20 °C). After discarding the supernatant, cell pellets were washed twice with 0.1% peptone water (Sigma-Aldrich, St. Louis, MO, USA) and finally harvested in 100 μL of 0.1% peptone water. This concentrated semiliquid cell suspension (approximately 9 log CFU) was subject to subsequent flour inoculation.

2.2. Wheat flour conditioning

All-purpose wheat flour from the same batch was purchased from a local supermarket in Singapore. It was equally divided into three portions and dried at 55 °C (Memmert Incubator I, Singapore). The a_w of all flour portions was monitored by the a_w meter (Aqua Lab model 3TE; Decagon Devices, Pullman, WA). Upon reaching around a_w 0.3, 0.5 and 0.7 at room temperature (24.7 ± 0.3 °C), respectively, the three portions were separately transferred to air-tight jars containing saturated salt solutions to control relative humidity (RH) for three weeks (Tadapaneni et al., 2017b). The saturated salt solutions used in the study were $MgCl_2$ (32.8% RH), $Mg(NO_3)_2$ (52.9% RH) and KI (68.9% RH), which equilibrated the three portions to $a_{w,25\ °C}$ of 0.33, 0.53 and 0.69, respectively (Greenspan, 1977).

2.3. Inoculation

To minimise the influence of inoculation on the thermal resistance of *Salmonella* in wheat flour (Hildebrandt et al., 2016), a concentrated cell suspension inoculation method which has been proven to barely change the a_w of wheat flour was used to inoculate the conditioned flour (Forghani et al., 2018; Forghani et al., 2019). The three portions of flour were inoculated separately. The 100-μL cell suspension prepared in 2.1 was aseptically spot deposited into 10 g of flour in a sterile stomacher bag and hand mixed for 5 min. Subsequently, another 90 g of flour was added to the seed, followed by a 3-min manual mix, two sets of 3-min mastication (Masticator Stomacher, IUL Instruments, Germany) and another 3-min manual mix. The procedure resulted in homogeneous initial microbial counts of approximately 7 log CFU/g based on the enumeration results of three 1-g samples taken from random locations in the inoculated batch. The post-inoculation $a_{w,25\ °C}$ of the three flour portions were checked, which were 0.33 ± 0.01, 0.53 ± 0.01 and 0.69 ± 0.01, respectively, representing no change in flour a_w by the inoculation process. The inoculated flour was weighed into 0.6-g samples and sealed in 1.5 mL centrifuge tubes to avoid moisture absorption from the environment. The samples (0.60 ± 0.03 g) were subject to thermal treatment directly to prevent any possible pre-treatment bacterial loss.

2.4. Thermal treatment

Thermal treatment was performed at 65 °C for up to 20 min. This moderate treatment temperature was chosen because it was unlikely to affect the functional properties of wheat flour (Keppler, Bakalis, Leadley, Sahi, & Fryer, 2018; Van Steertegem et al., 2013), thus ensuring that a_w would be the only variable influencing the inhibition effects. Samples at different a_w levels were simultaneously immersed in a hot-water bath (Julabo SW22, Singapore) that had been preheated to 65 °C and kept in agitation. The sample temperature was monitored by a non-inoculated blank with a thermometer located at the centre (Smith et al., 2016). The come-up time (150 s) for the sample core to reach within 0.5 °C of the target temperature was used as the zero time point (0 min). Starting from the 0 min samples, samples were subsequently taken out from the water bath at equally spaced time intervals and immediately put in an ice-water bath to quench the inactivation (Paéz et al., 2012; Suehr, Anderson, & Keller, 2019; Villa-Rojas et al., 2013; Ye et al., 2012).

2.5. Inactivation kinetics modelling

Each sample was transferred to 5.4 mL of 0.1% peptone water aseptically to establish a 10-fold dilution. Suitable 10-fold serial dilutions were plated on nalidixic-acid supplemented tryptic soy agar (TSA, Oxoid Limited, Hampshire, UK), followed by 24-h incubation at 37 °C (Ferreira, Horvath, & Tondo, 2013). The viable counts were enumerated and log-transformed. The data were fit to the Weibull model, $N = N_0 - (t/\delta)^{\beta}$, where N is the surviving population (log CFU/g) at time t (min), N_0 is the surviving population (log CFU/g) at 0 min, δ is the time (min) required for the first decimal reduction and β is the shape parameter (Chen et al., 2019). The adjusted R^2 values and root mean standard errors (RMSE) were calculated to determine the model's fitness to the inactivation data (Coe et al., 2022).

A

Serotype	a_w	δ (min)	δ (SE)	β	β (SE)	t_{5D} (min)	Adjusted R^2	RMSE
S. Enteritidis	0.33	^{A}19.77	2.37	0.82	0.06	^{A}146.64	0.887	0.103
	0.53	^{B}8.96	0.64	0.61	0.01	^{A}127.70	0.888	0.144
	0.69	^{B}7.95	1.09	0.73	0.16	^{A}85.47	0.868	0.201
S. Newport	0.33	^{A}18.66	1.37	0.67	0.09	^{A}224.09	0.918	0.081
	0.53	^{B}10.06	0.40	0.63	0.04	^{A}130.00	0.900	0.134
	0.69	^{B}9.26	0.06	0.58	0.02	^{A}146.42	0.934	0.105
S. Typhimurium	0.33	^{A}30.87	1.03	0.71	0.01	^{A}294.07	0.909	0.063
	0.53	^{B}8.08	0.77	0.57	0.04	^{B}141.30	0.890	0.145
	0.69	^{B}8.19	1.41	0.83	0.14	^{B}60.58	0.914	0.175

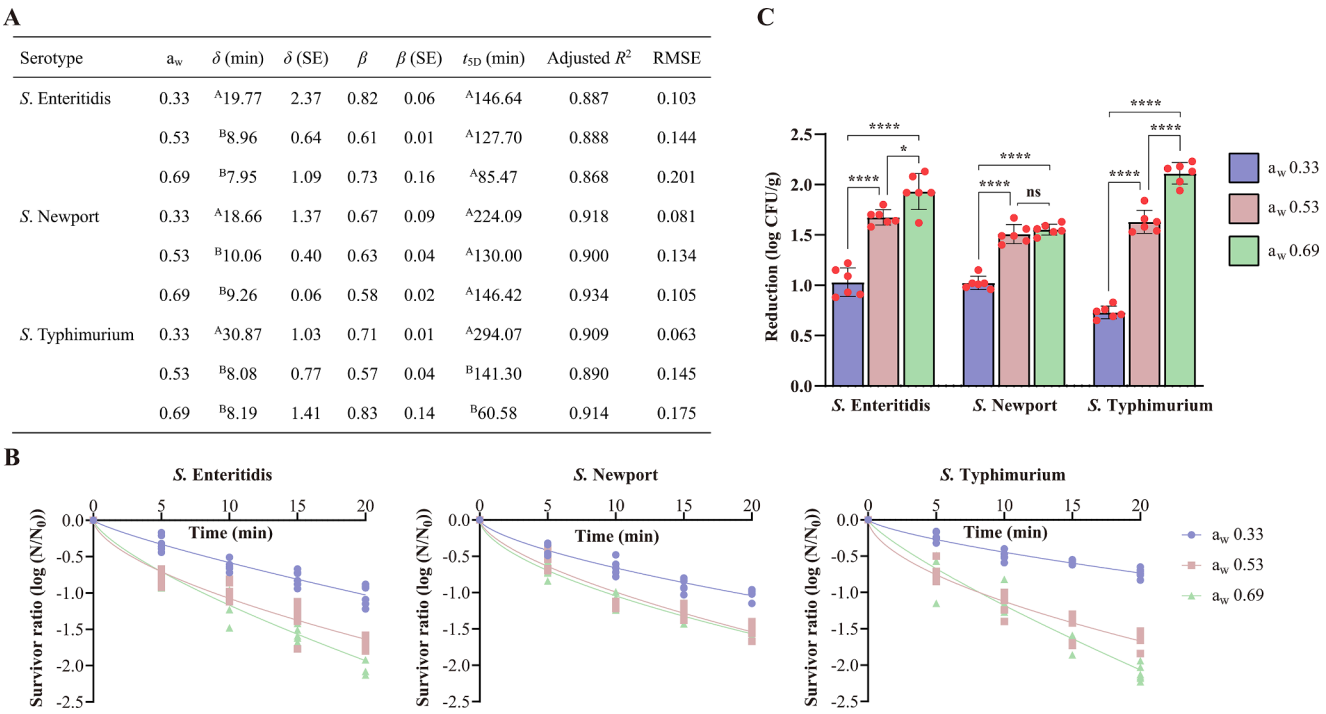

Fig. 1. Parameters of thermal inactivation kinetics of *Salmonella* Enteritidis, Newport and Typhimurium strains in a_w 0.33–, 0.53- and 0.69- wheat flour at 65 °C for 20 min calculated by Weibull model (A), the corresponding inactivation curves (B), and the total population reduction of each strain after the treatment (C). Note: δ and t_{5D} values for the same serotype preceded by different uppercase letters are significantly different ($P < 0.05$). Reductions are compared based on the mean results of 6 replicates (n = 6). *: $P < 0.05$; ****: $P < 0.0001$; ns: not significant.

2.6. Metabolite extraction

Based on our experience in recovering *E. coli* cells from wheat flour (Wang et al., 2022), a proper reduction in the size of wheat flour sample being inoculated could greatly enhance the recovery rate of bacterial cells. Therefore, to make sure the *Salmonella* cells would be recovered in sufficiency for NMR analysis, the 100-uL concentrated cell suspension prepared in 2.1 was inoculated into 10 g of wheat flour at each a_w for metabolomics. After being treated at 65 °C for 20 min in 50 mL centrifuge tubes, the 10-g flour samples were each diluted in 0.1% peptone water, thoroughly vortexed and centrifuged at low speed (800 × g) for 2 min (4 °C) to precipitate flour. The supernatant was collected, and the precipitate was diluted and centrifuged again. Same rounds of centrifugation were conducted for all samples and the process was repeated until no cell pellets were visible on the top of the flour precipitate. Afterwards, the pooled supernatants were subject to a final round of low-speed centrifugation to precipitate the remaining flour debris. *Salmonella* cells were then harvested by centrifuging the liquids in new tubes at 12,000*g* for 10 min (4 °C).

The collected cells were immediately suspended in 1 mL of ice-cold methanol-d_4 (Cambridge Isotope Laboratories, Tewksbury, MA, USA). Upon frozen in liquid nitrogen and thawed on ice for three cycles to destroy the cell membrane, the mixtures were stored at −20 °C for overnight extraction. Intracellular metabolites were obtained by centrifugation at 12,000 × g for 20 min (4 °C). The supernatants were spiked with 1 mmol/L of trimethylsilyl propanoic acid (TSP, Sigma-Aldrich, Singapore) as internal standard and transferred into NMR tubes (Sigma-Aldrich, St. Louis, MO, USA) for immediate NMR analysis (Chen et al., 2022b; Zhao, Chen, Wu, He, & Yang, 2020).

2.7. NMR analysis

NMR analysis was performed by the Bruker DRX-500 NMR spectrometer (Bruker, Rheinstetten, Germany) via a Triple Inverse Gradient probe (He, Zhao, Chen, Zhao, & Yang, 2021). The ^{1}H spectra (0–10 ppm) of all samples were obtained using the standard Bruker NOESY pulse sequence (noesypr1d). Besides, the 2D ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) spectrum (H: 0–10 ppm; C: 0–180 ppm) of a representative sample was also acquired, using the Bruker hsqcedetgpsisp2.3 pulse sequence.

Metabolites on the ^{1}H spectra were assigned by collectively referring to the 2D ^{1}H–^{13}C spectrum, databases and relevant studies (Guo et al., 2022; Wang, Wu, & Yang, 2022; Wang et al., 2022). To prepare the spectral data for multivariate analysis, binned datasets of the spectra were created by Mestrenova (Mestrelab Research SL, Santiago de Compostela, Spain). Principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were then performed on SIMCA (version 14.1, Umetrics, Umeå, Sweden) for an overall and pairwise comparison of the samples, respectively. The correlation coefficients and VIP values of metabolites were obtained from OPLS-DA. The criteria of |correlation coefficient| of > 0.602, VIP value of > 1, and P of < 0.05 were combinedly used to screen the metabolites that significantly discriminated each paired samples (Chen et al., 2020). These metabolites were then collectively used for constructing the metabolic network perturbation schematic based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to rationalise the different thermal resistance of *Salmonella* at different flour a_w.

2.8. Statistical analysis

The viable counts of each *Salmonella* strain at each time point were calculated from two separately inoculated 100-g flour samples each sampled in triplicate 0.6-g portions (n = 6). The NMR analysis was performed in independent triplicates using separately prepared inocula (n = 3). Analysis of variance (ANOVA) and the least significant difference (LSD) were conducted in SAS 9.4 (Statistical Analysis System, Cary, NC, USA) for sample comparison. The significance of difference was defined at $P < 0.05$.

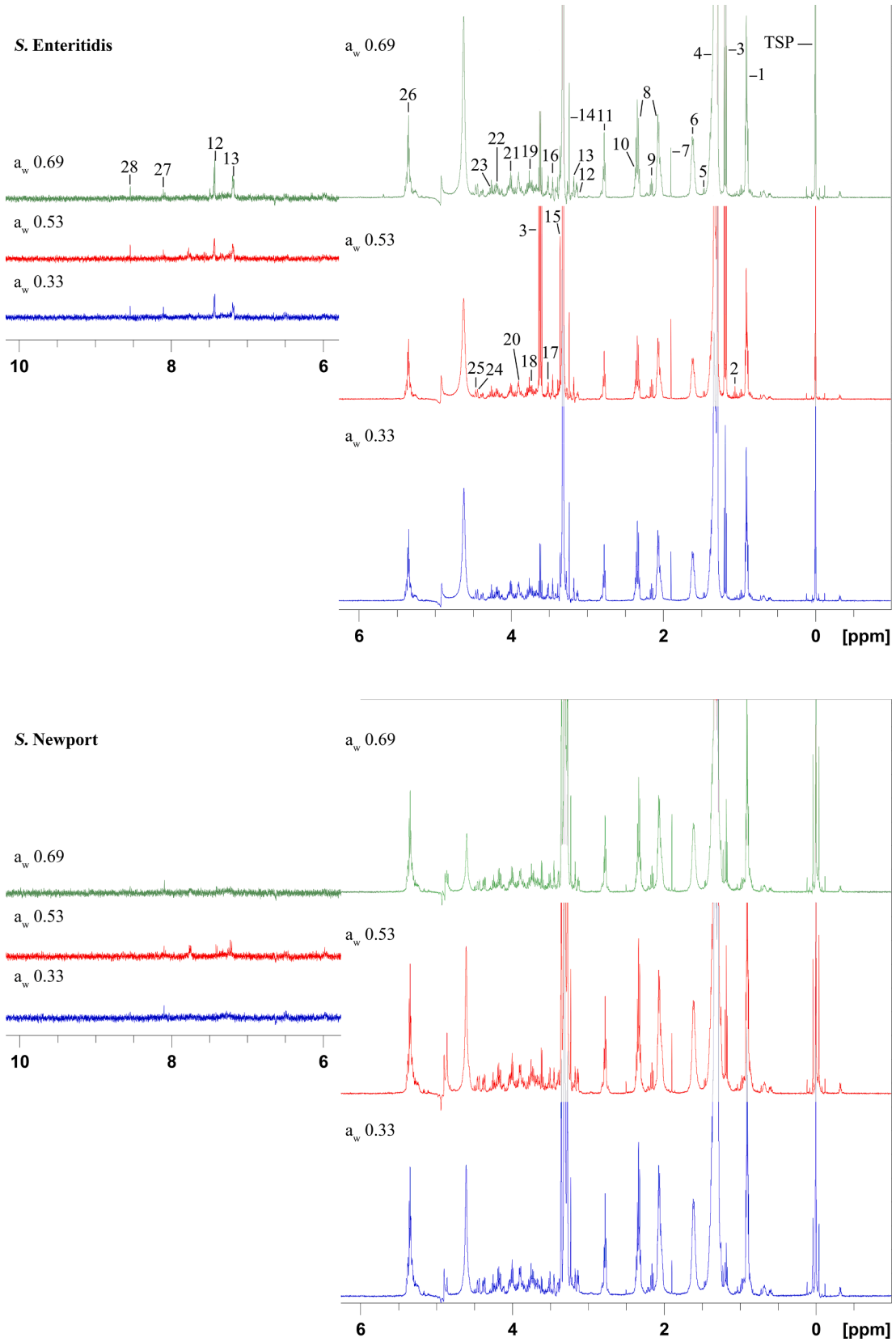

Fig. 2. Typical ^{1}H nuclear magnetic resonance (NMR) spectra of *Salmonella* Enteritidis, Newport and Typhimurium strains after being treated at 65 °C for 20 min in a_w 0.33–, 0.53- and 0.69- wheat flour.

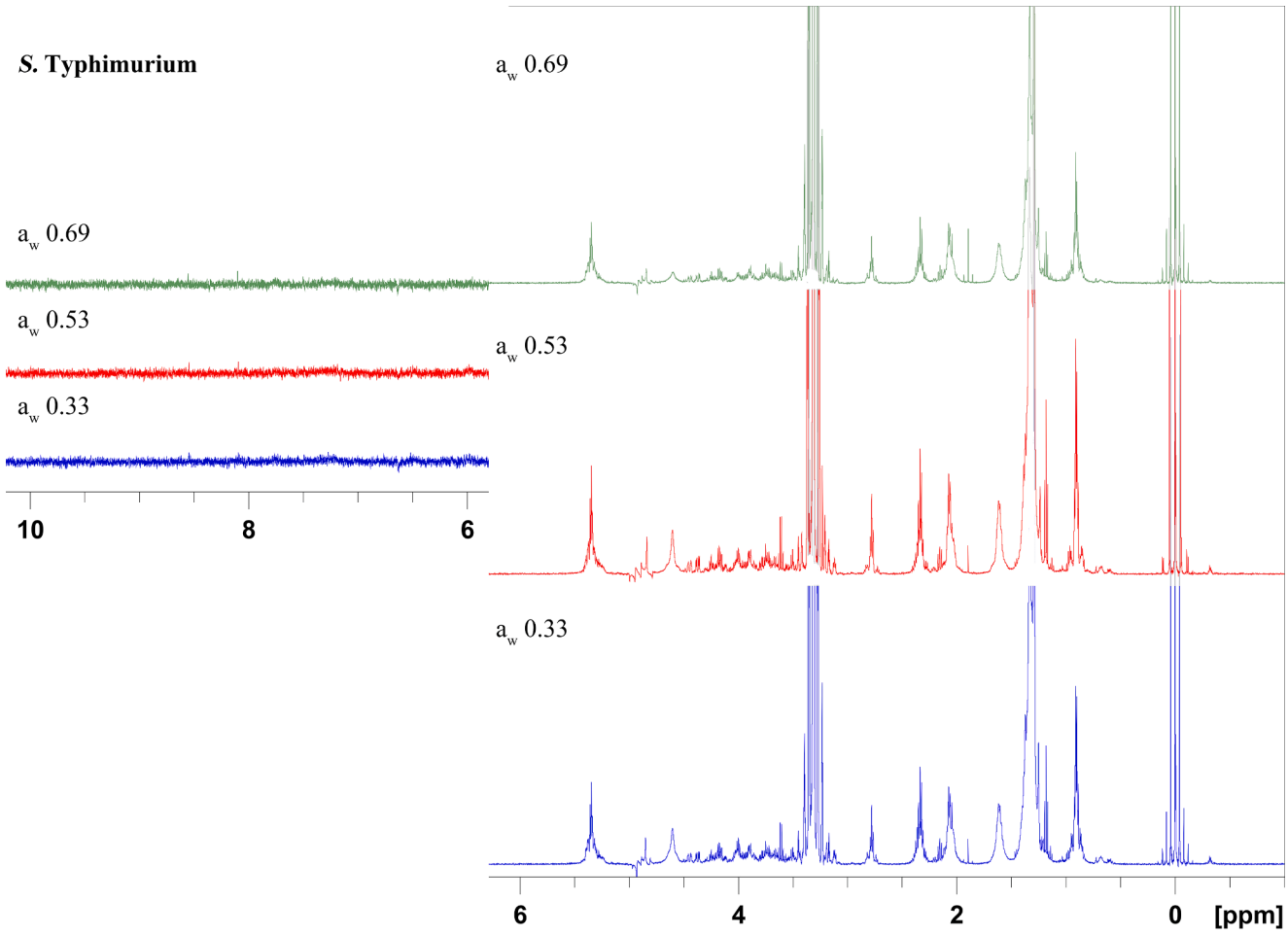

Fig. 2. (*continued*).

3. Results and discussion

3.1. Thermal inhibition kinetics of Salmonella strains at different flour a_w levels

Numerous studies have shown that the Weibull model is the most appropriate model for describing the inactivation kinetics of bacteria in LMFs (Chen et al., 2022a; Ma et al., 2009; Rachon, Peñaloza, & Gibbs, 2016; Santillana Farakos, Schaffner, & Frank, 2014). In this study, with high adjusted R^2 values ($\geq$0.868) and low RMSE ($\leq$0.201) obtained (Fig. 1A), an exceptionally good fit of the Weibull model to the thermal inactivation data of three *Salmonella* strains, namely Enteritidis (ATCC 13076), Newport (ATCC 6962) and Typhimurium (ATCC 14028), in wheat flour was also demonstrated (Forghani et al., 2019; Gurtler, Juneja, Jones, & Purohit, 2019; Liu et al., 2019).

The inactivation patterns of the strains from different a_w of flour, 0.33, 0.53 and 0.69, can be easily diagnosed from the resulting inactivation curves (Fig. 1B). It can be seen that all strains in the driest wheat flour (a_w 0.33) maintained the highest viability throughout the 20-min treatment process, which implied a superb protection effect of the low a_w on the *Salmonella* cells under the heat stress. Besides, the a_w-0.53 wheat flour, which was the second driest, also seemed to offer some protection to its *Salmonella* residents, though the protective effect did not appear until the *S. Enteritidis* and *S. Typhimurium* strains were treated for about 8 min based on the curves. Interestingly, this time coincided with the comparable δ values of the two strains at 0.53 and 0.69 flour a_w (Fig. 1A). Hence, an inference could be drawn that the protective effect attributed to this a_w difference took place only after the first log CFU was inhibited. From the Weibull model, the times required for a 5-log reduction (t_{5D}) were also calculated (Fig. 1A) (Cho, Kim, & Kang, 2020; Pala & Zorba, 2015; Pereira, Prestes, Silva, & Nascimento, 2020). Despite some insignificance ($P > 0.05$), the largest t_{5D} values were demonstrated by the three strains in the driest flour, which from another aspect, indicated the enhanced thermal resistance of *Salmonella* at lower a_w.

Furthermore, the total inhibition of *Salmonella* cells by the 20-min heat treatment is summarised in Fig. 1C. Overall, except for the insignificant difference between the reductions of *S.* Newport from the a_w 0.53- and a_w 0.69- wheat flour ($P > 0.05$), a remarkable decreasing trend in cell reduction with decreasing flour a_w was observed among the strains, which adds to the evidence for the cross-protection of low-a_w exposure to the heat stress in wheat flour.

3.2. Overall differences in Salmonella metabolomes after heat treatment at different flour a_w levels

Representative ^{1}H NMR spectra of the *Salmonella* strains in wheat flour stressed at 65 °C for 20 min are shown in Fig. 2. By referring to the two-dimensional (2D) ^{1}H–^{13}C spectrum, metabolic databases as well as the literature, a total of 28 metabolites, which covered the main bacterial cell components, including amino acids (e.g., leucine, valine, and alanine), organic acids (e.g., lactic acid and acetic acid), ethanol, betaine, sugars (e.g., α-D-glucose, β-D-glucose, and glucose 1-phosphate) and nucleotide-related compounds (e.g., ATP, ADP, and adenosine), were identified (Table S1).

Despite the same metabolite composition, the metabolic profiles of

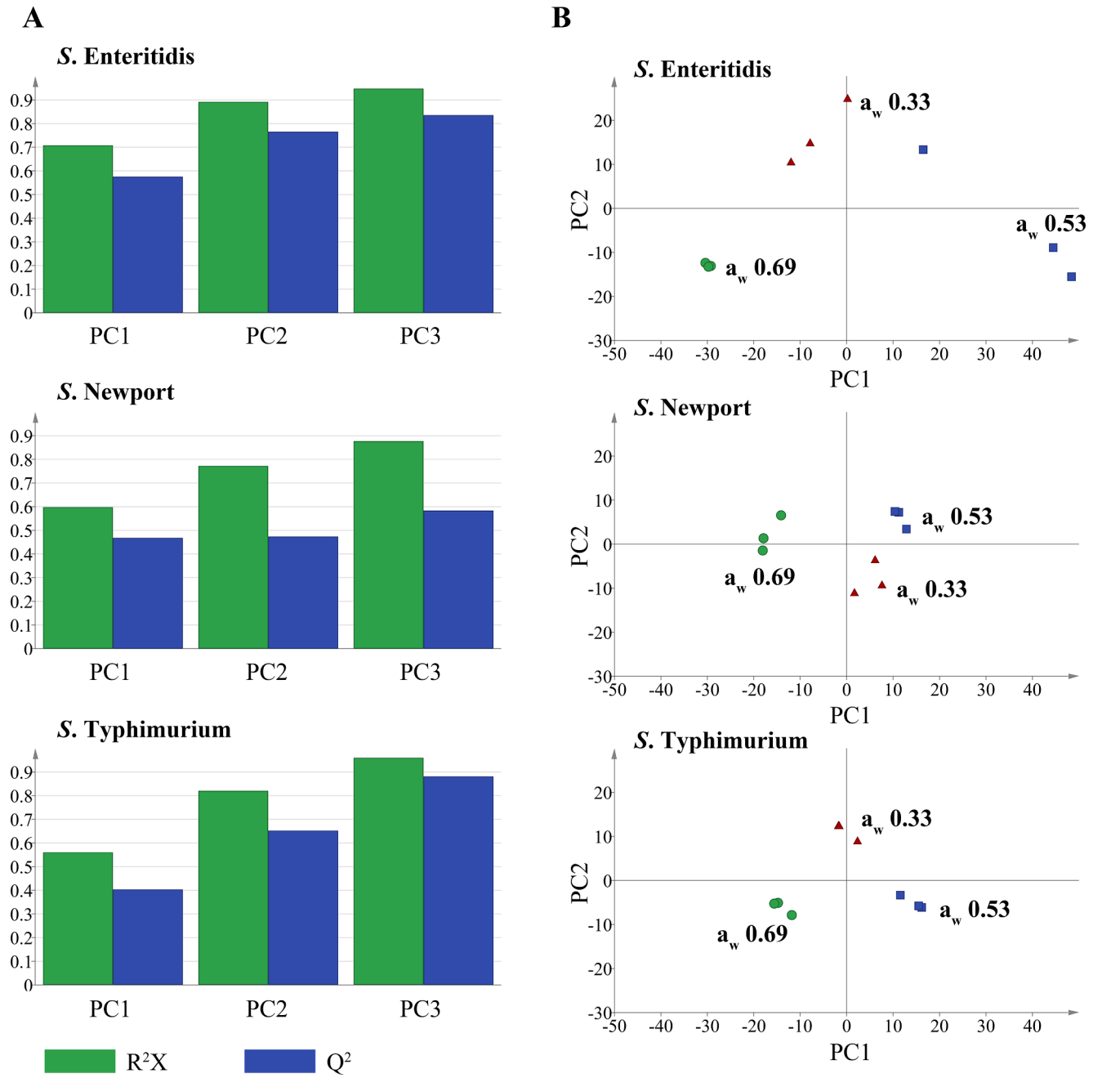

Fig. 3. Principal-component analysis (PCA) of ^{1}H NMR spectra of *Salmonella* Enteritidis, Newport and Typhimurium strains after being treated at 65 °C for 20 min in a_w 0.33–, 0.53- and 0.69- wheat flour. Principal components explaining the variances (A) and score plots (B). Note: triangles represent a_w 0.33, squares represent a_w 0.53 and circles represent a_w 0.69 on score plots.

each strain at different flour a_w may vary by the metabolite concentration. To illustrate the overall metabolic differences in the strains induced by the 20-min thermal treatment, metabolomes of the treated strains recovered from the a_w 0.33–, a_w 0.53- and a_w 0.69- flour samples were first compared via PCA. Three PCA models were built for the *S.* Enteritidis, *S.* Newport and *S.* Typhimurium strains separately, all of which demonstrated good predictability and interpretability based on the high R^2X (0.88–0.96) and Q^2 values (>0.5) accumulated by the first three principal components (PCs) (Fig. 3A).

Score plots were generated based on the PC1 and PC2 of each model (Fig. 3B). On each plot, clear separation of metabolomes from different a_w levels was observed, which affirmed us that the difference in thermal resistance due to flour a_w could be manifested at the metabolic level. Besides, for all strains, an obvious triangle shape was formed by the points denoting the metabolomes from the different flour a_w levels, where those from a_w 0.69 and a_w 0.53 were typically separated by the PC2 and those from a_w 0.53 and a_w 0.33 were further separated by the PC1. This observation, on one hand, implied that the thermal resistance enhancement pattern caused by the decrease in flour a_w was similar among the three *Salmonella* strains. On the other hand, it also revealed that although the thermal resistance of *Salmonella* may be continuously improved along with the decrease in a_w, the underlying metabolic mechanism was not a linear process but was rather complex.

3.3. Alterative metabolites in Salmonella after heat treatment at different flour a_w levels

A more supervised multivariate analysis tool, OPLS-DA, may provide

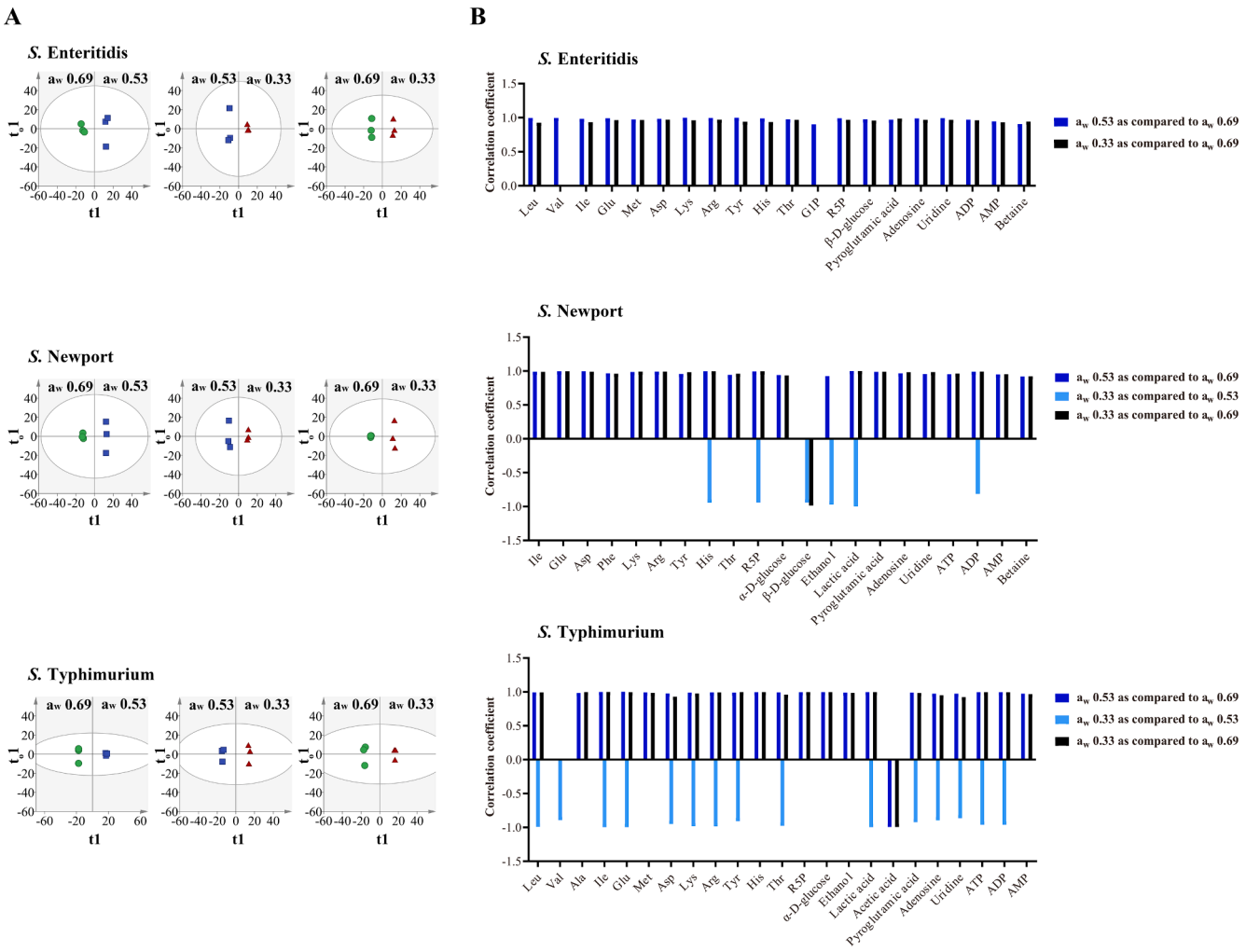

Fig. 4. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) of ^{1}H NMR spectra of *Salmonella* Enteritidis, Newport and Typhimurium strains after being treated at 65 °C for 20 min in a_w 0.33–, 0.53- and 0.69- wheat flour. Score plots (A) and coefficient plots (B). Note: G1P: glucose 1-phosphate; R5P: ribose 5-phosphate. Note: triangles represent a_w 0.33, squares represent a_w 0.53 and circles represent a_w 0.69 on score plots.

a deeper view of the complex mechanism. For each *Salmonella* strain, through pairwise comparison of its metabolomes after being treated in the a_w 0.33–, a_w 0.53- and a_w 0.69- flour samples, the metabolic activities characterising its thermal resistance at the specific a_w level could be indicated. A total of nine OPLS-DA models were built for the comparisons and all of them showed good fitness to the NMR spectral data based on the high R^2Y and Q^2 values (Table S2) (Pu, Vinitchaikul, Gu, Mao, & Zhang, 2021. Windarsih, Wijayanti, Irnawati, & Rohman, 2021; Ye et al., 2012). Corresponding to the well-separated metabolomes on the score plots in PCA, evident pairwise discriminations were illustrated on the OPLS-DA score plots as well (Fig. 4A). Based on the criteria of correlation coefficient of > 0.602, VIP value of > 1 and P of < 0.05, the significantly discriminative metabolites contributing to the pairwise discriminations were screened out and summarised in Fig. 4B.

For *S.* Enteritidis, compared to the cells recovered from the a_w-0.69 flour, cells from the two lower flour a_w (0.33 and 0.53) had significantly higher contents of an array of metabolites, including amino acids (e.g., Glutamic acid, Arginine and Tyrosine), sugars (e.g., glucose 1-phosphate and β-D-glucose) as well as nucleotides (adenosine and uridine) ($P <$ 0.05) (Fig. 4B). The *S.* Newport and *S.* Typhimurium strains from the lower a_w levels (0.33 and 0.53) also had higher contents of these metabolites compared to their respective counterparts from a_w 0.69, and in addition, the former also showed higher possession of ethanol and lactic acid. In general, when the flour a_w decreased from 0.69 to either 0.53 or 0.33, a conspicuous metabolite increasing trend was demonstrated by all three *Salmonella* strains.

In contrast to the huge metabolic differences between cells from the a_w-0.69 flour sample and the two lower-a_w (0.33 and 0.53) flour samples, the metabolic differences of cells within the two lower-a_w samples themselves were far less obtrusive. Specifically, in *S.* Enteritidis, none of the identified metabolites showed significantly differential contents at the two lower a_w levels ($P > 0.05$) (Fig. 4B). In *S.* Newport and *S.* Typhimurium, while some metabolites' contents did decrease when the flour a_w dropped from 0.53 to 0.33, the change was not huge enough to reverse the metabolite increasing trend when the a_w decreased from 0.69 to 0.33. Overall, as the metabolic differences between each strain from a_w 0.69 and a_w 0.53 and between the same strain from a_w 0.69 and a_w 0.33 were essentially the same, these differences may represent the major metabolic changes contributing to the increased thermal resistance as the flour a_w decreased in this study. Regarding this, in the following section, we would mainly focus on these metabolic changes to demystify the thermal resistance improvement mechanisms in the *Salmonella* strains.

3.4. *Differences in heat-induced metabolic network perturbations in Salmonella at 0.69 and lower (0.53 and 0.33) flour a_w levels*

The significantly discriminative metabolites in the heat-stressed cells from the a_w-0.69 and the two lower-a_w (0.53 and 0.33) flour samples were mapped onto the metabolic pathways in *Salmonella* to indicate the

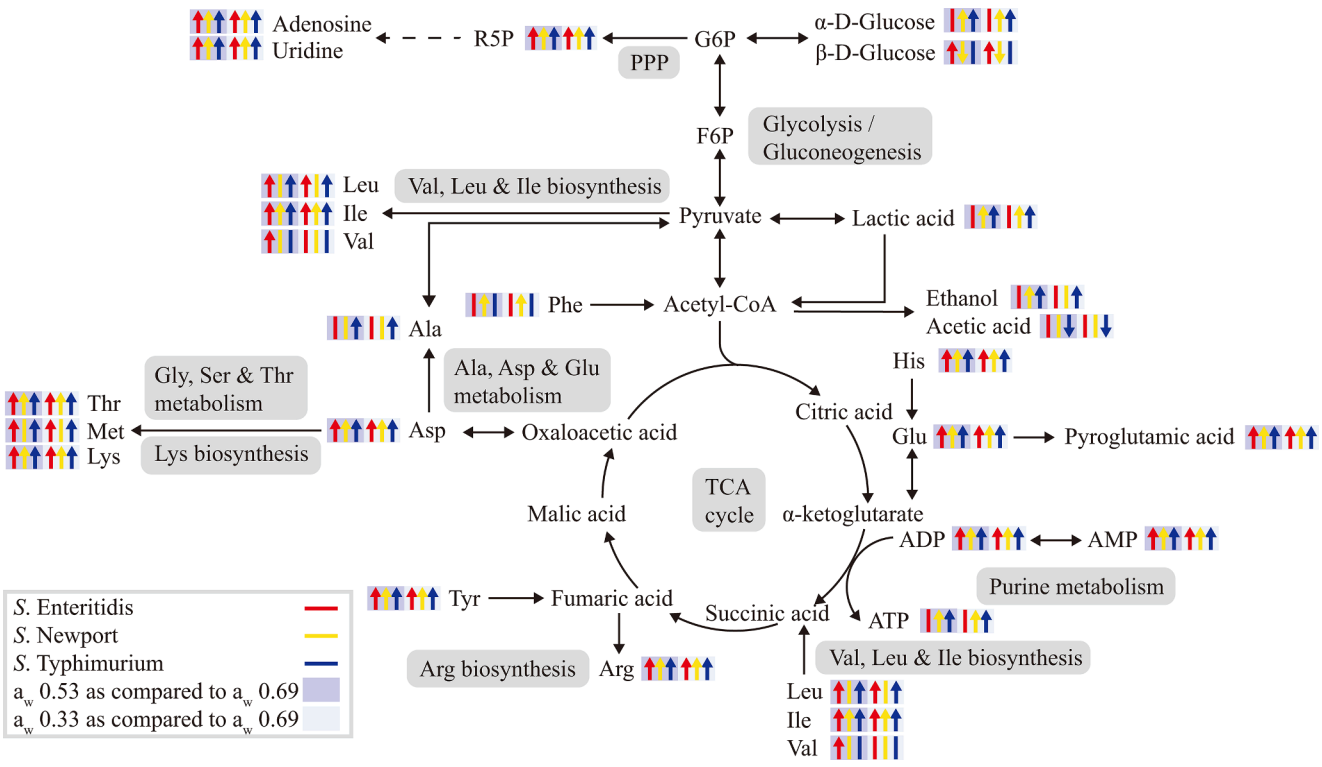

Fig. 5. Differences in heat-induced metabolic network perturbations in *Salmonella* Enteritidis, Newport and Typhimurium strains from a_w 0.69- wheat flour and lower-a_w (0.53 and 0.33) wheat flour. Note: R5P: ribose 5-phosphate; G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; PPP: pentose phosphate pathway.

major differences in *Salmonella*'s metabolic network perturbations at these a_w levels (Fig. 5). Based on this, an assumptive schematic diagram rationalising *Salmonella*' increased thermal resistance at the lower flour a_w was also proposed (Fig. 6).

3.4.1. Amino acid metabolism

In all three strains, as amino acids accounted for a substantial portion of the significantly changed metabolites, their metabolism was believed to play a pivotal role in discriminating the thermal resistance of *Salmonella* at a_w 0.69 and the two lower flour a_w (Fig. 5; Fig. 6). Two mechanisms in line with these metabolic changes were presumed. Firstly, the amino acid contents may reflect the strains' capability to deplete the abnormal proteins formed as a result of heat stress. During thermal treatment, some intracellular proteins which are rendered abnormal and misfolded can aggregate in the bacterial cell (Jozefczuk et al., 2010; Xie et al., 2021). Uncontrolled accumulation of these aggregated proteins may induce a cascade of toxic effects on the cell, which eventually leads to apoptotic or necrotic cell death (Bednarska, Schymkowitz, Rousseau, & Van Eldere, 2013). In this case, prevention of aggregate accumulation via protein degradation is critical for cell survival (Mandelstam, 1963; Willetts, 1967). In this study, from a vast elevation in a spectrum of amino acids (e.g., isoleucine, glutamic acid and threonine) in the lower-a_w cells as compared to the a_w-0.69 cells of all three strains, a more efficient protein degradation process in the former was suggested, which potentially explained the higher *Salmonella* viability achieved by the lower-a_w samples. Meanwhile, with more free amino acids released from abnormal protein degradation, the building blocks for new proteins and enzymes vital at the elevated temperature would be more sufficient, which may strengthen the cells in another way (Jozefczuk et al., 2010; Willetts, 1967).

The second speculated mechanism was that the amino acid-related metabolic differences may underly the different ability to cope with osmotic stress (Fig. 6). Osmotic stress is a secondary stress always accompanying heat stress (Qu, Ding, Jiang, & Zhu, 2013). In this study, it may be induced by the slight uplifting of sample a_w during heating due to the sorption isothermal effect (Syamaladevi et al., 2016; Xu et al., 2019). Many amino acids (e.g., leucine, alanine, and glutamic acid) are natural osmotic regulators in bacterial cells (Wiesenthal, Müller, Harder, & Hildebrandt, 2019; Zhao, Zhao, Wu, Lou, & Yang, 2019), which protect bacteria cells through sustaining cytoplasmic osmolarity and preventing the collapse of subcellular structures. As a result, with more amino acids accumulated as compared to the a_w-0.69 cells, the thermal resistance of cells from the lower flour a_w may also be reinforced by the enhanced osmotic protection.

3.4.2. Energy metabolism

Through various metabolic pathways, amino acids can finally enter the TCA cycle for energy production (Fig. 5; Fig. 6). Therefore, concomitant with the higher amino acid levels, a higher yield of ATP, the energy storage molecule, was generated in the lower-a_w samples. However, the higher amino acid contents cannot be credited alone for the high ATP production. In fact, while the amino acids provide carbon skeletons to keep the TCA cycle in operation, the ATP formation itself is carried out by the ATP synthase, a molecular machine that catalyses the formation of ATP from the lower-energy molecule, ADP, and inorganic phosphate (P_i). According to a transcriptomics study by Mandal and Kwon (2017), all genes encoding the 9 subunits of ATP synthase were shown to be important for S. Typhimurium's survival under desiccation. Moreover, the ATP synthase genes were also abundantly expressed under desiccation in Plectus murrayi and chickpeas (Adhikari, Wall, & Adams, 2009; Jaiswal et al., 2014). In this study, the lower wheat flour a_w provided a more desiccated environment for the bacteria, and thus, a more stimulated transcription of these ATP synthase genes was speculated. Consistent with the speculation, a higher level of ADP was observed in parallel to the higher ATP content in the lower-a_w cells, which suggested an elevated ADP-ATP conversion throughput potentially associated with the enhanced expression of the ATP synthase genes. Overall, with more amino acids maintaining the function of the TCA cycle and increased expression of the ATP synthase genes, higher energy production in the *Salmonella* strains from the lower-a_w samples

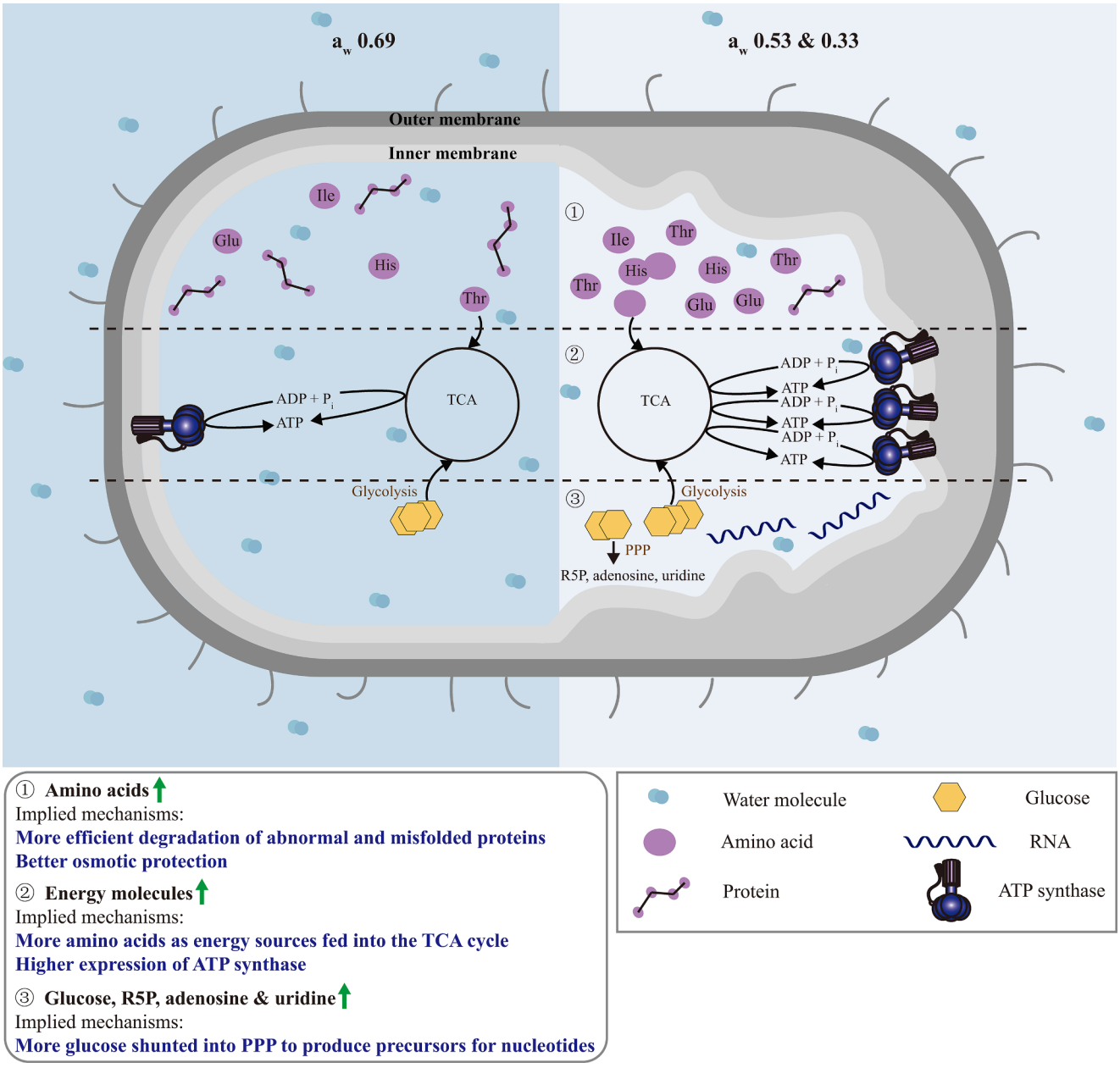

Fig. 6. Proposed mechanisms underlying *Salmonella*'s increased thermal resistance at lower-flour a_w (0.53 and 0.33) as compared to a_w 0.69. Note: R5P: ribose 5-phosphate; PPP: pentose phosphate pathway.

compared to that from the a_w-0.69 samples was unsurprisingly resulted, which provided the lower-a_w cells with more fuels to sustain their physiological functions and their lives.

3.4.3. *Glucose metabolism*

As amino acids provided abundant substrates for energy production, there remained less burden on glucose for energy production in cells at lower-a_w levels. Consequently, more glucose from these cells can be shunted from glycolysis to other metabolic pathways to support additional metabolic activities (Fig. 5; Fig. 6). The pentose phosphate pathway (PPP) was one of the pathways to shunt glucose (Ge et al., 2020), as evidenced by the significantly higher level of ribose 5-phosphate detected in the lower-a_w cells ($P < 0.05$). Ribose 5-phosphate is a key precursor for nucleotide synthesis in bacteria (Stincone et al., 2015), which, in this study, resulted in increased levels of adenosine and uridine to support RNA synthesis in the lower-a_w cells.

3.5. *Differences in heat-induced metabolic network perturbations in Salmonella at 0.53 and 0.33 flour a_w levels*

Amino acids were the most noteworthy metabolites discriminating cells at the two lower flour a_w (0.53 and 0.33) in both *S.* Newport and *S.* Typhimurium (Fig. 4B). Differences in the concentration of these amino acids might be attributed to the different extents of "unintentional" degradation of normal proteins. Based on a common perspective, during a heating process, while the misfolded proteins can be intentionally degraded to avoid aggregation, there are also chances for some normal proteins to be "unintentionally" degraded by the vibration of the surrounding water molecules (Earnshaw, Appleyard, & Hurst, 1995). Compared to samples with high a_w, this "unintentional" protein degradation is less likely to happen in the more desiccated samples, partly because of the reduced amount of vibrating water molecules and partly because of the conformational modification (rigidification) of proteins induced by low water availability (Klibanov, 1989; Laroche et al., 2005;

Tadapaneni et al., 2017a). In this study, from the significantly lower contents of certain amino acids in the most desiccated (a_w-0.33) cells ($P < 0.05$), the greater cell viability achieved at 0.33 a_w than that achieved at a_w 0.53 may be explained by this mechanism.

4. Conclusions

In this study, the increase in thermal resistance of *Salmonella* associated with the decrease in the a_w of wheat flour was verified in our experimental setting. Besides, through NMR-based metabolomics, the differences in the heat-induced metabolic responses of the *Salmonella* strains, including the efficiency of misfolded protein degradation, the availability of osmoprotectant molecules, the ATP generating throughput by the ATP synthase and the allocation of glucose in the metabolic pathways, were found to underly the varying thermal resistance at the different flour a_w. Overall, our study is the first attempt to unravel the mechanisms of *Salmonella*'s enhanced thermal resistance at lower flour a_w. The results would enable the development of measures to remove the relevant metabolic changes artificially so as to reduce the thermal resistance of *Salmonella* in future wheat flour production.

CRediT authorship contribution statement

Yue Wang: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Resources, Software, Visualization, Writing – original draft. **Hongshun Yang:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112203.

References

Adhikari, B. N., Wall, D. H., & Adams, B. J. (2009). Desiccation survival in an Antarctic nematode: Molecular analysis using expressed sequenced tags. *BMC Genomics, 10*(1), 69.

Bednarska, N. G., Schymkowitz, J., Rousseau, F., & Van Eldere, J. (2013). Protein aggregation in bacteria: The thin boundary between functionality and toxicity. *Microbiology (Reading), 159*(Pt 9), 1795–1806.

Beuchat, L. R., Komitopoulou, E., Beckers, H., Betts, R. P., Bourdichon, F., Fanning, S., … Ter Kuile, B. H. (2013). Low–water activity foods: Increased concern as vehicles of foodborne pathogens. *Journal of Food Protection, 76*(1), 150–172.

Bugarel, M., Tudor, A., Loneragan, G. H., & Nightingale, K. K. (2017). Molecular detection assay of five *Salmonella* serotypes of public interest: Typhimurium, Enteritidis, Newport, Heidelberg, and Hadar. *Journal of Microbiological Methods, 134*, 14–20.

Canakapalli, S. S., Sheng, L., & Wang, L. (2022). Survival of common foodborne pathogens on dates, sundried tomatoes, and dried pluots at refrigerated and ambient temperatures. *LWT, 154*, Article 112632.

Centers for Disease Control and Prevention. (2018). National enteric disease surveillance: *Salmonella* annual report, 2016. https://www.cdc.gov/nationalsurveillance/pdfs/2016-Salmonella-report-508.pdf.

Chen, L., Liu, Q., Zhao, X., Zhang, H., Pang, X., & Yang, H. (2022). Inactivation efficacies of lactic acid and mild heat treatments against Escherichia coli strains in organic broccoli sprouts. *Food Control, 133*, Article 108577.

Chen, L., Zhang, H., Liu, Q., Pang, X., Zhao, X., & Yang, H. (2019). Sanitising efficacy of lactic acid combined with low-concentration sodium hypochlorite on Listeria innocua in organic broccoli sprouts. *International Journal of Food Microbiology, 295*, 41–48.

Chen, L., Zhao, X., Li, R., & Yang, H. (2022). Integrated metabolomics and transcriptomics reveal the adaptive responses of Salmonella enterica serovar Typhimurium to thyme and cinnamon oils. *Food Research International, 157*, Article 111241.

Chen, L., Zhao, X., Wu, J., Liu, Q., Pang, X., & Yang, H. (2020). Metabolic characterisation of eight *Escherichia coli* strains including "Big Six" and acidic responses of selected strains revealed by NMR spectroscopy. *Food Microbiology, 88*, Article 103399.

Cho, E. R., Kim, S. S., & Kang, D. H. (2020). Inactivation kinetics and membrane potential of pathogens in soybean curd subjected to pulsed ohmic heating depending on applied voltage and duty ratio. *Applied and Environmental Microbiology, 86*(14), e00656–00620.

Coe, C., Boltz, T., Stearns, R., Foster, P., Taylor, R. L., Moritz, J., … Shen, C. (2022). Thermal inactivation of Salmonella Typhimurium and surrogate Enterococcus faecium in mash broiler feed in a laboratory scale circulated thermal bath. *Poultry Science, 101*(8), Article 101976.

Earnshaw, R. G., Appleyard, J., & Hurst, R. M. (1995). Understanding physical inactivation processes: Combined preservation opportunities using heat, ultrasound and pressure. *International Journal of Food Microbiology, 28*(2), 197–219.

Eglezos, S. (2010). Microbiological quality of wheat grain and flour from two mills in Queensland. *Australia. Journal of Food Protection, 73*(8), 1533–1536.

Emwas, A. H. (2015). The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. In B. J. (Ed.), Metabonomics. Methods in Molecular Biology. (2015/02/14 ed., Vol. 1277, pp. 161-193): Humana Press, New York, NY.

Ferreira, F. S., Horvath, M. B., & Tondo, E. C. (2013). Assessing the growth and recovery of Salmonella Enteritidis SE86 after sodium dichloroisocyanurate exposure. *Brazilian Journal of Microbiology, 44*(3), 785–790.

Finn, S., Condell, O., McClure, P., Amézquita, A., & Fanning, S. (2013). Mechanisms of survival, responses and sources of Salmonella in low-moisture environments. *Frontiers in Microbiology, 4*, 331.

Fong, K., & Wang, S. (2016). Heat resistance of Salmonella enterica is increased by pre-adaptation to peanut oil or sub-lethal heat exposure. *Food Microbiology, 58*, 139–147.

Forghani, F., Bakker, M. d., Futral, A. N., & Diez-Gonzalez, F. (2018). Long-term survival and thermal death kinetics of enterohemorrhagic *Escherichia coli* serogroups O26, O103, O111, and O157 in wheat flour. Applied and Environmental Microbiology, 84 (13), e00283-00218.

Forghani, F., den Bakker, M., Liao, J. Y., Payton, A. S., Futral, A. N., & Diez-Gonzalez, F. (2019). Salmonella and enterohemorrhagic *Escherichia coli* serogroups O45, O121, O145 in wheat flour: Effects of long-term storage and thermal treatments. *Frontiers in Microbiology, 10*, 323.

Ge, T., Yang, J., Zhou, S., Wang, Y., Li, Y., & Tong, X. (2020). The role of the pentose phosphate pathway in diabetes and cancer. *Frontiers in Endocrinology, 11*(365), 365.

Greenspan, L. (1977). Humidity fixed points of binary saturated aqueous solutions. Journal of Research of the National Bureau of Standards. *Section A, Physics and Chemistry, 81*(1), 89.

Guo, C., He, Y., Wang, Y., & Yang, H. (2022). NMR-based metabolomic investigation on antimicrobial mechanism of Salmonella on cucumber slices treated with organic acids. *Food Control, 137*, Article 108973.

Gurtler, J. B., Juneja, V. K., Jones, D. R., & Purohit, A. (2019). Thermal inactivation kinetics of three heat-resistant Salmonella strains in whole liquid egg. *Journal of Food Protection, 82*(9), 1465–1471.

He, Y., Zhao, X., Chen, L., Zhao, L., & Yang, H. (2021). Effect of electrolysed water generated by sodium chloride combined with sodium bicarbonate solution against Listeria innocua in broth and on shrimp. *Food Control, 127*, Article 108134.

Hildebrandt, I. M., Marks, B. P., Anderson, N. M., & Grasso-Kelly, E. M. (2020). Reproducibility of Salmonella thermal resistance measurements via multilaboratory isothermal inactivation experiments. *Journal of Food Protection, 83*(4), 609–614.

Hildebrandt, I. M., Marks, B. P., Ryser, E. T., Villa-Rojas, R., Tang, J., Garces-Vega, F. J., & Buchholz, S. E. (2016). Effects of inoculation procedures on variability and repeatability of Salmonella thermal resistance in wheat flour. *Journal of Food Protection, 79*(11), 1833–1839.

Jaiswal, D. K., Mishra, P., Subba, P., Rathi, D., Chakraborty, S., & Chakraborty, N. (2014). Membrane-associated proteomics of chickpea identifies Sad1/UNC-84 protein (CaSUN1), a novel component of dehydration signaling. *Scientific Reports, 4* (1), 4177.

Jarvis, N. A., O'Bryan, C. A., Dawoud, T. M., Park, S. H., Kwon, Y. M., Crandall, P. G., & Ricke, S. C. (2016). An overview of *Salmonella* thermal destruction during food processing and preparation. *Food Control, 68*, 280–290.

Jozefczuk, S., Klie, S., Catchpole, G., Szymanski, J., Cuadros-Inostroza, A., Steinhauser, D., … Willmitzer, L. (2010). Metabolomic and transcriptomic stress response of *Escherichia coli*. *Molecular Systems Biology, 6*(1), 364.

Keppler, S., Bakalis, S., Leadley, C. E., Sahi, S. S., & Fryer, P. J. (2018). Evaluation of dry heat treatment of soft wheat flour for the production of high ratio cakes. *Food Research International, 107*, 360–370.

Kharel, K., Adhikari, A., Graham, C. J., Prinyawiwatkul, W., & Yemmireddy, V. K. (2018). Hot water treatment as a kill-step to inactivate *Escherichia coli* O157:H7, Salmonella enterica, Listeria monocytogenes and Enterococcus faecium on in-shell pecans. *LWT - Food Science and Technology, 97*, 555–560.

Klibanov, A. M. (1989). Enzymatic catalysis in anhydrous organic solvents. *Trends in Biochemical Sciences, 14*(4), 141–144.

Laroche, C., Fine, F., & Gervais, P. (2005). Water activity affects heat resistance of microorganisms in food powders. *International Journal of Food Microbiology, 97*(3), 307–315.

Liu, Q., Wu, J., Lim, Z. Y., Lai, S., Lee, N., & Yang, H. (2018). Metabolite profiling of Listeria innocua for unravelling the inactivation mechanism of electrolysed water by nuclear magnetic resonance spectroscopy. *International Journal of Food Microbiology, 271*, 24–32.

Liu, Q., Jin, X., Feng, X., Yang, H., & Fu, C. (2019). Inactivation kinetics of *Escherichia coli* O157: H7 and Salmonella Typhimurium on organic carrot (Daucus carota L.) treated with low concentration electrolyzed water combined with short-time heat treatment. *Food Control, 106*, Article 106702.

Liu, S., Tang, J., Tadapaneni Ravi, K., Yang, R., Zhu, M. J., & Elkins Christopher, A. (2018). Exponentially increased thermal resistance of Salmonella spp. and Enterococcus faecium at reduced water activity. *Applied and Environmental Microbiology, 84*(8), e02742–02717.

Liu, Z., Sheng, L., Canakapalli, S. S., & Wang, L. (2022). Evaluation of the microbial control efficacies of commonly used home-drying and storage practices of dried peaches. *LWT, 165*, Article 113734.

Ma, L. I., Zhang, G., Gerner-Smidt, P., Mantripragada, V., Ezeoke, I., & Doyle, M. P. (2009). Thermal inactivation of Salmonella in peanut butter. *Journal of Food Protection, 72*(8), 1596–1601.

Manchester, M., & Anand, A. (2017). Chapter Two - Metabolomics: strategies to define the role of metabolism in virus infection and pathogenesis. In M. Kielian, T. C. Mettenleiter & M. J. Roossinck (Eds.), Advances in Virus Research (Vol. 98, pp. 57-81): Academic Press.

Mandal, R. K., & Kwon, Y. M. (2017). Global screening of Salmonella enterica serovar Typhimurium genes for desiccation survival. *Frontiers in Microbiology, 8*, 1723.

Mandelstam, J. (1963). Protein turnover and its function in the economy of the cell. *Annals of the New York Academy of Sciences, 102*(3), 621–636.

McCallum, L., Paine, S., Sexton, K., Dufour, M., Dyet, K., Wilson, M., … Hope, V. (2013). An outbreak of Salmonella Typhimurium phage type 42 associated with the consumption of raw flour. *Foodborne Pathogens and Disease, 10*(2), 159–164.

McDonough, F. E., & Hargrove, R. E. (1968). Heat resistance of Salmonella in dried milk. *Journal of Dairy Science, 51*(10), 1587–1591.

Paéz, R., Lavari, L., Vinderola, G., Audero, G., Cuatrin, A., Zaritzky, N., & Reinheimer, J. (2012). Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion. *Food Research International, 48*(2), 748–754.

Pala, Ç. U., Zorba, N. N. D., & ÖZcan, G. (2015). Microbial inactivation and physicochemical properties of ultrasound processed pomegranate juice. Journal of Food Protection, 78 (3), 531-539.

Parnell, T. L., Harris, L. J., & Suslow, T. V. (2005). Reducing Salmonella on cantaloupes and honeydew melons using wash practices applicable to postharvest handling, foodservice, and consumer preparation. *International Journal of Food Microbiology, 99*(1), 59–70.

Pereira, A. A. M., Prestes, F. S., Silva, A. C. M., & Nascimento, M. S. (2020). Evaluation of the thermal resistance of Salmonella Typhimurium ATCC 14028 after long-term blanched peanut kernel storage. *LWT, 117*, Article 108701.

Petsong, K., Benjakul, S., Chaturongakul, S., Switt, A. I. M., & Vongkamjan, K. (2019). Lysis profiles of Salmonella phages on Salmonella isolates from various sources and efficiency of a phage cocktail against S. *Enteritidis and S. Typhimurium. Microorganisms, 7*(4), 100.

Pu, J., Vinitchaikul, P., Gu, Z., Mao, H., & Zhang, F. (2021). The use of metabolomics to reveal differences in functional substances of milk whey of dairy buffaloes raised at different altitudes. *Food & Function, 12*(12), 5440–5450.

Qu, A.-L., Ding, Y.-F., Jiang, Q., & Zhu, C. (2013). Molecular mechanisms of the plant heat stress response. *Biochemical and Biophysical Research Communications, 432*(2), 203–207.

Rachon, G., Peñaloza, W., & Gibbs, P. A. (2016). Inactivation of Salmonella, *Listeria monocytogenes* and Enterococcus faecium NRRL B-2354 in a selection of low moisture foods. *International Journal of Food Microbiology, 231*, 16–25.

Santillana Farakos, S. M., Schaffner, D. W., & Frank, J. F. (2014). Predicting survival of Salmonella in low–water activity foods: An analysis of literature data. *Journal of Food Protection, 77*(9), 1448–1461.

Smith, D. F., Hildebrandt, I. M., Casulli, K. E., Dolan, K. D., & Marks, B. P. (2016). Modeling the effect of temperature and water activity on the thermal resistance of Salmonella enteritidis PT 30 in wheat flour. *Journal of Food Protection, 79*(12), 2058–2065.

Smith, D. F., & Marks, B. P. (2015). Effect of rapid product desiccation or hydration on thermal resistance of Salmonella enterica serovar Enteritidis PT 30 in wheat flour. *Journal of Food Protection, 78*(2), 281–286.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M. C., Campbell, K., Cheung, E., … Ralser, M. (2015). The return of metabolism: Biochemistry and physiology of the pentose phosphate pathway. *Biological Reviews, 90*(3), 927–963.

Suehr, Q. J., Anderson, N. M., & Keller, S. E. (2019). Desiccation and thermal resistance of *Escherichia coli* O121 in wheat flour. *Journal of Food Protection, 82*(8), 1308–1313.

Syamaladevi, R. M., Tang, J., Villa-Rojas, R., Sablani, S., Carter, B., & Campbell, G. (2016). Influence of water activity on thermal resistance of microorganisms in low-moisture foods: A review. *Comprehensive Reviews in Food Science and Food Safety, 15*(2), 353–370.

Tadapaneni, R. K., Syamaladevi, R. M., Villa-Rojas, R., & Tang, J. (2017). Design of a novel test cell to study the influence of water activity on the thermal resistance of Salmonella in low-moisture foods. *Journal of Food Engineering, 208*, 48–56.

Tadapaneni, R. K., Yang, R., Carter, B., & Tang, J. (2017). A new method to determine the water activity and the net isosteric heats of sorption for low moisture foods at elevated temperatures. *Food Research International, 102*, 203–212.

U.S. Department of Agriculture. (2022). Handling flour safely: what you need to know. https://www.fda.gov/food/buy-store-serve-safe-food/handling-flour-safely-what-you-need-know.

U.S. Food and Drug Administration. (2015). Voluntary recall for Navajo Pride bleached flour due to possible health risk. https://wayback.archive-it.org/7993/20170723005828/https://www.fda.gov/Safety/Recalls/ArchiveRecalls/2015/ucm445036.htm.

Van Steertegem, B., Pareyt, B., Slade, L., Levine, H., Brijs, K., & Delcour, J. A. (2013). Impact of heat treatment on wheat flour solvent retention capacity (SRC) profiles. *Cereal Chemistry, 90*(6), 608–610.

Villa-Rojas, R., Tang, J., Wang, S., Gao, M., Kang, D. H., Mah, J. H., … López-Malo, A. (2013). Thermal inactivation of Salmonella Enteritidis PT 30 in almond kernels as influenced by water activity. *Journal of Food Protection, 76*(1), 26–32.

Wang, Y., Gao, X., & Yang, H. (2022). Integrated metabolomics of "big six" *Escherichia coli* on pea sprouts to organic acid treatments. *Food Research International, 157*, Article 111354.

Wang, Y., Wu, J., & Yang, H. (2022). Comparison of the metabolic responses of eight *Escherichia coli* strains including the "big six" in pea sprouts to low concentration electrolysed water by NMR spectroscopy. *Food Control, 131*, Article 108458.

Wang, Y., Zhou, D., & Yang, H. (2022). Metabolic responses of "big six" *Escherichia coli* in wheat flour to thermal treatment revealed by nuclear magnetic resonance spectroscopy. *Applied and Environmental Microbiology, 88*(7), e00098–00022.

Wiesenthal, A. A., Müller, C., Harder, K., & Hildebrandt, J. P. (2019). Alanine, proline and urea are major organic osmolytes in the snail Theodoxus fluviatilis under hyperosmotic stress. *The Journal of Experimental Biology, 222*(3), jeb193557.

Willetts, N. J. B. J. (1967). Intracellular protein breakdown in non-growing cells of *Escherichia coli*. *Intracellular protein breakdown in non-growing cells of Escherichia coli, 103*(2), 453.

Windarsih, A., Wijayanti, T., Irnawati, I., & Rohman, A. (2021). The use of ^{1}H-NMR spectroscopy coupled with chemometrics for authentication of Curcuma xanthorrhiza adulterated with Curcuma aeruginosa. *Key Engineering Materials, 884*, 320–326.

Wu, J., Zhao, L., Lai, S., & Yang, H. (2021). NMR-based metabolomic investigation of antimicrobial mechanism of electrolysed water combined with moderate heat treatment against *Listeria monocytogenes* on salmon. *Food Control, 125*, Article 107974.

Xie, Y., Xu, J., Yang, R., Alshammari, J., Zhu, M.-J., Sablani, S., … Elkins Christopher, A. (2021). Moisture content of bacterial cells determines thermal resistance of *Salmonella enterica* serotype Enteritidis PT 30. *Applied and Environmental Microbiology, 87*(3), e02194–02120.

Xu, J., Tang, J., Jin, Y., Song, J., Yang, R., Sablani, S. S., & Zhu, M.-J. (2019). High temperature water activity as a key factor influencing survival of *Salmonella* Enteritidis PT30 in thermal processing. *Food Control, 98*, 520–528.

Ye, Y., Zhang, L., Hao, F., Zhang, J., Wang, Y., & Tang, H. (2012). Global metabolomic responses of *Escherichia coli* to heat stress. *Journal of Proteome Research, 11*(4), 2559–2566.

Zhang, G., Ma, L. I., Patel, N., Swaminathan, B., Wedel, S., & Doyle, M. P. (2007). Isolation of *Salmonella* Typhimurium from outbreak-associated cake mix. *Journal of Food Protection, 70*(4), 997–1001.

Zhao, L., Zhao, X., Wu, J., Lou, X., & Yang, H. (2019). Comparison of metabolic response between the planktonic and air-dried *Escherichia coli* to electrolysed water combined with ultrasound by ^{1}H NMR spectroscopy. *Food Research International, 125*, Article 108607.

Zhao, X., Chen, L., Wu, J., He, Y., & Yang, H. (2020). Elucidating antimicrobial mechanism of nisin and grape seed extract against *Listeria monocytogenes* in broth and on shrimp through NMR-based metabolomics approach. *International Journal of Food Microbiology, 319*, Article 108494.