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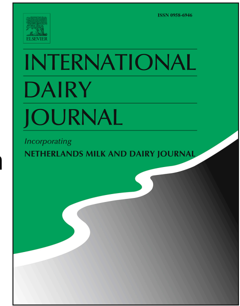

The effects of sequential heat treatment on microbial reduction and spore inactivation during milk processing

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1 **The effects of sequential heat treatment on microbial reduction and spore inactivation**
2 **during milk processing**
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27 ABSTRACT

28

29 Sequential heating processes are commonly applied to milk by the dairy industry as part of
30 their microbiological control strategy. Often pasteurisation at 72 °C is followed by a sequential
31 high heat treatment step of up to 125 °C; however, such severe heat treatment can lead to
32 reduced protein quality. Nine temperature combinations (80–90 °C) were evaluated to assess
33 microbial reduction and whey protein nitrogen index values during pilot scale milk processing.
34 A total of 110 bacterial isolates were identified to species level by 16S rDNA sequencing, with
35 *Bacillus licheniformis* identified as the dominant species. While the experimental treatments
36 did not achieve microbial reductions comparable with the control heating process, the results
37 of this study provide a benchmark for milk processors relative to the effects of sequential heat
38 treatments on milk and their impact on the survival of both thermally resistant microbial
39 populations and thermally labile milk components during processing.

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42 **1. Introduction**

43

44 With the increasing acceptance of pasteurisation and improvements in processing
45 equipment, high temperature-short time (HTST) pasteurisation has become one of the most
46 widely used heat treatments in liquid milk production. Currently, a commercial HTST usually
47 refers to a heat treatment ranging between 72 °C and 75 °C for 15–20 s (Reich et al., 2017),
48 which reduces the numbers of vegetative pathogenic bacteria, such as *E. coli, Salmonella* spp.,
49 *L. monocytogenes* and *Y. enterocolitica,* by > 5 log cfu mL^{-1} (Claeys et al., 2013; Juffs & Deeth,
50 2007). Thermoduric and spore-forming bacteria, however, can survive HTST pasteurisation,
51 and grow even at refrigeration temperatures, which can lead to spoilage of pasteurised liquid
52 milk products during storage (Buehler, Martin, Boor, & Wiedmann, 2018; Gupta & Brightwell,
53 2017).

54 Thermoduric bacteria refer to those that can withstand a heat treatment of 63.5 °C for
55 35 min (Buehner, Anand, & Djira, 2015) including some spore-forming bacteria such as
56 *Bacillus* species. Bacterial spores can withstand a heat treatment of 80 °C for 10 min or 100 °C
57 for 30 min for highly heat resistant (HHR) spores. Spore-forming bacteria are a large group of
58 microbiological hazards and *Bacillus, Clostridium, Geobacillus, Anoxybacillus* spp. are
59 commonly found in dairy products (Doyle et al., 2015; Gopal et al., 2015; Li et al., 2019;
60 McHugh et al., 2018). In unpasteurised liquid milk, mesophilic spores are the predominant
61 spore type, whereas thermophilic spores are the most common type found in milk powders
62 (Kent, Chauhan, Boor, Wiedmann, & Martin, 2016). Some spore formers can produce heat
63 stable toxins that threaten food safety and consumers' health. For example, an outbreak of
64 bovine botulism was identified on a dairy farm in France in 2015, which was caused by spore
65 contamination of grass silage (Relun et al., 2017).

66 To reduce spore numbers, many dairy processes consist of sequential heat treatments,
67 whereby the first heat-treatment will reduce vegetative cells to an acceptable number and may
68 promote spore germination (Burgess, Lindsay, & Flint, 2010; Ghosh, Zhang, Li, & Setlow, 2009)
69 whereby the second heat treatment will then inactivate the germinated spores, and accordingly
70 reduce the spore count in the final product.

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71 Typically, the unpasteurised milk will be pasteurised at 71–74 °C for 15 s prior to
72 separation, with the skim milk stream thereof normally subjected to a sequential additional
73 heat treatment ranging from 75–125 °C for 5–15 s, depending on customer requirements, prior
74 to evaporative concentration. While these sequential heat treatments address microbiological
75 safety, some severe heat treatment (up to 125 °C for 15 s) causes deleterious effects to the
76 nutritional quality of the subsequent product, including protein structural changes and the
77 formation of Maillard compounds, as well as changes in physical properties such as viscosity,
78 colour and organoleptic properties (Van Boekel, 1998). The whey protein nitrogen index
79 (WPNI) is one of the indicators used for monitoring thermal loads applied to whey protein
80 containing dairy products, where heat damage is quantified based on the amount of native
81 whey proteins present in liquid or powdered milk (Alan, Wang, & Schmidt, 2017). Higher WPNI
82 values are indicative of lower thermal loads applied and retention of higher levels of heat labile
83 components in their native form. According to the WPNI, skim milk powders are classified as
84 low heat (> 6), medium heat (1.51–5.99), or high heat (< 1.5), which determines their end use
85 in the food industry (Singh, 2007).

86 In this study, unpasteurised milk was subjected to primary and secondary heat
87 treatments using a 3 × 3 matrix of 80, 85 and 90 °C for 15 s. A typical high heat treatment
88 regime of 73 °C for 15 s followed by 125 °C for 15 s was used as a control process for
89 evaluation of microbial inactivation. The aim of this study was to evaluate the microbial
90 inactivation of sequential heat treatments at intermediate temperatures to determine if
91 combinations of lower temperatures could result in similar bacterial and spore inactivation as
92 the control temperature profile, while maintaining a higher WPNI.

93

94 **2. Materials and Methods**

95

96 *2.1. Unpasteurised milk source*

97

98 Batches of unpasteurised milk (100 L) were sourced on a monthly basis from June to
99 September 2018 from a local milk processor for four independent trials. The unpasteurised

100 milk was delivered in clean plastic storage containers and refrigerated at 4 ± 1 °C overnight
101 before processing.
102
103 *2.2. Sequential heat-treatment trials*
104
105 To each of the batches of unpasteurised milk, two heat treatments were applied on
106 successive days using an indirect tubular ultra-high temperature (UHT) pilot plant
107 (MicroThermics, NC, USA), consisting of pre-heating, final heating, holding and cooling
108 sections.
109 A schematic flow chart of the sequential heating process is shown in Fig. 1. On the first
110 day of each trial, the unpasteurised milk was heat-treated at four different temperatures: 73.5
111 (control), 80, 85 and 90 °C for 15 s, respectively. The heat-treated whole milk was then cooled
112 to 55 °C and 25 ± 5 L were collected in 50 L sealed stainless-steel vessels. The milk contact
113 surfaces of the containers were sanitised using a 2% Lactrin N (Biocel, Cork, Ireland) caustic
114 solution overnight, rinsed with process water and steam cleaned before use. The
115 stainless-steel vessels were used for all processed milk collection and storage prior to further
116 use. After the initial heat treatment, whole milk was immediately transferred to a bench-top
117 centrifugal separator Milky FJ130 ERR (Milky, Hrdejovice town, Czech Republic), which
118 separated the milk into a skim milk and cream fractions. Prior to use, the milk contact surfaces
119 of the separator, metal bowl and discs, were sterilised at 121 °C for 15 min in an autoclave.
120 Between separation of each whole milk batch processed at different temperatures, the metal
121 separator bowl was removed and sanitised using a 2% Lactrin N solution, rinsed with process
122 water and steam cleaned before further processing. The centrifugal separation of milk was
123 undertaken at 55 °C and the separated cream was sampled and then discarded, 20 L of skim
124 milk was stored overnight at 4 °C.
125 On the second day of processing, the overnight-stored skim milk was heat-treated for
126 a second time in the Microthermics unit at either 80, 85, 90 or 125 °C (control) for 15 s.
127
128 *2.3. Sampling*
129

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130 The trials were repeated in independent quadruplicate runs. At each sampling time
131 point, two samples for microbiological analysis were collected and another one sample was
132 taken for WPNI analysis using 50 mL sterile tubes. All samples were kept on ice and were
133 analysed on the day of sampling.
134
135 *2.4. Whey protein nitrogen index measurement*
136
137 The WPNI was measured by a modified method from GEA Niro analytical method A 21
138 a (Niro, 2009). As per the specifications, a saturated NaCl (Merck, New Jersey, US) solution
139 was prepared by adding 1 kg of NaCl to 2 L of deionised water and filtering the solution
140 through filter paper No. 6 (Whatman, Buckinghamshire, UK). NaCl (8.0 ± 0.1 g) was added to
141 22 mL of each milk sample in a 100 mL beaker and placed in a water bath at 37 ± 0.5 °C for 30
142 min. The tubes were stirred intermittently during the first 15 min to ensure complete saturation
143 of the NaCl. The NaCl-saturated samples were filtered through No. 6 filter paper, into 50 mL
144 Erlenmeyer flasks (Merck), with cloudy filtrates re-filtered through the same filter paper. One
145 millilitre of each sample filtrate was placed in a 50 mL Erlenmeyer flask and diluted with 10 mL
146 of the saturated NaCl solution. Parafilm (Bemis, Neenah, USA) was placed over the opening of
147 the flask which was inverted to mix the contents. A plastic pipette was used to transfer 2.5 mL
148 sample to each of two plastic cuvettes, one of which had 2–3 drops of 10% HCl added (blind
149 sample). A UV-Visible spectrophotometer (Agilent, CA, USA) was adjusted to 100%
150 transmittance at a wavelength of 420 nm using the blind sample. Both a low heat and high
151 heat standard reference non-fat dry milk powder, purchased from American Dairy Products
152 Institute (ADPI), were used to form the standard curve for the index. The WPNI index was
153 used to determine the heat classification of each sample. Samples were analysed in duplicate.
154
155 *2.5. Microbiological enumeration tests*
156
157 Samples of unpasteurised whole milk, heat-treated whole milk, heat-treated skim milk,
158 cream, skim milk stored overnight and skim milk heat-treated a second time were tested for
159 total bacterial count (TBC), thermoduric bacteria, thermophilic bacteria, mesophilic aerobic

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160 bacterial spores and thermophilic aerobic bacterial spores. Presumptive *B. cereus* group
161 bacteria were only tested in unpasteurised whole milk as an indicator of the *B. cereus* group
162 level in the unpasteurised milk. The details of spore and bacterial testing methods used are
163 described in Table 1. For the TBC and thermophilic bacteria, 1 mL of sample was directly
164 pour-plated and incubated for the required time at 30 °C and 55 °C, respectively. For
165 thermoduric bacteria, the samples were heated at 63.5 °C for 35 min before being plated and
166 incubated. For all spore counts, the samples were heat-treated at 80 °C for 10 min. From each
167 sample, 1 mL was pour-plated in duplicate on the appropriate agar for the test. For
168 presumptive *Bacillus cereus* group bacteria, BACARA agar (Biomerieux, Marcy-l'Étoile,
169 France) was used by spreading 3 × 0.33 mL of each sample on the plates. Tryptic soy agar
170 (TSA; Becton Dickinson, NJ, USA) was used for all bacterial analyses and plate count skimmed
171 milk agar (PCSMA; Merck, NJ, USA) was used for spore tests. Five millilitres of overlay of the
172 same medium was applied on the plate surface for thermoduric bacteria and spore tests post
173 agar solidification, to prevent the spread of colonies. The agar plates were incubated at the
174 required time, temperature and atmosphere conditions as outlined in Table 1. After incubation,
175 the colonies were counted. The results were expressed as cfu mL^{-1} of milk. Spreading colonies
176 were counted as single colonies if less than one quarter of the agar surface was covered; if
177 more than one quarter of the agar surface was covered, the result was discarded and recorded
178 as a plate with spreading colonies.

179

180 *2.7. Isolation and purification of bacterial strains for sequencing*

181

182 Samples for sequencing were taken from three sampling points - the unpasteurised
183 whole milk samples (Fig. 1, sampling point 1-A), pasteurised skim milk samples after overnight
184 storage at 4 °C (Fig. 1, sampling point 1-B) and skim milk heat-treated samples at 90 °C and
185 stored overnight at 4 °C (Fig. 1, sampling point 4-B). Samples were only taken from Trials 3
186 and 4. From the appropriate agar plates, four colonies of variable morphology, where possible,
187 from each microbial test were isolated and purified. For purification, the bacterial isolates were
188 aseptically streaked onto TSA plates which were incubated for 18 h at the temperature of
189 isolation. A single colony was aseptically transferred, using a 10 µL loop, into 10 mL of tryptic

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190 soy broth (TSB; Becton Dickinson) which was incubated for 18 h at the temperature of isolation.
191 Two millilitres of the growth culture were centrifuged using a benchtop centrifuge at 14,000 × *g*
192 for 1 min and the supernatants were discarded. The pellet was resuspended in cryovial
193 solution using Cryoinstant tubes 822075ZA (VWR, Pennsylvania, US) and the cryovials were
194 frozen at –20 °C until required. A total of 110 bacterial isolates were obtained.
195
196 *2.8. DNA extraction*
197
198 For each of the 110 isolates obtained, a cryobead was added aseptically to 10 mL of
199 brain heart infusion (BHI) broth and incubated for 18 h at the temperature of isolation. DNA
200 was extracted from 2 mL of bacterial culture using the Qiagen DNeasy UltraClean Microbial Kit
201 (Qiagen, Venlo, Netherlands), as per the manufacturer's instructions. DNA purity and
202 concentration were measured using a Biodrop µLITE^{TM} (Novex Electrophoresis GmbH,
203 Heidelberg, Germany). The extracted DNA from each isolate was stored in 1.5 mL micro tubes
204 (SARSTEDT, Nümbrecht, Germany) at –20 °C and analysed within 3 months.
205
206 *2.9. 16S rDNA sequencing*
207
208 The DNA of the 110 bacterial isolates was used for 16S sequencing as a primary
209 species identification. The universal primer set 27F/1492R (27F -
210 AGAGTTTGATCMTGGCTCAG, 1492R – GGTTACCTTGTTGTTAC-GACTT) was used to
211 amplify the bacterial 16S rRNA region (Sim et al., 2012). The amplicon size was approximately
212 1.4 kbp. Entire 16S rDNA sequences were determined by Sanger sequencing (Fellner &
213 Sanger, 1968). Each pair of 16S sequenced reads was aligned and the consensus was
214 extracted by the Geneious Prime® 2019.1.3 software. NCBI database searching was
215 performed on each consensus using the BLAST tool embedded in this software. Where the
216 species was reported as identified, the top 20 blast 'hits' for species identification were found
217 to be the same.
218

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219 *2.10. 16S sequencing dendrogram analysis*

220

221 BioNumerics software version 7.6 (Applied-Maths, BioMérieux Marcy-l'Étoile, France)
222 was used to construct a similarity dendogram of the isolates. The dendrogram was
223 constructed using the standard algorithm with Unweighted Pair Group Method with Arithmetic
224 Mean (UPGMA).

225

226 *2.11. Statistical analysis*

227

228 The bacterial and spore counts from each sample were converted to log cfu mL^{-1}. The
229 average and standard deviation of the values at each sampling point were calculated and
230 graphed using GraphPad Prism 7.02 (California, USA). Where the numbers were below the
231 detection limit an arbitrary value of 0 log cfu mL^{-1} was applied.

232 The results of the bacterial and spore numbers were analysed using GraphPad Prism
233 7.02 (California, USA) to generate a Boxplot for each test method, except for the presumptive
234 *B. cereus* group on BACARA plates as the numbers were too low for statistical analysis. In Fig.
235 2 to Fig. 5, each rectangular box consists of the median (as a horizontal line), the 25th and 75th
236 percentile (as the lower and upper lines of the rectangle, respectively), and the maximum and
237 minimum values as whiskers.

238 The ANOVA tests including two-way ANOVA and one-way ANOVA were carried out
239 using RStudio (Version 1.2.1335) to analyse the significant differences in spore counts with
240 processing temperatures for each specific microbiological test. Linear and multiple linear
241 regression were applied to analyse the relation between WPNI results and processing
242 temperatures.

243

244 **3. Results**

245

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246 *3.1. Microbial enumeration results*

247

248 *3.1.1. Unpasteurised milk*

249 In the unpasteurised milk (Fig. 2), the median of the TBC, thermoduric bacteria and
250 thermophilic bacteria counts were 5.27, 4.11 and 1.41 log cfu mL^{-1}, respectively. For
251 mesophilic and thermophilic aerobic bacterial spores, the median counts were 4.12 and 1.12
252 log cfu mL^{-1}, respectively. The range of the TBC was from 5.12 to 5.67 log CFU mL^{-1}. For
253 thermoduric bacteria, the results ranged from 3.64 to 4.24 log cfu mL^{-1} and for thermophilic
254 bacteria, the range was from 1.12 to 1.55 log cfu mL^{-1}. For bacterial spores, mesophilic aerobic
255 spores ranged from 3.39 to 4.14 log cfu mL^{-1} while thermophilic aerobic spores ranged from
256 0.30 to 1.37 log cfu mL^{-1}.

257

258 *3.1.2. Microbial counts after the first heat treatment and separation*

259 For whole milk after the first heat treatment (Fig. 3A), the median of the TBC
260 decreased from 3.73 to 1.39 log cfu mL^{-1} as the heating temperature increased from 73.5 to 90
261 °C ($P < 0.01$). For thermoduric bacteria and mesophilic aerobic bacterial spores, the median
262 decreased from 3.66 to 1.52 log cfu mL^{-1} ($P < 0.05$), and from 3.88 to 1.54 log cfu mL^{-1} ($P <$
263 0.05), respectively. There was no significant difference ($P > 0.05$) in thermophilic bacteria or
264 thermophilic spore counts with increasing temperature.

265 Heat treated skim milk (Fig. 3B) had a very similar microbiological profile to the heat
266 treated whole milk. After centrifugal separation, the TBC ($P < 0.1$), thermoduric bacteria ($P <$
267 0.001) and mesophilic spores ($P < 0.001$) decreased considerably with increasing
268 temperature. Compared with the heat-treated whole milk, the TBC in 85 and 90°C heat treated
269 skim increased by about 1 log cfu mL^{-1}. In heat treated skim, the median of thermophilic
270 bacteria and thermophilic spores did not show any significant difference ($P > 0.05$) and
271 remained at a low level of less than 1.5 log cfu mL^{-1}. In separated cream (Fig. 3C), the results of
272 all microbial tests followed similar trends but were about 1–1.5 log cfu mL^{-1} lower than the
273 results observed for heat treated skim, with the exception of 73.5 °C pasteurised cream, of
274 which TBC in cream was greater than that of in 73.5 °C pasteurised skim.

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276 *3.1.3. Microbial counts after overnight storage*

277 Following overnight storage of the skim milk, thermoduric bacteria and mesophilic
278 spores showed a similar pattern to the values recorded immediately after skimming (Fig. 4).
279 However, thermophilic bacteria increased considerably post storage, for skim milk samples
280 heated at the higher heating temperatures (85 and 90 °C) ($P < 0.001$). In overnight stored skim
281 milk, thermoduric bacteria ($P < 0.05$) and mesophilic spores ($P < 0.01$) decreased with
282 increasing temperature, ranging from 4.31 to 2.26 log cfu mL^{-1} and from 3.76 to 2.01 log cfu
283 mL^{-1}, respectively. Thermophilic bacteria significantly ($P < 0.05$) increased with higher heating
284 temperatures, from 1.19 (overnight stored 73.5 °C pasteurised skim) to 2.66 log cfu mL^{-1}
285 (overnight stored 90 °C heat-treated skim). For thermophilic aerobic bacterial spore counts,
286 there was no significant difference ($P > 0.05$) between temperature treatments following
287 overnight storage.

288

289 *3.1.4. The effectiveness of sequential heat treatment steps on microbial inactivation*

290 As shown in Fig. 5, none of the other combinations of heat treatment used in this study
291 (80, 85 or 90 °C for the first heat treatment followed by 80, 85 or 90 °C for the second heat
292 treatment) reduced the microbial load for the bacterial groups tested to the same extent as
293 73.5/125 °C sequential treatment. The 75/125 °C sequential treatment reduced the median
294 values of all the bacterial groups tested to below the detection limit.

295 For 80 °C (first heating) followed by 90 °C (second heating) and 90 °C (first heating)
296 followed by 90 °C (second heating), the microbial results were the lowest where the median
297 values of all tests were below 1.25 log cfu mL^{-1}.

298 For the different temperature combinations used (with the exception of the
299 73.5/125 °C sequential treatment), there was no significant ($P > 0.05$) difference in TBC,
300 thermoduric bacteria, thermophilic bacteria or thermophilic spores. However, the median
301 values of the various bacterial groups at the most severe heat treatment (90/90 °C) was 1.18,
302 1.13, 0.93 and 0.98 log cfu mL^{-1}, respectively. For mesophilic spores, the numbers decreased
303 with increasing temperature.

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305 *3.2. The effect of different heat treatment combinations on WPNI*

306

307 Although WPNI is traditionally a method used for the heat classification of powders,
308 the method was used in this study to represent the WPNI in liquid skim milk post heat
309 treatment. The control sample treated at a maximum temperature of 125 °C for 15 s (for the
310 second heat treatment) resulted in a WPNI of 1.82 ± 0.90 (Table 2), which represents typical
311 values for commercial high heat treated skim. A WPNI of greater than 2.5 was obtained in all
312 the other experimental temperature combinations tested which fall within the classification
313 range for medium heat treated skim. Multiple linear regression indicated that WPNI decreased
314 significantly ($P < 0.001$) with increasing temperature of first and second heat treatment
315 temperature. Increasing heat treatment temperatures resulted in lower WPNI values, with the
316 highest temperature combination of 90/90 °C having a WPNI of 3.56 ± 1.22, and the lowest
317 temperature combination of 80/80 °C giving a WPNI of 6.89 ± 0.45.

318

319 *3.3. Results of 16S sequencing*

320

321 A total of 110 isolates were obtained from the agar plates of unpasteurised whole milk
322 (Fig. 1, sampling point 1-A), 73.5 °C pasteurised skim milk after overnight storage (Fig. 1,
323 sampling point 1-B) and 90 °C heat-treated skim milk after overnight storage (Fig. 1, sampling
324 point 4-B) in Trial 3 and Trial 4. Of the isolates obtained, 95 were identified by 16S sequencing
325 while 15 could not be identified due to DNA contamination and errors during BLAST and were
326 excluded from further analysis. The isolates identified were predominantly *B. licheniformis* (31%
327 of total identified isolates), *Enterococcus* spp., (24% of the isolates), other *Bacillus* species
328 including *B. cereus* group, *B. coagulans* and other *Bacillus* species (11.6%) and other bacterial
329 species, including *Kurthia*, *Staphylococcus* spp. and *E. coli* (<10%) (Fig. 6).

330 The relative percentage of all bacterial species is shown in Fig. 7. For each of the
331 three sampling points, *B. licheniformis* was the predominant species. In overnight-stored skim
332 milk samples, the proportions of bacteria changed greatly compared with the unpasteurised
333 milk.

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334 There were 40 *Bacillus* isolates collected, of which 72.5% were *B. licheniformis*, 22.5%
335 were other *Bacillus* spp. and 5% were *B. cereus* group. Fig. 8 shows the percentage of *B.*
336 *licheniformis* identified by different microbial test methods in Trial 3 and 4 at varying sampling
337 points. In the unpasteurised milk, 50% of *B. licheniformis* were identified from the thermoduric
338 bacteria test, followed by 30% from the thermophilic spore test, and 10% from each of
339 mesophilic spore and thermophilic bacterial tests. In overnight-stored skim milk that was
340 pasteurised at 73.5 °C, 75% of *B. licheniformis* were from the thermophilic spore test, 13%
341 were from the mesophilic spore test and 12% were form the thermophilic bacterial test. In
342 90 °C heat treated overnight-stored skim milk, the percentage of *B. licheniformis* identified
343 from the thermophilic bacterial test increased to 46%, whereas from the thermophilic spore
344 test, the proportion decreased to 36%, compared with 73.5 °C pasteurised skim milk. The
345 mesophilic spore test contributed to 18% of identified *B. licheniformis* in 90 °C heat-treated
346 skim. The number of isolates identified as *B. cereus* group was too low for a statistical
347 analysis.
348
349 **4. Discussion**
350
351 To reduce spores during milk powder production, processors typically use multiple
352 sequential heating steps, with temperature combinations of 73.5 °C for 15 s followed by up to
353 125 °C for 15 s prior to evaporation being applied in the manufacture of high heat skim powder.
354 Application of this heat treatment, as a control heating process, resulted in reductions in the
355 five tested microbial groups, including spores, to undetectable levels. However, intensive heat
356 treatments also had a deleterious effect on native whey protein levels in milk with a low WPNI
357 value indicative of protein thermal damage and a high heat classification should the milk
358 transition to finished powder. Although the other heat treatments used in this study resulted in
359 WPNI values within the range of medium heat skim milk powder, the microbial reductions of
360 the experimental sequential heating temperatures were not comparable with the control
361 heating samples, although this does not necessarily indicate that the products exceed the
362 microbial specifications of a given dairy processor.

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363 A significant increase ($P < 0.05$) of thermophilic bacteria was observed with increasing
364 initial heat treatment temperature post overnight storage, while other microbial groups tested
365 were similar before and after overnight storage. This increase in thermophilic bacteria could be
366 attributed to the germination of spores after heat treatment followed by outgrowth of vegetative
367 cells during cold storage. The increased proportions of *B. licheniformis* identified using the
368 thermophilic bacteria method in overnight-stored skim showed that thermophilic bacteria
369 increased as the result of germination of *B. licheniformis* spores (Fig. 8). Such germination of
370 spore-forming bacteria after heat treatment has been described previously, resulting in higher
371 bacterial counts in heat treated materials (Júnior, Tamanini, de Oliveira, Ribeiro, & Beloti, 2018;
372 Martin et al., 2011). In another study on the effect of HTST pasteurisation on bacterial and
373 spore counts (Ranieri, Huck, Sonnen, Barbano, & Boor, 2009), there was also higher microbial
374 counts in 85.2 °C heat-treated milk than that of 72 °C pasteurised milk after 1 day of holding at
375 6 °C. In that study, *B. licheniformis* was the most commonly identified species one day
376 post-pasteurisation. Ranieri et al. (2009) concluded this was as a result of psychrotolerance of
377 this spore-forming species, whereby spores were activated by heating and germinated into
378 cells that multiplied during cold storage. The germinated cells no longer maintained the heat
379 resistance of the spore form and therefore could not be detected using the spore testing
380 method but were shown as an increase in thermophilic bacteria counts. Another hypothesis
381 which could be applied to the increased bacterial counts observed in higher temperature
382 treated milk post overnight storage could be linked to heat inactivation of naturally occurring
383 antimicrobial components, such as lactoperoxidase (Barrett, Grandison, & Lewis, 1999), or
384 from a change in competitive inhibition scenarios based on the microbial communities present
385 post heating at increasing thermal loads.

386 Cream is often considered a high microbial load milk fraction due to the enrichment of
387 bacteria post centrifugal separation, which can result in a TBC of up to 6 log cfu mL^{-1} in raw
388 cream (Rodarte, Zamora, Trujillo, & Juan, 2018). In this study, the cream was separated after
389 the first heat treatment, and the results of TBC, thermoduric bacteria and mesophilic spores
390 were lower than that in skim milk, while the thermophilic bacteria and thermophilic spores were
391 not significantly altered (Fig. 3). This result demonstrates that bacteria do not necessarily
392 concentrate in heat-treated cream, but can be found in relatively equal proportions in the skim

393 milk and the cream. Similar results were reported in another study in ohmic-heated and
394 conventional heat-treated skim milk and cream (Kim & Kang, 2015). It would be counter
395 intuitive to expect microorganisms to be preferentially enriched in the cream phase based on
396 their density, with equipment cleanliness throughout operational cycles likely a key factor in
397 maintaining low microbial loads in cream. In this study the microbial observations on the cream
398 stream are likely due to the fact that the separator bowl and discs were autoclaved to remove
399 microbial contamination prior to cream separation, coupled with the short run times which
400 would limit outgrowth during processing.

401 From three sampling points in Trials 3 and 4, 110 isolates were obtained with *B.*
402 *licheniformis* identified as the dominant species. *B. licheniformis* was identified from several
403 different spore tests employed, confirming its wide-ranging growth characteristics. The
404 prevalence of *B. licheniformis* has been reported frequently in dairy production chains, from
405 farm and manufacturing environments to finished products (Buehner, Anand, & Garcia, 2014;
406 Burgess et al., 2010; Gopal et al., 2015; Li et al., 2019). While not being considered a
407 pathogenic species, it has been associated with pathogenicity (Caamaño-Antelo et al., 2015)
408 and food poisoning incidents due to its toxin producing ability. Reduction of *B. licheniformis* in
409 unpasteurised milk, through awareness and hygiene measures on-farm, is a critical control
410 point to reduce total spore counts in downstream powder production (Gupta & Brightwell, 2017;
411 Kumari & Sarkar, 2014).

412 It should be noted that *Enterococcus* was also identified at 24% of total 16S-identified
413 isolates in this study, which indicated that heat-resistant *Enterococcus* were able to survive
414 heat treatment at 90 °C for 15 s. Heat-resistant *Enterococcus* which were classified as
415 thermoduric *Enterococcus*, were reported in some studies (McAuley, Gobius, Britz, & Craven,
416 2012). From these authors, the heat resistance of *Enterococcus* isolates is greatly variable,
417 with the D value varying from 0.3 to 5.0 min at 72 °C, with the most heat resistant isolates
418 withstanding 78 °C for 0.5 min. In this study, *Enterococci* isolates were identified after the heat
419 treatment at both 73.5 and 90 °C. Additionally, it has a strong ability to produce biofilms, which
420 can be challenging in dairy processing, whereby the heat resistance of entrapped microbes
421 are greater, as they are imbued with a protective effect (Cherif-Antar et al., 2016). In a
422 comparative study on microwave heat treatment, *Enterococcus faecalis* PCM896 was still

423 detectable after treatment at 72 °C with a D value at 1.54 min (Malinowska-Pańczyk et al.,
424 2019), which confirmed the common survival of *Enterococcus* after pasteurisation
425 temperatures.
426 Although this study endeavoured to recreate the heating conditions in a typical high
427 heat treated skim milk concentrate/powder process, the fact that the work was completed at
428 pilot plant scale, means that the results reflect indirect tubular heating technologies more so
429 than the direct steam injection/infusion systems used in commercial installations.
430 Notwithstanding, it is assumed that the observations from this study reflect the expected
431 microbiological behaviour in a typical dairy factory, applying the conditions used in this study.
432 The control sequential heating regime of 73.5/125 °C was able to successfully eliminate all
433 tested microbial groups with a concomitant low WPNI value. The complete inactivation of the
434 microflora present in the unpasteurised milk indicated that subsequent microbial populations in
435 finished products are likely attributed to post sequential heating process re-contamination
436 (Miller et al., 2015). Other temperature combinations maintained a higher WPNI and
437 inactivated thermophilic bacteria and thermophilic aerobic bacteria spores to low levels < 1.0
438 log cfu mL^{-1}; however, the results were not equivalent to the control samples. It is likely that to
439 achieve equivalent microbial reductions as a high heat treatment step the logical conclusion is
440 that a cascade approach utilising a non-thermal mechanical (bacterial clarifier) or size
441 (microfiltration) based approach in combination with a lower thermal load, would be required to
442 meet requirements focused on both food safety and nutritional quality (Wiegers, 2018).
443
444 **5. Conclusions**
445
446 The experimental sequential heat treatments used in this study resulted in greater
447 inactivation of TBC, thermoduric bacteria and mesophilic spores with increasing processing
448 temperature, however the microbial inactivation was not comparable with the control heating
449 process, which achieved a complete inactivation in all tested microorganisms, albeit with a
450 lower WPNI than any other heating combinations. Thermophilic spores were not influenced by
451 the experimental heating temperatures at any processing point sampled. However, post
452 overnight storage there was higher thermophilic bacteria outgrowth associated with a

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453 temperature induced germination of certain pyschrotrophic species present in milk.
454 Additionally, after the first heat treatment, there was no preferential enrichment of bacteria or
455 spores, post centrifugal cream separation. From the bacteria isolates obtained in
456 unpasteurised whole milk and overnight-stored skim heat-treated at 73.5 °C and 90 °C, the
457 results of 16S sequencing showed that *B. licheniformis*, *Enterococcus* spp. and other
458 *Bacillus* spp. were the most prevalent species.

459 This study provides valuable information on the impact of multiple sequential heating
460 conditions on microbial and spore inactivation relative to heat induced protein damage as
461 indicated by the WPNI. The outcome of this work also highlights the complexity of
462 microbiological behaviour during milk processing and offers insights for dairy manufacturers
463 seeking to reduce the thermal load they are applying to milk without compromising microbial
464 safety or nutritional quality in finished products.

465

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467

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Figure legends

Fig. 1. A schematic diagram of sequential heating process procedures and sampling points. Sampling points for identification of isolates by 16S sequencing are highlighted in orange.

Fig. 2. Boxplots of microbial results (cells and spores) in the unpasteurised milk used in the heating trials.

Fig. 3. Boxplots of microbial results (cells and spores) in whole milk (A), skim milk (B) and cream (C) after the first heat treatment at 73.5 (■), 80 (■), 85 (■) and 90 °C (■).

Fig. 4. Boxplots of microbial results (cells and spores) in overnight stored skim milk (B) compared with heat treated skim (A); heat treatment temperatures at 73.5 (■), 80 (■), 85 (■) and 90 °C (■).

Fig. 5. Boxplots of microbial results (cells and spores) in sequentially heat-treated skim milk depending on initial and second heat treatment temperature.

Fig. 6. Bar chart of 16S-identified bacteria genera/species for isolates recovered from different steps in the sequential heating trial on different species.

Fig. 7. Pie chart representation of 16S identified bacteria genera/species for isolates recovered from individual sampling points in Trial 3 and Trial 4.

Fig. 8. Pie chart representation of 16S identified *B. licheniformis* distribution percentage from various test methods at individual sampling points.

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1 **Table 1**

2

3 Details of the methods used for microbial and spore enumeration. [a]

4

Test #	Name	Heat treatment		Plate incubation			Agar medium	Reference
		temperature (°C)	time (min)	atmosphere	temperature (°C)	time (days)		
1	TBC	none	none	aerobic	30	3	TSA|	IDF 100B:1991
2	Thermoduric bacteria	63.5	35	aerobic	30	2	TSA	Wehr & Frank (2004)
3	Thermophilic bacteria	none	none	aerobic	55	2	TSA	ISO/TS 27265:2009
4	Presumptive *B. cereus*	none	none	aerobic	30	2	BACARA	FDA BAM; ISO 7932:2004
5	Mesophilic aerobic bacterial spores	80	10	aerobic	30	3	Skim milk PCA	Wehr & Frank (2004)
6	Thermophilic aerobic bacterial spores	80	10	aerobic	55	2	Skim milk PCA	Wehr & Frank (2004)

5

6 [a] Abbreviations are: TBC, total bacterial count; TSA: Tryptic soy agar, PCA, plate count agar

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Table 2

Whey protein nitrogen index (WPNI) with different heat treatment combinations. [a]

Sample	Treatment	Average
Unpasteurised Skim Milk	No heat	8.01 ± 0.88
First heat treatment		
Control	73.5 °C × 15 s	7.72 ± 0.43
Experimental	80 °C × 15 s	7.22 ± 0.37
Experimental	85 °C × 15 s	6.52 ± 0.51
Experimental	90 °C ×15 s	5.36 ± 0.60
Second heat treatment		
Control	(73.5) 125 °C × 15 s	1.82 ± 0.90
Experimental	(80) 80 °C × 15 s	6.89 ± 0.45
Experimental	(80) 85 °C × 15 s	6.45 ± 0.73
Experimental	(80) 90 °C × 15 s	5.38 ± 0.72
Experimental	(85) 80 °C × 15 s	6.08 ± 0.61
Experimental	(85) 85 °C × 15 s	5.23 ± 0.52
Experimental	(85) 90 °C × 15 s	4.45 ± 0.67
Experimental	(90) 80 °C × 15 s	5.26 ± 0.88
Experimental	(90) 85 °C × 15 s	4.21 ± 0.80
Experimental	(90) 90 °C × 15 s	3.56 ± 1.22

[a] Values for the average are means ± std deviation; duplicate samples were tested in each of four trials, i.e., the average of 8 values of the same sampling point. For the second heat treatment values, the first heat treatment temperature is given in parentheses.

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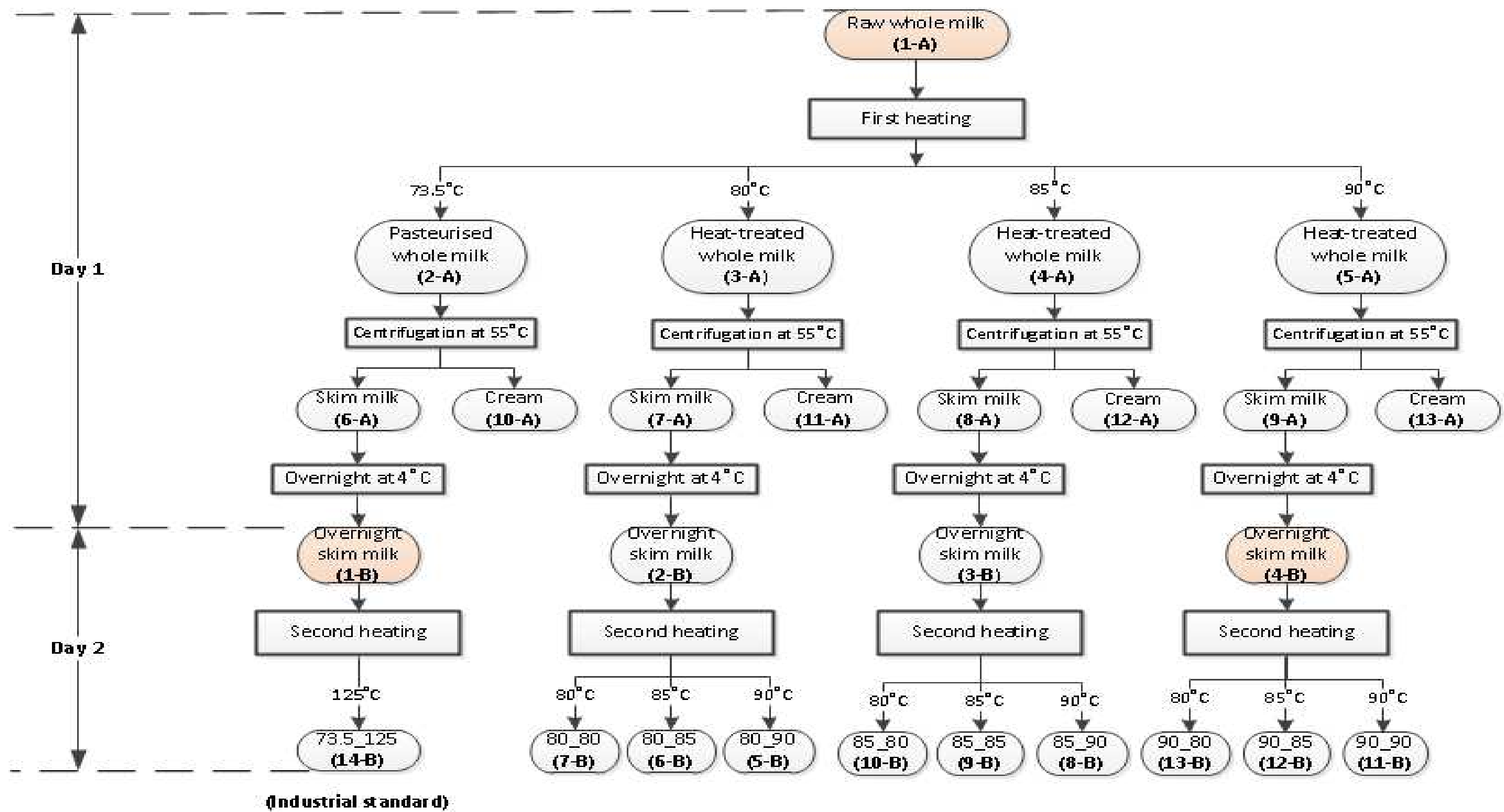

Figure 1.

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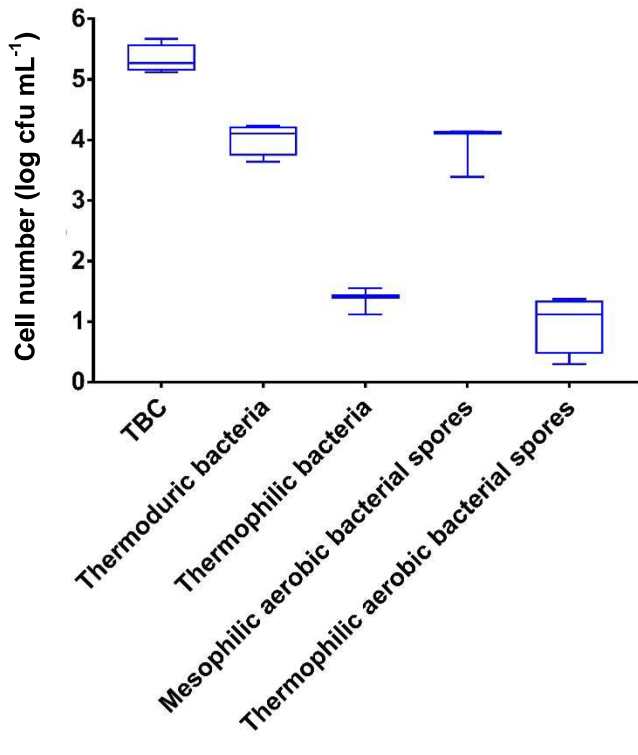

Figure 2.

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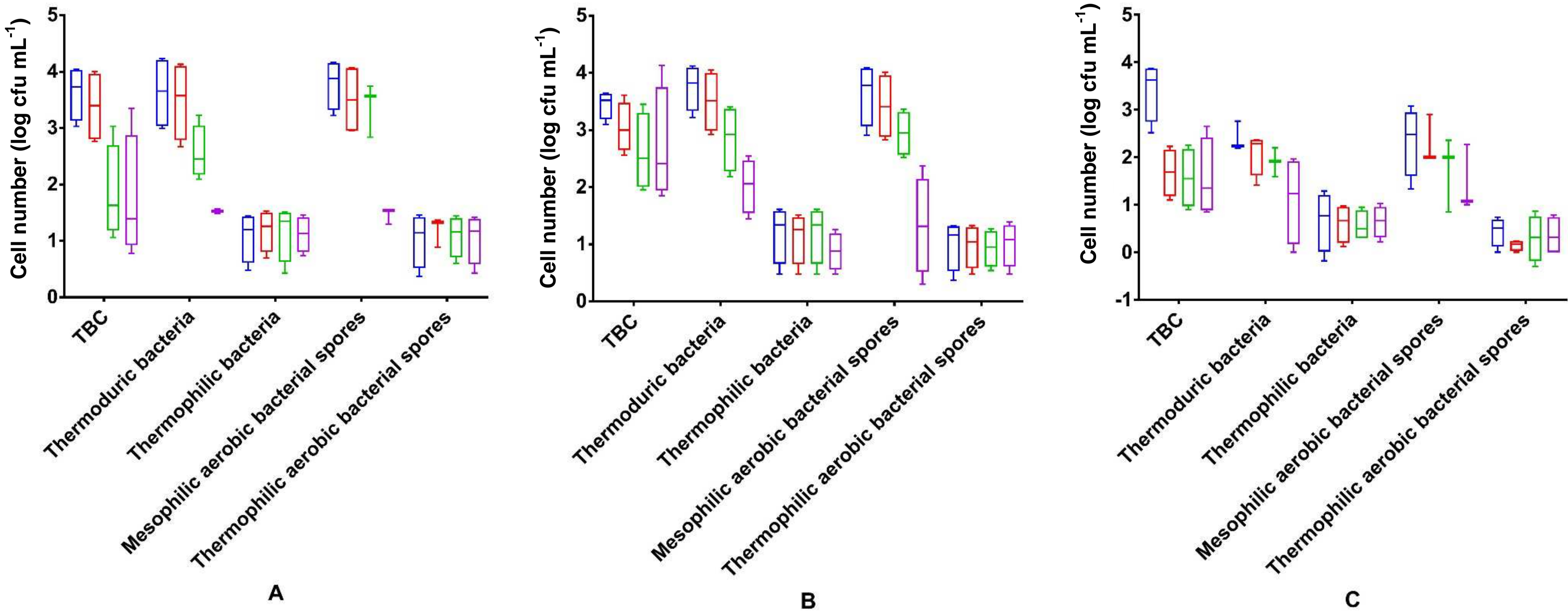

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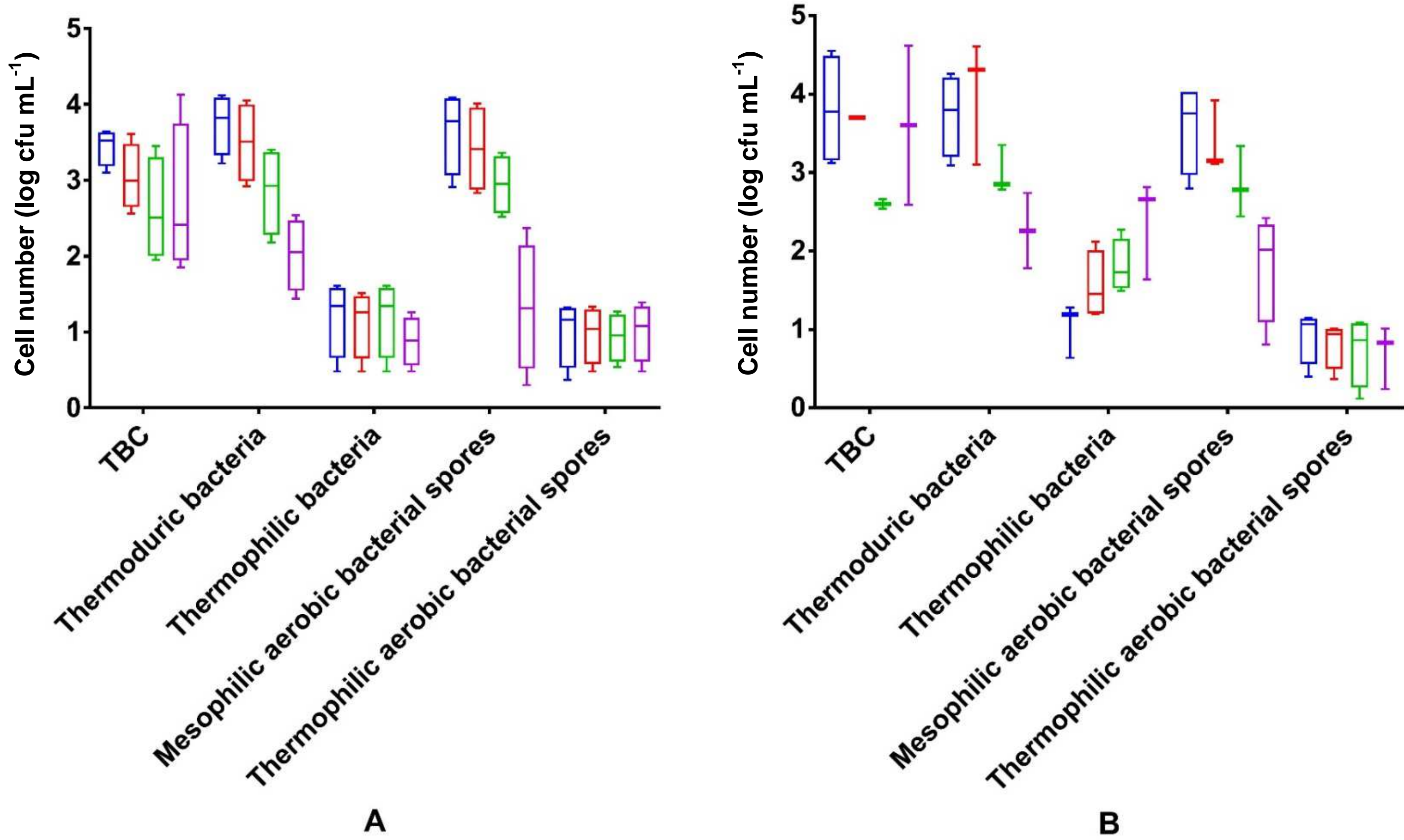

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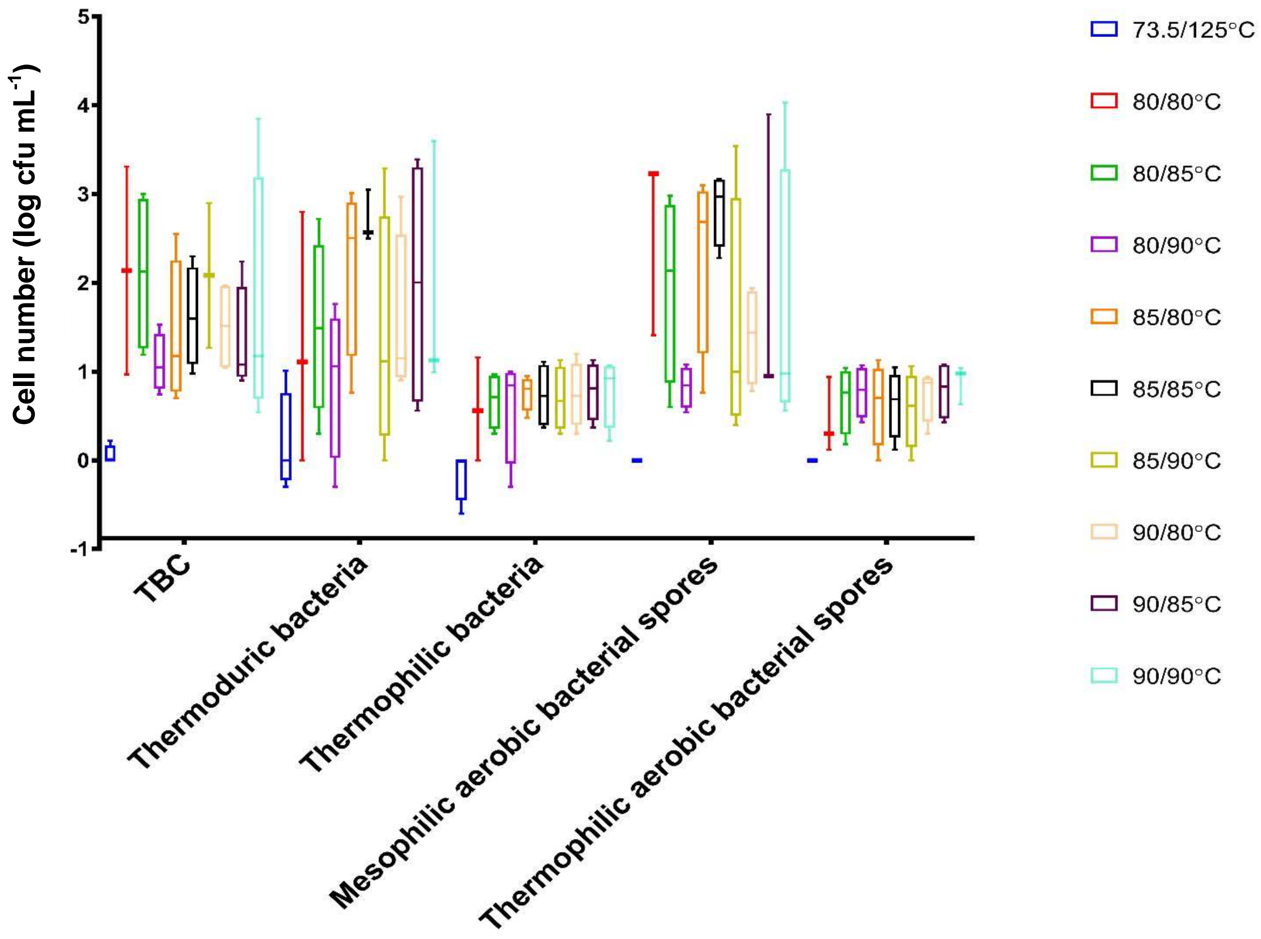

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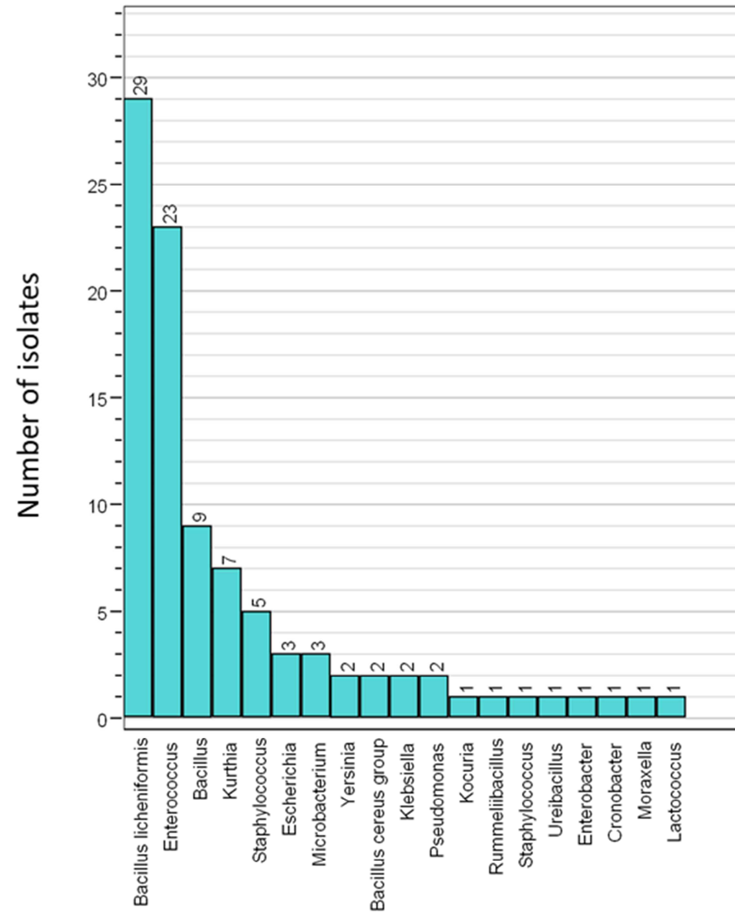

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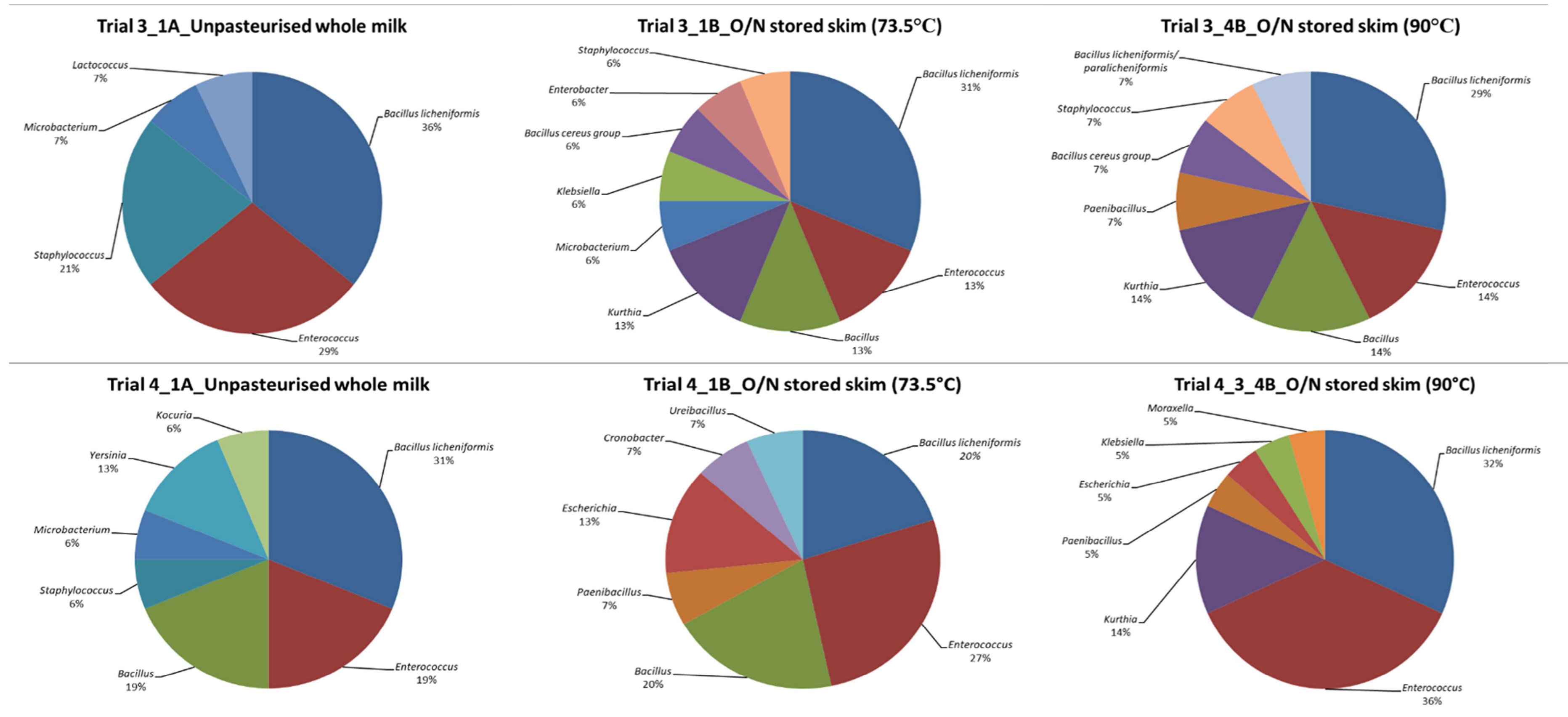

Figure 7

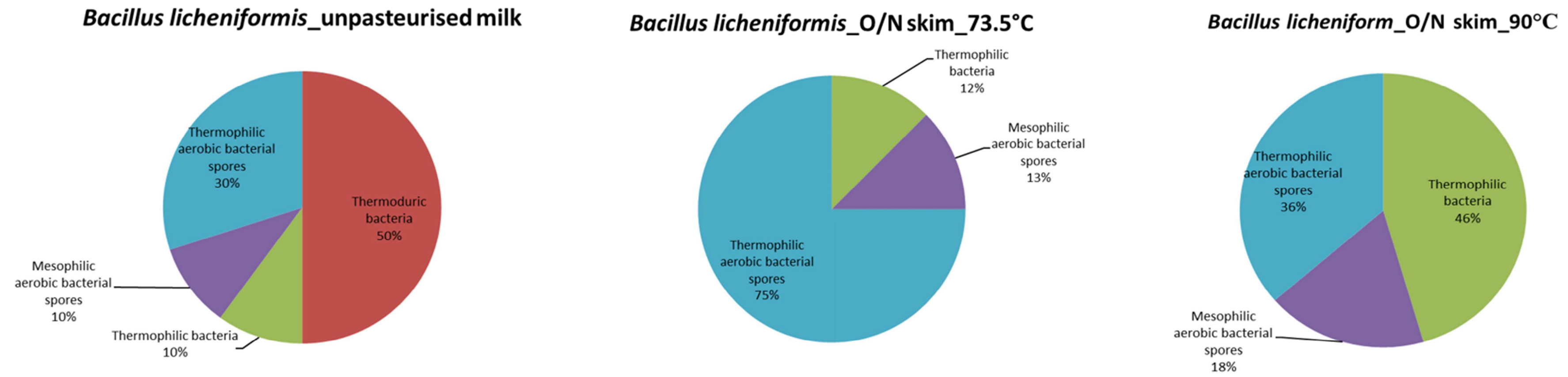

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Figure 8