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Impact of High Hydrostatic Pressure on the stability of lytic bacteriophage cocktail Salmonelex™ towards potential application on *Salmonella* inactivation

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Abstract

This work consisted in the first comprehensive study in which the potential to exploit the *Salmonella* lytic bacteriophages' cocktail, Salmonelex™, in association with high hydrostatic pressure (HHP) towards potential application in egg matrices decontamination was evaluated. The impact of HHP (200-600 MPa) on the bacteriophages' viability pointed out a stability in the range of 200 to 400 MPa. From 400 MPa onwards, the inactivation was potentiated by an increase in the pressure magnitude, being matrix dependent. Salmonelex™ possessed a prominent baroresistance, requiring 600 MPa to completely lose its infectivity. Egg yolk presented the highest baroprotective effect, followed by whole egg and egg white. Transmission electron microscopy unveiled that 500 and 600 MPa elicited a detrimental impact on the bacteriophages' structural integrity. It was noteworthy the barotolerance (200-300 MPa) of Salmonelex™, previously exposed to different pH conditions (5-9), which proved not to undermine its infectivity. Regarding the influence of ovalbumin, lysozyme, L-α-phosphatidylcholine, palmitic and oleic acids on the mild HHP-induced inactivation of Salmonelex™, a baroprotective effect was observed, particularly conferred by those compounds comprising egg yolk. The promising results highlighted the feasibility of combining Salmonelex™ as an adjuvant to mild HHP processing of egg matrices.

Keywords

High Hydrostatic Pressure (HHP), *Salmonella*, Bacteriophage Salmonelex™, Egg, matrix protection

1. Introduction

Bacterio(phages) are viruses that specifically infect bacterial cells and present a narrow spectrum towards a particular bacterial species (Loc-Carrillo & Abedon, 2011). The incorporation of lytic phages in food systems as a biocontrol approach is an emerging field of study. It has been argued that these natural antimicrobial agents do not promote alterations in the nutritional and organoleptic properties of foods, presenting a scarce impact on the endogenous microbiota, which represent prominent advantages of their application (Hagens & Offerhaus, 2008; Perera, Abuladze, Li, Woolston, & Sulakvelidze, 2015). Nevertheless, physicochemical factors, namely pH, temperature, and osmotic pressure (Jończyk, Kłak, Międzybrodzki, & Górski, 2011), along with food components and processing technologies, influence the stability of phages. Hence, all factors must be considered when seeking to integrate a bacteriophage in an inherently complex system such as a food product towards its decontamination or preservation. Salmonelex™ is an example of a cocktail of lytic phages – S16 and Felix O1-like phage (FO1a), belonging to the *Myoviridae* family – generally recognized as safe (GRAS) and approved in the US, Australia, and New Zealand as a biocontrol agent targeting *Salmonella* in foodstuffs, namely meat and poultry products (FSANZ, 2016; U.S FDA, 2016). The application of Salmonelex™ was previously investigated in fresh-cut lettuce (Oliveira, Abadias, Colás-Medà, Usall, & Viñas, 2015) and ground meat (Grant, Parveen, Schwarz, Hashem, & Vimini, 2017; Yeh et al., 2017), being documented a *Salmonella* population inactivation of *ca.* 1 logarithmic cycle.

The quest for alternative biological approaches to guarantee the safety of food products has been addressed, namely the exploitation of multi-hurdle technologies based on the association of lytic bacteriophages with high hydrostatic pressure (HHP) to enhance the inactivation of the target bacterium. Despite the promising bactericidal effect attained

with HHP-phage systems, the phages´ stability (i.e., bioactivity/infectivity) under high pressure environment is highlighted as the first stage to be addressed in the implementation of those processes (Ahmadi, Anany, Walking-Ribeiro, & Griffiths, 2015; Komora et al., 2020; Tabla et al., 2012). Moreover, these bio-engineered systems are claimed to be a more energy-efficient, environmental-friendly, minimally processing option in comparison to the conventional thermal processes.

High hydrostatic pressure is a non-thermal processing technology, operating at pressure magnitudes between 100 and 1,000 MPa, eliciting a minimal impact on the nutritional and sensorial features of foods, maintaining the structures of amino acids, vitamins and elements of taste and aroma (Avelar, Vicente, Saraiva, & Rodrigues, 2021; Pereira & Vicente, 2010).

The purpose of the present study was to evaluate the feasibility of combining *Salmonella* lytic bacteriophages' cocktail (Salmonelex^{TM}) as an adjuvant to mild high pressure processing of whole egg and its components (egg white and egg yolk); the factors which could affect this process and the phage's stability (i.e., pressure magnitude, individual egg compounds, pH range, lytic spectrum), as well as mechanistic insights on Salmonelex^{TM} inactivation by high pressure, are first unraveled.

2. Materials and methods

2.1 *Microorganisms and inoculum preparation*

Salmonella enterica serovar Typhimurium DT104 was utilized as the propagating host (Marti et al., 2013; O'Flynn, Coffey, Fitzgerald, & Ross, 2006). In order to determine the bacteriophage cocktail lytic spectrum, eight additional strains of *S. enterica* deposited in the Culture Collection of CBQF were selected (Table 1).

The bacterial stock cultures were streaked onto Tryptic Soy Agar (TSA, Biokar Diagnostics, France) supplemented with 6 g L^{-1} of yeast extract (Biokar Diagnostics) (TSAYE) and incubated at 37 ºC for approximately 24 h. Subsequently, a single colony was inoculated into 5 mL of Tryptic Soy Broth (TSB, Biokar Diagnostics) (TSBYE), incubated overnight at 37 ºC, and sub-cultured (1% v/v) into fresh TSBYE under the abovementioned conditions.

The commercial bacteriophage cocktail Salmonelex™ (Micreos Food Safety, The Netherlands) presented an initial titre of 11 log plaque forming units (PFU) mL^{-1}, and was stored and maintained in the original saline stock solution, at 4 ºC, until further use.

2.1.1 Determination of the bacteriophage titre

The phage titre was determined by the double-layer plaque assay as previously described by Kropinski, Mazzocco, Waddell, Lingohr, & Johnson (2009). In brief, the phage samples were decimal serially diluted in 0.1 M phosphate buffered saline solution, pH 7.4 (PBS; VWR Chemicals, USA), added (100 µL) to the early stationary bacterial culture (300 µL) and afterwards incorporated into TSBYE containing 0.7% (w/v) of bacteriological agar (Pronadisa, Spain). This mixture constituted the overlay, which was subsequently poured onto a bottom agar plate, TSAYE (underlay). The plates were gently swirled and incubated overnight at 37 ºC. Plaques formed by Salmonelex™ infection of *S.* Typhimurium DT104 were enumerated and the titre of the phage expressed as PFU mL^{-1} determined.

2.2 Impact of HHP on the stability/infectivity of Salmonelex™ in egg matrices

In order to investigate the pressure stability of the bacteriophage in egg matrices, samples of egg white (EW), egg yolk (EY) and liquid whole egg (LWE) were inoculated with Salmonelex™ to a final titre of 8 $\log_{10}$ PFU g^{-1}, followed by homogenous distribution of

the inoculum through thorough agitation. Prior to each challenge, detection of *Salmonella* spp. was performed according to the ISO 6579-1/2017.

One millilitre aliquots of each egg component were transferred to pressurization microtubes (Microtube PE 0.5 mL Beckmann) using a sterile syringe, and double vacuum sealed in low permeability polyamide-polyethylene bags (PA/PE-90, Penta Ibérica Lda., Portugal). Samples were loaded in a high pressure equipment (Hiperbaric 55, Spain), utilizing water as the pressure-transmitting fluid and a pressurization rate of approximately 14 MPa s^{-1}, whilst depressurization occurred in less than 3s. The pressure treatment was set within the range of 200-600 MPa, for 5 min at 10 ºC. Afterwards, the samples were immediately cooled in an ice-water bath and then transferred to refrigerated storage (4 ºC) until being analysed. Non-pressure treated phage samples (0.1 MPa, 4 ºC) in both PBS and egg matrices were used as controls.

The HHP parameters (pressurization rate, holding time, temperature and pressure magnitude) were selected based on previous studies concerning the pressure stability of lytic phages, being similar to those commercially applied (Komora et al., 2018; Tomasula et al., 2014).

Phage titres were determined by the double-layer plaque assay as previously detailed (2.1.1). Three independent experiments were performed.

2.3 Influence of pH and egg compounds on pressure stability of Salmonelex™

The impact of pH on Salmonelex™ stability upon exposure to a pressure processing of 200 and 300 MPa (5 min, 10 ºC) was also assessed. For this purpose, sodium citrate (pH 5), sodium acetate (pH 6), potassium phosphate (pH 7 and 8) and sodium carbonate-bicarbonate (pH 9 and 10) buffers (Sigma-Aldrich, Germany) at a final concentration of

10 mM were initially prepared. In order to evaluate the effect of egg compounds, the following solutions were prepared: (i) 3.5% (w/v) of lysozyme (Sigma-Aldrich); (ii) 54% (w/v) albumin (Merck, Germany); (iii) lysozyme (3.5% (w/v)) and albumin (54% (w/v)) (Abeyrathne, Lee, & Ahn, 2013), with a final pH value of 8.0; (iv) 8% (w/v) L-α-Phosphatidylcholine (L-α- lecithin from egg yolk) (Sigma-Aldrich); (v) 7.5% (w/v) palmitic acid (Sigma-Aldrich); (vi) 7.5% (v/v) oleic acid (Sigma-Aldrich) (Walczak, Bocian, Kowalkowski, Trziszka, & Buszewski, 2017).

Afterwards, the solutions were inoculated with 8 $\log_{10}$ PFU mL^{-1} of Salmonelex^{TM}, transferred to microtubes, following the same protocol aforementioned (section 2.3), and submitted to 200 and 300 MPa (10 ºC, 5 min). Controls for each sample were maintained at atmospheric pressure (0.1 MPa, 4 ºC). Three independent experiments were performed.

2.4 Lytic spectra and efficiency of plating (EOP) determination

The lytic activity of Salmonelex^{TM}, artificially inoculated in the egg matrices and PBS (8 $\log_{10}$ PFU mL^{-1}) and submitted to 200 MPa and 300 MPa (5 min, 10 ºC), was evaluated against 7 *S. enterica* strains, representatives of the most prominent serovars (Table 1) through double-layer plaque assay, as described in section 2.2.1, and the efficiency of plating was determined (Barros et al., 2019). The relative EOP was calculated as the ratio of the phage titre (PFU mL^{-1}) of each target host strain and that of the reference propagating host. Three independent experiments were performed.

2.5 Effect of HHP exposure on the morphology and structural integrity of Salmonelex^{TM}

The morphology and structural integrity of the two *Salmonella*-specific bacteriophages, S16 and FO1a, were evaluated through transmission electron microscopy (TEM), as previously described by Komora et al. (2018), in order to better understand the pressure stability of Salmonelex^{TM} subjected to HHP treatments. Briefly, non- and pressure-treated

phage suspensions were mounted on Formvar/carbon film-coated 300 mesh nickel grids (Electron Microscopy Sciences, USA). The excess liquid was removed with filter paper, and 10 µL of 1% uranyl acetate (BDH, UK) was added onto the grids. Visualization was carried out on a JEOL JEM 1400 TEM at 120 kV (Japan). Images were digitally recorded using a CCD digital camera (Orious 1100W, Japan).

2.6 Statistical analysis

One-way analysis of variance (ANOVA), with Tukey's test as post-hoc for multiple comparisons, was used to assess differences between samples, after homoscedasticity and normality of data were verified (Levene's and Shapiro-Wilk tests, respectively). The significance level assumed was 5%.

3. Results and discussion

3.1 Evaluation of the impact of HHP on the stability of Salmonelex™ in egg matrices

The HHP-induced inactivation of Salmonelex™, experimentally inoculated in egg white, egg yolk, and liquid whole egg, at different pressure magnitudes (200-600 MPa), is depicted in Figure 1.

It was possible to observe that in the range of 200 to 300 MPa, no significant differences ($P > 0.05$) were identified between the bacteriophage logarithmic reductions, *ca*. 1 $\log_{10}$ cycle. Upon exposure to 400 MPa, Salmonelex™ demonstrated capability to endure HHP when incorporated in egg components (phage titre reductions of *ca*. 1 $\log_{10}$ cycle in egg yolk and liquid whole egg), albeit to a lower extent concerning egg white (1.7 $\log_{10}$ cycles), comparatively to PBS suspension (3.9 $\log_{10}$ cycles). The observed matrix-dependent pressure susceptibility of the bacteriophage was found to be more pronounced

191 at 500 MPa, with phage titre reductions ranging from 0.8 to 7 log cycles - in the latter
192 case, PBS, to values below the detection limit of the enumeration technique (1 $\log_{10}$ PFU
193 mL^{-1}) - being attained. Whilst in egg yolk the barotolerance of the virion particles was
194 maintained (0.8 $\log_{10}$ cycles), a higher inactivation was obtained in liquid whole egg and
195 egg white (2.2 and 3.4 $\log_{10}$ cycles, respectively). As a whole, the results herein presented
196 pointed to a prominent baroresistance of Salmonelex^{TM} up to 500 MPa. When submitted
197 to the highest pressure (600 MPa), the phage viability/infectivity was completely
198 impaired in all the assayed matrices.
199 The bacteriophage stability towards high pressure processing appeared to be related with
200 the food system environment (physicochemical properties, namely salinity, fat and
201 protein content, pH) along with the matrix physical state (García-Anaya et al., 2020;
202 Komora et al., 2018).
203 This correlation was previously documented by Sharma et al. (2008), who observed that
204 coliphages (T4; phiX174; MS2) susceptibility to HHP lessened when attached to meat
205 sausage in comparison to a liquid suspension.
206 The emulsifying ability, along with the higher viscosity, of the pseudoplastic non-
207 *Newtonian* fluid, egg yolk (Kumbár, Strnková, Nedomová, & Buchar, 2015), may
208 contribute to explain the notable baroprotective effect observed, hampering the impact of
209 HHP on phage´s protein denaturation and consequent structural damage. Concerning the
210 colloidal structure of egg white, albeit at mild HHP conferred a shielding effect, the
211 processing at the highest pressure magnitudes may elicit the proteins unfolding - leading
212 to a loss of the solubility, owing to the formation of small aggregates (Van der Plancken,
213 Van Loey, & Hendrickx, 2007) - and hence a higher degree of virus susceptibility to HHP
214 inactivation was attained.

This hypothesis was corroborated by rheological analysis (data not shown), in which it was observed that the viscosity of the matrices increased concomitantly with HHP magnitude. Egg yolk, egg white and liquid whole egg complex viscosity ranged from 0.80, 0.10 and 0.11 Pa s at 200 MPa, respectively, to 7.30, 0.20, 0.23 at 400 MPa and 31.5, 0.46 and 0.42 Pa s at 500 MPa. The high pressure processing from 400 MPa onwards induced proteins denaturation, resulting in aggregation and coagulation, leading to a more compact, rigid gel-like structure. This was more prominent in egg yolk, which presented a higher elastic modulus (31.4-780.7 Pa, 400-600 MPa) in comparison to egg white (1.2-260.0 Pa). The above described viscoelastic profiles were in agreement with the previous findings of Lee, Heinz, & Knorr (2001) and Ahmed, Ramaswamy, Alli, & Ngadi (2003). The higher viscosity along with the enhanced stiffness attained at higher pressures resulted in a noticeable shielding effect towards HHP induced phage damage, particularly in egg yolk, and to a lower extent in liquid whole egg and egg white, respectively. This matrix-provided baroprotection was translated in a higher phage viability in comparison to PBS. A putative phage entrapment/immobilization conferred by the egg yolk-based emulsions (ascribable to the phospholipidic content) may likely positively impact the maintenance of the virion particle integrity and hence bactericidal efficacy. In fact, the higher lipidic content of egg yolk (32.6%) along with the lower water percentage (*ca.* 50%) in comparison to egg white (0.03 and 87%, respectively) (Yamamoto, Juneja, Hatta, & Kim, 1996) may contribute to the observed baroprotective effect. Liquid whole egg encompasses 58% egg white (albumen) and 31% yolk (Livney, 2012), and hence the shielding effect towards HHP inactivation is a consequence of such proportion/ratio. In this sense, at 500 MPa, liquid whole egg conferred an intermediate degree of phage protection amongst the egg matrices.

To the best of our knowledge, this is the first study documenting the impact of high hydrostatic pressure processing on the stability/infectivity of Salmonelex™ in egg matrices. Nonetheless, some studies evaluated the effect of the HHP in the inactivation of other bacteriophages, and a putative protective role of food systems, namely, on temperate lactococcal bacteriophages (c2, P001 and P008) (Moroni, Jean, Autret, & Fliss, 2002; Müller-Merbach, Rauscher, & Hinrichs, 2005), and few have addressed the lytic/virulent phages, amongst which a report of the listeriophage P100 (Komora et al., 2018). With respect to the latter and concerning mild pressures, the results obtained in the present study are consistent with those documented by Komora et al. (2018), in which pressures of 200 and 300 MPa (5 min, 10 ºC) did not elicit substantial viability loss of the bacteriophage P100 in PBS and in a heterogeneous spectrum of food matrices with distinct physicochemical and rheological features, namely fermented sausage, semi-soft cheese, and whole milk. The authors stated that processing at 400 MPa promoted the complete inactivation of the phage (to values below the detection limit) irrespective of the matrix. This fact highlighted the notable barotolerance of Salmonelex™, since the bacteriophage cocktail required 500 MPa to completely lose its infectivity once in PBS, while when incorporated in egg components such abolishment was only observed at 600 MPa.

The influence of the initial Salmonelex™ load on the inactivation triggered by mild pressure (200 and 300 MPa) exposure was also evaluated in the current work (Figure 1), indicating that there were no significant differences ($P > 0.05$) in HHP impact whether the phage titre was 8 or 11 $\log_{10}$ PFU mL^{-1}. This result corroborated the previously documented by Komora et al. (2018) concerning P100 titre ranging from 6 to 8 $\log_{10}$ PFU mL^{-1}, in which no correlation was found between the virion particles load and the extent of HHP-induced inactivation at 300 MPa. Nonetheless, with respect to higher pressures

264 (400 and 500 MPa), the lowest initial Salmonelex™ titre resulted in a higher HHP
265 detrimental impact. Moroni et al. (2002), evaluating the efficiency of a dynamic pressure
266 process on the inactivation of c2 temperate phage in PBS, stated that the efficacy of the
267 treatment is affected by the initial phage titre - the higher the initial load, the less effective
268 the process becomes.
269 Müller-Merbach et al. (2005) investigated the effect of pressures in the range of 300 to
270 600 MPa on the viability of temperate phages, namely P001 and P008 (9 $\log_{10}$ PFU mL^{-1})
271 in liquid suspension. In agreement with the results herein documented, and concerning
272 the phage P001, processing at 300 MPa elicited a slight inactivation, whilst higher
273 pressures, such as 450 and 600 MPa, resulted in an inactivation of 1 and 5 $\log_{10}$ cycles,
274 respectively. This indicates a higher baroresistance of phage P001, in comparison with
275 Salmonelex™, since at the highest pressure (600 MPa) the observed reduction was lower.
276 Regarding the phage P008, pressures of 550 and 600 MPa originated reductions of 2 and
277 5 $\log_{10}$ cycles, respectively, after 2 hours of processing, also demonstrating higher
278 tolerance to pressure than Salmonelex™.
279 The pressure stability of bacteriophages submitted to HHP processing is heterogeneous.
280 Indeed, studies conducted by Guan et al. (2007, 2006), in which six coliphages (8 $\log_{10}$
281 PFU mL^{-1}) were processed at 600 MPa (5 min, 21 ºC), demonstrated a dissimilar pressure
282 response. The authors reported an inactivation of <1 $\log_{10}$ cycle for one of the coliphages
283 (the most baroresistant), <4 $\log_{10}$ cycles for three of the viral particles and >7 $\log_{10}$ cycles
284 for the remaining two phages (presenting themselves as the most barosensitive). The same
285 heterogeneity was observed for lower pressures (350-550 MPa). These results proved that
286 HHP impact is phage-specific and should therefore be assessed each time a different
287 phage is intended to be used.

3.2 Impact of the principal egg compounds on pressure stability of Salmonelex™

The effect of the pH (a factor which may hamper phage effectiveness) on the pressure stability of Salmonelex™ was evaluated, with the purpose of mimicking the alkalinity/acidity of distinct food systems, namely egg yolk (pH 6.4) and egg white (pH 7-9) (U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition, 1992), in which the bacteriophage is intended to be incorporated. Pressure magnitudes of 200 and 300 MPa were selected to conduct these experiments owing to the fact that within this HHP range, a scarce impact on the bacteriophage viability, whether inoculated in PBS or egg components, was observed. Moreover, considering the aforementioned viscoelastic profiles of the egg matrices (section 3.1), no relevant alterations were developed.

In the range of pH values of 5 to 8, the bacteriophage cocktail underwent a maximum of 1 $\log_{10}$ cycle reduction following HHP processing (200 MPa) and no significant differences ($P > 0.05$) were observed (Figure 2). Nonetheless, a pH value of 9, which may correspond to that of egg white of an older egg (8.8) (U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition, 1992), promoted a slightly higher viability loss (1.7 $\log_{10}$ cycles) upon pressure treatment. Concerning processing at 300 MPa, similar inactivation values were obtained. It was noteworthy the baroresistance of Salmonelex™, previously exposed to different pH values, particularly those mimicking the egg components, which proved not to undermine its stability. These findings were in accordance with the previously reported concerning the bacteriophages LPSE1 (*Siphoviridae*) (Huang et al., 2018) and SE07 (*Podoviridae*) (Thung et al., 2017) targeting *Salmonella* spp. which maintained their stability at pH values intervals of 4 to 11 and 4 to 12, respectively.

312 The bacteriophage demonstrated capability to endure the hostile environment of the egg
313 inner milieu, namely the harsh physicochemical features (Baron et al., 2016). Moreover,
314 the milieu, particularly some specific egg proteins and lipids, appeared to shield the phage
315 from HHP. In this sense, we sought to investigate the influence of the principal egg white
316 (lysozyme and albumin) and egg yolk compounds (phosphatidylcholine (L-α- lecithin
317 from egg yolk), palmitic and oleic acids) on the Salmonelex^{TM} inactivation by HHP
318 (Figure 2). The individual effect of lysozyme and albumin originated similar reductions
319 of 1.1 and 1.3 $\log_{10}$ cycles ($P < 0.05$), correspondingly, whilst their association with the
320 alkaline pH condition (pH 8) elicited a slight inactivation of 0.3 $\log_{10}$ cycles. The scarce
321 impact when the three components were combined, in comparison to the inactivation
322 attained using lysozyme and albumin, independently, may have been a consequence of
323 the alkaline pH. Speroni et al. (2005) investigating the effect of high-pressure on Low-
324 Density Lipoproteins (LDL) from hen egg yolk, found that, particularly at pH 8, the
325 pressure processing enhanced the protein aggregation and denaturation.
326 Likewise, the formation of aggregates was documented by Quirós, Chichón, Recio, &
327 López-Fandiño (2007) in ovalbumin, when submitted to HHP (200-400 MPa), also at pH
328 8. The pressure magnitude at which bovine serum albumin (BSA) forms a gel has been
329 demonstrated to be lower when the protein solution is alkaline, and results in more
330 compact gels (De Maria, Ferrari, & Maresca, 2015). In accordance with these findings,
331 one may hypothesize that the denaturation, aggregation and, eventually, gel formation of
332 albumin may have been triggered by the alkaline pH (8), which in turn could have
333 hindered lysozyme activity towards Salmonelex^{TM}. These findings highlighted a putative
334 protective effect towards the bacteriophage stability provided by the food matrix (egg).
335 L-α-Phosphatidylcholine (L-α- lecithin from egg yolk), which represents 71.1% of the
336 phospholipid fraction of egg yolk lipids (Blesso, 2015), provided a prominent shielding

337 effect, eliciting a low degree of phage inactivation (0.24 $\log_{10}$ cycles) (Figure 2). One
338 may hypothesize that interactions may be established between the polar head groups of
339 the phospholipids, which may lead to the viral particle immobilization in the inner moiety
340 of this vesicle-like structure. This phospholipid has been employed in the entrapment of
341 bacteriophages in liposome-based formulations via encapsulation enhancing its stability,
342 availability, and hence efficacy (Chhibber, Kaur, & Kaur, 2018).
343 Phosphatidylcholine is mainly composed of palmitic acid (16:0) and oleic acid (18:1), the
344 major saturated and unsaturated fatty acids in egg yolk, which were individually assessed.
345 In the case of the mentioned fatty acids and given the considerable phage infectivity
346 maintenance (0.26 $\log_{10}$ cycles), it is feasible to speculate the interaction between the
347 hydrophobic acyl-chains, surrounded by the polar heads, forming a micelle-like structure,
348 which may entrap the virion particles in the inner cavity (Berg, Tymoczko, & Stryer,
349 2002; Blesso, 2015).
350

351 *3.3 Lytic spectrum and EOP determination*

352 A panel encompassing eight *S. enterica* strains (including reference strains) was utilized
353 to determine the lytic activity of Salmonelex^{TM} inoculated in egg components and
354 submitted to 200 and 300 MPa. The two-phages cocktail possessed a broad lytic
355 spectrum, being able to infect five out of the eight assayed strains (belonging to 7 serovars
356 of *S. enterica*), displaying a high EOP value (Table 1), irrespective of the matrix and
357 pressure magnitude. This is of utmost importance concerning *S.* Enteritidis, *S.*
358 Typhimurium, and the monophasic variant, *S.* Typhimurium 1,4,[5],12:i:- since,
359 according to the European Food Safety Authority & European Centre for Disease
360 Prevention and Control (2021), those serovars were the most commonly reported in
361 Europe, representing 78.3 % of the confirmed human salmonellosis cases in 2019.

362 Moreover, eggs and egg-derived products were responsible for most of the documented
363 outbreaks, with *S.* Enteritidis being the most reported serovar.
364 Noticeably, the bacteriophage (8 $\log_{10}$ PFU mL^{-1}) incorporated in the different matrices
365 was not capable to infect *S.* Senfteberg ATCC 43845, *S.* Infantis M2016, and *S.* Derby.
366 Nonetheless, at a higher phage titre (11 $\log_{10}$ PFU mL^{-1}), in saline buffer, these three
367 serovars were susceptible to the bacteriophage cocktail. The bacteriophage S16 (whose
368 receptor molecule is LPS) has been described as possessing a broader host range of
369 *Salmonella* strains than FO1 (which specifically recognizes OmpC) (Marti et al., 2013).
370 Fong et al. (2019) documented, concerning FO1 (9 $\log_{10}$ PFU mL^{-1}) lytic spectrum, an
371 absence of susceptibility of *S.* Senftenberg FSL S5-658 and S270, whilst *S.* Braenderup
372 FSL S5-373 and *S.* Infantis S198 presented scarce and intermediate sensitivity,
373 respectively.
374

375 *3.4 Analysis of the mechanism underlying Salmonelex™ HHP – induced inactivation*

376 The knowledge concerning bacteriophages' inactivation through HHP and the
377 mechanism(s) underlying their viability loss is scarce. Given the simplicity of the
378 macromolecular structure of bacteriophages, which are units predominantly composed of
379 nucleic acids and coat proteins, termed capsids, it is feasible to investigate their
380 inactivation process. In this sense, in order to acquire mechanistic insights into
381 Salmonelex™ infectivity loss, TEM was performed to analyse the structural integrity and
382 the morphological features of the bacteriophage cocktail before and after being submitted
383 to high-pressure treatments within the range of 200-600 MPa.
384 Transmission electron microscopy micrographs of non- and pressure-treated
385 Salmonelex™, a cocktail of lytic double-stranded DNA phages – S16 and Felix O1a – in
386 saline buffer are presented in Figure 3. The microscopic analysis enabled to distinguish

387 between the two phages comprising the cocktail (in a 1:1 ratio), based on the
388 distinct/characteristic morphology of each virion particle. In accordance with the
389 documented in the literature, while S16 presented an elongated head (*ca*. 113 nm in length
390 and 88.5 nm width), Felix O1 structure was overall smaller and the capsid was icosahedral
391 (head averages *ca*. 83 nm diameter) (Marti et al., 2013).
392 In the sample pressurized at 300 MPa (Figure 3B), there were no relevant changes in the
393 canonical morphologies, in comparison to the control sample (Figure 3A), since intact
394 conformation of the bacteriophages was observed. Nonetheless, once exposed to a
395 pressure magnitude of 500 MPa (Figure 3E) a detrimental HHP impact on the
396 bacteriophage integrity was visualized. Indeed, in some of the S16 phage particles, the
397 morphological features were not conserved owing to a detachment between the capsid
398 and the long contractile tail. Concerning the HHP processing at 600 MPa (Figure 3F), the
399 structural damages were more pronounced, with the majority of the S16 observed phages
400 being completely disrupted - the particles lost their proteinaceous tails, which would
401 hamper the recognition and attachment to the surface of the host bacterium, and some of
402 the capsids presented ruptures with leakage of the genetic material. On the other hand,
403 damages to Felix O1 were not as evident, and only *ca*. 40% appeared to be disrupted,
404 comparatively to *ca*. 96% of S16. Nonetheless, Felix O1 must have also sustained protein
405 denaturation since Salmonelex^{TM} was completely inactivated.
406 In opposition, Komora et al. (2018) observed a considerable damage on some P100 phage
407 particles following HHP exposure at 300 MPa (5 min, 10 ºC), with a partial, or in some
408 cases total, tail loss and presenting apparently deformed heads; whilst processing at 400
409 MPa demonstrated to be completely destructive, with none of the phage particles
410 displaying tail. Solomon et al. (1966), evaluating the impact of a processing at 420 MPa
411 (5 min, 30 ºC) on the bacteriophage T4, reported that only 41% of the viral particles

conserved their morphological integrity. The results herein presented underlined the remarkable Salmonelex^{TM} baroresistance, in comparison to the abovementioned lytic bacteriophages. Moroni et al. (2002) hypothesized a correlation between phage morphology and stability, in which the small isometric phage head presented higher stability and resistance to high pressure in comparison to the prolate phage head. This hypothesis was later corroborated by Müller-Merbach et al. (2005). The results herein presented are in accordance with those findings, since the number of S16 phage particles that sustained morphological damages was higher than those of Felix O1.

It is conceivable that the phage infectivity may be compromised via essential phage protein HHP-mediated denaturation, eliciting a loss of functionality of structural proteins, which may originate a multitude of morphological alterations in virion particles. The mechanisms through which HHP impacts phage stability and infectivity may comprise: (i) partial inactivation, by subtle alterations in capsid and tail structures with maintenance of apparent structural integrity; (ii) loss of capability to attach to the host cell, potentially with detachment of the tail, maintaining an intact capsid; (iii) capsid disruption, eliciting genetic material release/leakage (Kingsley, 2013; Müller-Merbach et al., 2005; Tian et al., 2020).

The results previously detailed disclosed the unfeasibility of the application of higher pressures, such as 500 and 600 MPa, in a process in which bacteriophage stability, and infectivity, maintenance is mandatory.

4 Conclusion

To our knowledge, this is the first study documenting the impact of HHP on the stability of Salmonelex™ in egg matrices, along with its characterization. The promising results highlighted the notable potential of Salmonelex™ to be associated with HHP towards decontamination of egg and egg products. Salmonelex™ was determined to possess a prominent baroresistance, particularly when incorporated in egg matrices, requiring at least 600 MPa to completely lose its infectivity. Moreover, TEM analysis unraveled that 200 and 300 MPa were considered pressure magnitudes feasible to be used in a HHP-bacteriophage biocontrol system, since no structural or morphological damages were observed.

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Table 1. Efficiency of plating (EOP) of Salmonelex™ against *Salmonella enterica* strains, following exposure to HHP processing (200 and 300 MPa)

Species	**Serovar**	**EOP**[a]			
		PBS [b]	EW [b]	LWE [b]	EY [b]
Salmonella enterica	Enteritidis ATCC 13076	H	H	H	H
	Infantis M2016	0	0	0	0
	Braenderup H9812	H	H	H	H
	Weltevreden TA 428/97	H	H	H	H
	Senftenberg ATCC 43845 (775W)	0	0	0	0
	1,4,[5],12:i:-, monophasic variant of *Salmonella* Typhimurium	H	H	H	H
	Derby	0	0	0	0
	Wernigerode	H	H	H	H

[a] The EOP value was defined as high, representing >10% and 0 when inexistent

[b] The bacteriophage cocktail Salmonelex™ (8 $\log_{10}$ PFU mL^{-1} or g^{-1}) was previously inoculated in PBS, egg white (EW), liquid whole egg (LWE) and egg yolk (EY) and pressurized at 200 and 300 MPa

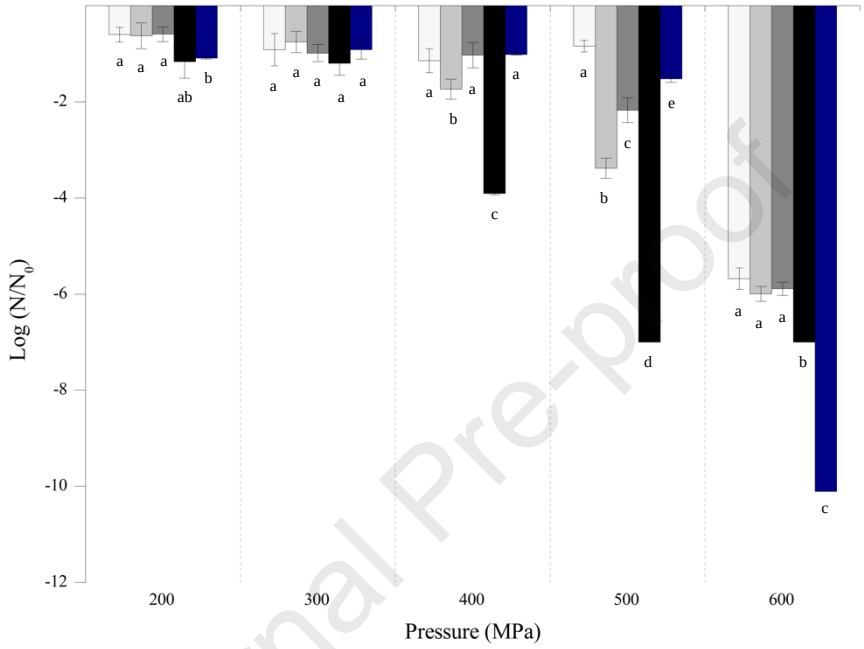

Figure 1. Impact of different HHP magnitudes (5 min, 10 °C) on Salmonelex™ inactivation (initial phage load of 8 $\log_{10}$ PFU g^{-1} or mL^{-1}) inoculated in egg yolk (□), egg white (■), liquid whole egg (■), PBS (■) and the bacteriophage stock solution (11 $\log_{10}$ PFU mL^{-1}) (■). N is the phage titre (PFU g^{-1} or mL^{-1}) at a particular pressure magnitude and N_0 is the initial phage titre. Data reported are mean values of three independent experiments ± standard deviation (error bars). Means with the same letter are not significantly different ($P > 0.05$).

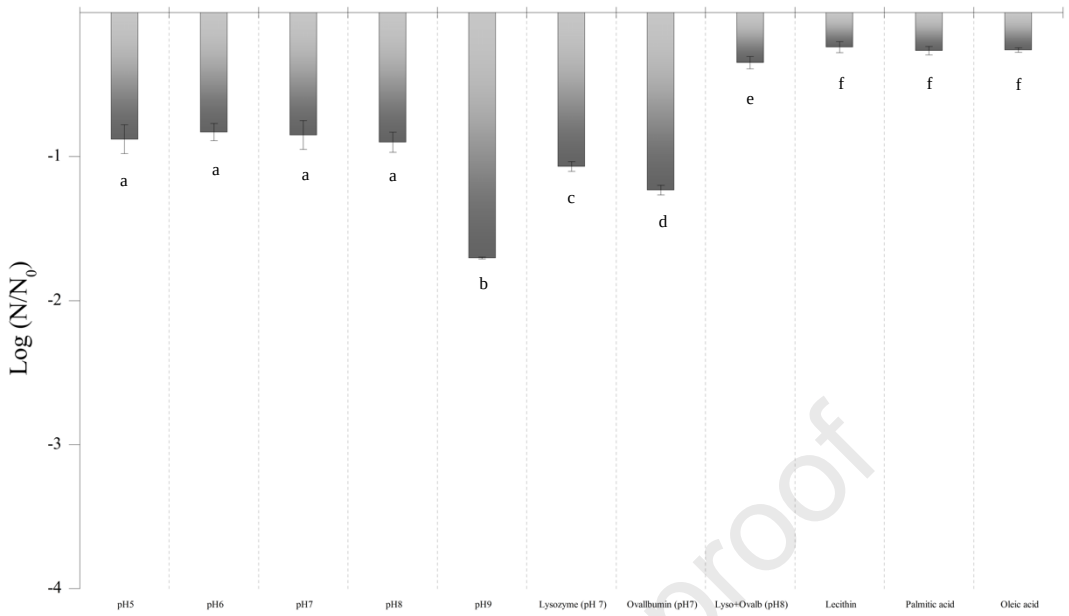

Figure 2. Impact of pH and egg compounds on the pressure (200 MPa, 5 min, 10 °C) stability of Salmonelex^{TM} (initial phage load of 8 $\log_{10}$ PFU mL^{-1}). N is the phage titre (PFU mL^{-1}) at a particular pressure magnitude and N_0 is the initial phage titre. Data reported are mean values of three independent experiments ± standard deviation (error bars). Means with the same letter are not significantly different ($P > 0.05$).

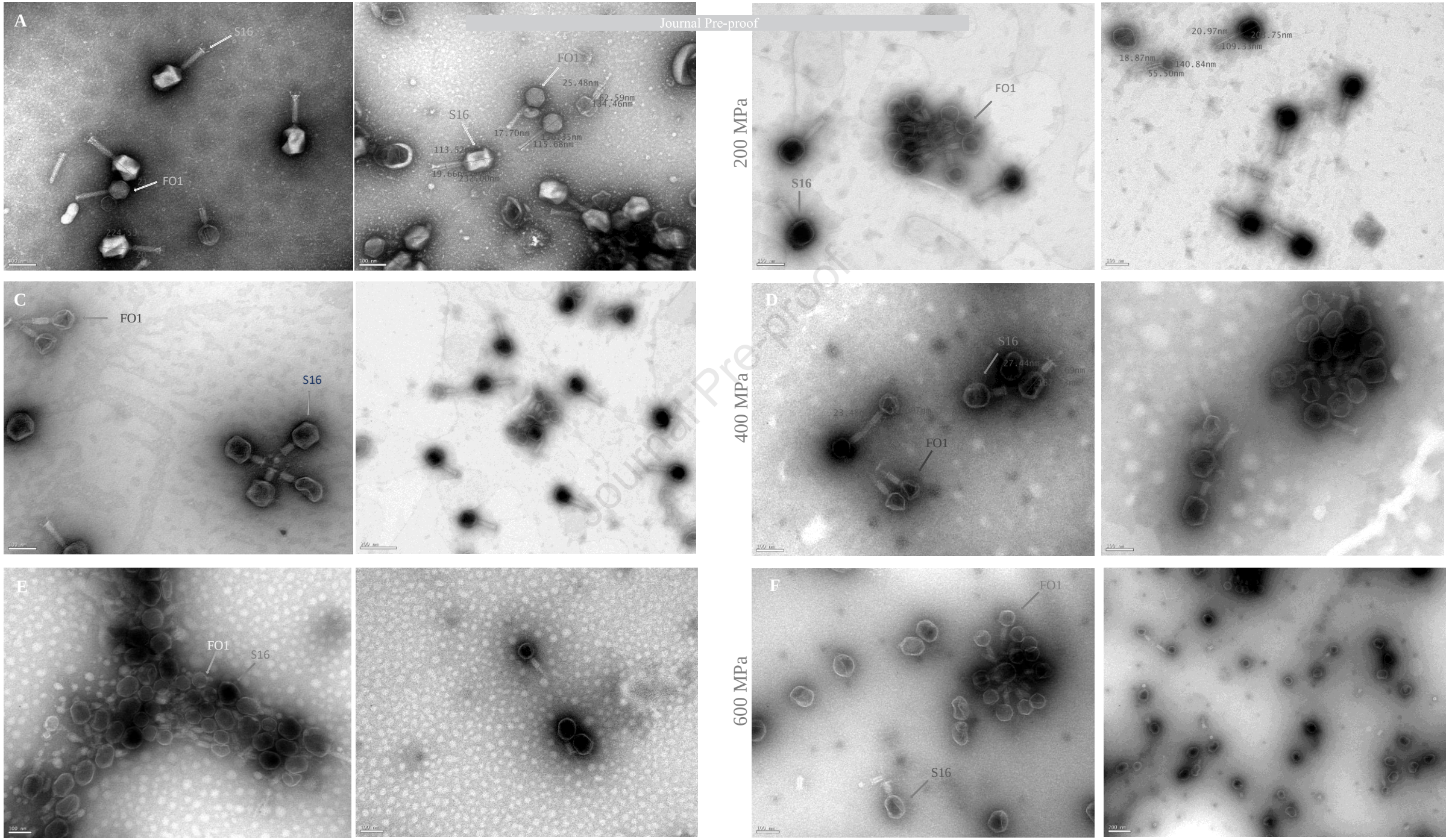

Figure 3. Transmission electron microscopy micrographs of non- and pressure-treated *Salmonella* bacteriophage cocktail Salmonelex™ - 0.1 (A), 200 (B), 300 (C), 400 (D), 500 (E) and 600 MPa (F). Scale bar represents 100 nm for all the micrographs (except in C2 and E2, in which represents 200 nm). Bacteriophages S16 and Felix O1a composing Salmonelex™ were identified.

Highlights

- Salmonelex™ notable baroresistance – 600 MPa to completely lose its infectivity
- Egg matrices conferred a baroprotective effect up to 500 MPa, particularly egg yolk
- pH values (5-9) and egg compounds did not hinder phage infectivity at mild HHP
- Structural damages only observed at 500/600 MPa, more notoriously in S16 phage
- Feasibility of Salmonelex™ as an adjuvant to mild HHP processing of egg matrices

No conflict of interest to declare