EXPANDING THE APPLICATIONS OF BACTERIOPHAGES IN FOOD SAFETY

ENGINEERING STRATEGIES FOR BROADENING BACTERIOPHAGE APPLICATION IN THE FOOD SUPPLY CHAIN

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TITLE: Engineering Strategies for Broadening Bacteriophage Application in the Food Supply Chain

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Dedication

For my love, Ed.

Lay Abstract

Bacterial contamination of food can lead to widespread outbreaks and subsequent preventable deaths. Our best tool against bacteria, antibiotics, cannot be widely applied to food for risk to the natural human biome and creation of resistant bacteria. Bacteriophages, viruses that infect bacteria, are a naturally occurring bactericide that offer an alternative solution. This thesis focuses on improving the application of bacteriophages in food. First, bacteriophages are selected for resistance to common food processing stresses, such as heat, drying, and acidity, to prepare future generations that are stress-resistant. Second, a protective sugar powder was designed that could be used to add bacteriophages to milk before pasteurization. Post-pasteurization, the sugar would dissolve and release bacteriophage into the milk to deal with any post-processing contamination. Lastly, an infant-safe bacteriophage powder was developed that could be intermixed with powdered infant formula in an effort to reduce infant death due to the ingestion of bacteria.

Abstract

Bacterial contamination of food is a global concern. Methods of treating bacterial contamination are limited. Bacteriophages, bacterial viruses, offer a promising solution. However, bacteriophages may have limited application for foods that undergo sterilization processing, are inhospitable to organisms, or must be maintained in a dry state. This thesis focused on methods to expand the application of bacteriophages.

First, bacteriophages were subjected to generalized stresses of desiccation, heat, and acidity, under laboratory conditions to propagate new populations with improved stress resistance. However, testing of these stress-resistant populations under real-world conditions failed to produce results comparable to generalized laboratory conditions. Success in the application of selected bacteriophages may require high situational specificity during selection, including in terms of food matrix and stress mechanics. The focus of our research shifted from the modification of bacteriophage populations themselves to the development of food-safe protective matrices.

Designed matrices encapsulate bacteriophage for integration with modern food production and even the food products themselves. A pullulan-trehalose sugar powder was developed for the protection of a model bacteriophage from pasteurization. Microparticles were engineered such that the majority of the particle would be composed of trehalose as a stabilizer and polysaccharide pullulan was designed to accumulate at the particle surface to slow dissolution. This structure resulted in a bacteriophage powder that remained intact and protective over short-term high-temperature pasteurization, whereas unprotected bacteriophage experienced significant loss in titer.

Leucine-lactose and leucine-lactose-maltodextrin microparticles were engineered for the inclusion of bacteriophage in powdered infant formula. The bacteriophage powder was designed as a dormant protection system that could activate upon reconstitution. The excipient system was formulated to not significantly affect the pH, composition, and dissolution of commercial infant formula. The bacteriophage powder was also engineered to match the shelf life and secondary shelf life of infant formula. Altogether, this thesis demonstrates that bacteriophage application in different foods can be expanded through particle engineering.

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List of Abbreviations

BLOQ	Below limit of quantification
BP	Before pasteurization
CDC	Centers for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit(s)
CM	Calcium-magnesium
DSC	Differential scanning calorimetry
FDA	Food and Drug Administration
GMO	genetically modified organism
GP	Gene protein
GRAS	Generally recognized as safe
HP	Hypothetical protein
HTST	High temperature short term pasteurization
LL	Leucine lactose
LML	Leucine lactose maltodextrin
MOI	multiplicity of infection
N.A.	Not available
N.S.	Not significant
NCBI	National Center for Biotechnology Information
NCR	Non-coding region
OD	Optical density
OXA	Oxford selective agar
P	Pullulan
PALCALM	Palcam selective agar
PD	Pasteurized powder
PDP	Pre-dissolved, pasteurized powder
PFU	Plaque forming unit(s)
Phage	Bacteriophage
PIF	Powdered infant formula
R5D	S5D sample that was propagated 5 times in the absence of stress
R5pH	S5pH sample that was propagated 5 times in the absence of stress
R5T	S5T sample that was propagated 5 times in the absence of stress
RH	Relative humidity
RPM	Rotations per minute
S1D	P100 sample that underwent 1 cycle of desiccation selection
S1pH	P100 sample that underwent 1 cycle of low-pH selection
S1T	P100 sample that underwent 1 cycle of heat selection
S2D	P100 sample that underwent 2 cycles of desiccation selection
S2pH	P100 sample that underwent 2 cycles of low-pH selection
S2T	P100 sample that underwent 2 cycles of heat selection
S3D	P100 sample that underwent 3 cycles of desiccation selection
S3pH	P100 sample that underwent 3 cycles of low-pH selection

S3T	P100 sample that underwent 3 cycles of heat selection
S4D	P100 sample that underwent 4 cycles of desiccation selection
S4pH	P100 sample that underwent 4 cycles of low-pH selection
S4T	P100 sample that underwent 4 cycles of heat selection
S5D	P100 sample that underwent 5 cycles of desiccation selection
S5pH	P100 sample that underwent 5 cycles of low-pH selection
S5T	P100 sample that underwent 5 cycles of heat selection
SD	Spray dried
SEM	Scanning electron microscopy
SM	Sodium-magnesium
T	Trehalose
TEM	Transmission electron microscopy
TSB	Tryptic soy broth
VD	Vacuum dried
UV	Ultraviolet
WHO	World Health Organization
YE	Yeast extract

List of Symbols

OD_{600}	Optical density at a 600 nm wavelength
C_{f}	Concentration of the feedstock
V	Volume of feedstock spray dried
m_{c}	Mass of powder collected after spray drying
D	Diffusion coefficient
k_B	Boltzmann's constant
T	Temperature
r	Radius
η	Dynamic viscosity
$[\eta]$	Intrinsic viscosity
M	Molecular weight
Pe	Peclet number
κ	Evaporation rate
t_{sat}	Time for a component to reach saturation at droplet surface
t_{d}	Droplet lifetime
c_0	Initial concentration of the component
c_{sol}	Component maximum solubility
t_{t}	Time to amorphous shell formation
ρ_{t}	True density
d_g	Geometric diameter
c_F	Solids concentration from feedstock
ρ_P	Particle density
d_0	Initial droplet diameter
$\mathrm{OD(t)}$	Optical density with respect to time
λ	Lag phase duration
μ	Bacterial growth rate
OD_λ	Optical density during lag phase
E^*_{Lab}	Colour change based on CIELAB system
L^*	Lightness, based on CIELAB system
a^*	Chromaticity – magenta to green axis
b^*	Chromaticity – blue to yellow axis
T_{in}	Temperature at spray dryer inlet
T_{out}	Temperature at spray dryer outlet

1. Bacteriophage Buffet - A Taste of Food Safety

1.1. Bacterial contamination of food

Compromised food safety due to bacterial contamination is a serious public health issue. The World Health Organization (WHO) last estimated over 10 million cases of foodborne illnesses, leading to over 85 000 deaths globally in a year[1]. The possibility of multi-country, and even multi-continent, outbreaks due to contaminated food[2–5] has increased with the rising globalization. A 2022 report by WHO linked a *Salmonella* Typhimurium outbreak to a widely distributed chocolate product[3]. An estimated 151 cases were linked to the aforementioned chocolate products across 11 countries. Similarly, in 2020 there were 123 cases of a *Salmonella* Typhimurium ST19 outbreak linked to Brazil nuts across 5 countries[5]. In addition to the possibility of geographically widespread foodborne pathogen outbreaks, ongoing years-long outbreaks can also occur at production sites with persistent contamination or recurring contamination[4,6]. For instance, 210 *Salmonella* Virchow ST16 cases between 2017 and 2023 have been linked to contaminated chicken products[4].

The consolidation of food products under a singular corporation can also lead to exacerbation of food contamination. One of the recalls given within Canada in 2024 was for Quaker granola products due to possible *Salmonella* contamination[7]. This recall encompassed 38 different Quaker products. The possible use of the contaminated granola in other yogurt products led to an additional recall encompassing another 34 products[8]. Recalls serve as a governmental tool to mitigate outbreaks. However, audits of both the Canadian Food Inspection Agency (CFIA)[9] and the Food and Drug Administration (FDA)[10] have found significant inadequacies in agencies' approach to food recalls. Investigation into the CFIA's practices found that while the agency consistently initiated food recalls swiftly, follow up was not completed in a timely manner[9]. The FDA audit revealed significantly worse practices, with the 57 days being the average number to initiate a recall[10]. In the worst cases, four different products contaminated with *Salmonella* took 121-165 days to initiate a recall. Lack of timeliness can contribute to the severity of an outbreak.

Even effective recalls still require a problem to be identified before the recall can be initiated. It is apparent that the current structures in place are not sufficient to prevent contamination from occurring in the first place[11]. Current processing methods, including pasteurization, high pressure processing, and irradiation, are established techniques for reducing bacteria but they affect sensory properties of food and do not protect against downstream contamination[12]. Complementary intervention methods should be considered as a fail safe to prevent foodborne pathogen outbreaks.

A possible intervention method is the use of a bactericide during food production or inclusion of a bactericide directly into food. There are food preservatives, including natural and synthetic compounds, that are permitted for inclusion in food to limit the growth of bacteria[13]. However, there are health concerns regarding the safety of these preservatives when consumed in large quantities[14–16]. An attractive alternative to preservatives are bacteriophages (phages), bacterial viruses.

1.2. Bacteriophages

Phages are considered to be the most populous and diverse biological group on Earth[17]. Phages are bacteria's natural predator, capable of infecting them through cell-specific receptors and using their host to replicate before rupturing the host cell (Figure 1). The replication method of phages allows them to self-amplify in the presence of their hosts, with no growth possible when in their absence. Phages have been successfully used in compassionate use therapy to treat bacterial infections that were resistant to antibiotics[18]. With the rise of antibiotic resistance, phage therapy has been considered as an alternative treatment of bacterial infections. Another application for phages that has gained more attention is the use of phages in food to prevent or reduce bacterial contamination. Studies have shown that phages are effective at reducing different bacterial contamination in a wide variety of foods, including vegetables[19–21], fruit[19], dairy products[19,21], and meat products[19,21–23].

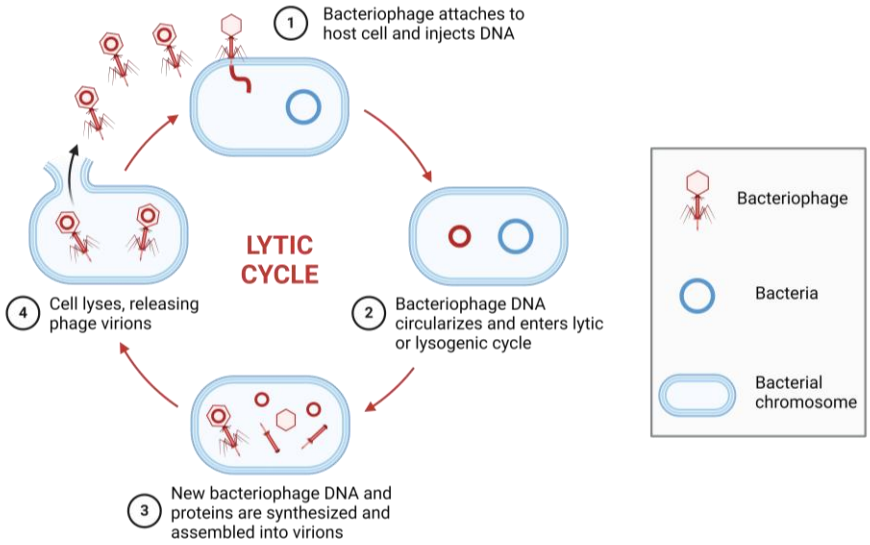

Figure 1 Self-amplifying lifecycle of lytic bacteriophage. Lytic bacteriophages infect their host and inject their DNA into the host bacterium. The bacteriophage DNA then hijacks the host's reproduction machinery to replicate more bacteriophage. The newly replicated bacteriophage then ruptures the cell, killing the host and releasing more bacteriophage into the surrounding environment for further bacteria elimination. Figure adapted from Biorender.com template on lytic vs. lysogenic cycles of bacteriophage.

Phages offer several advantages over traditional preservatives. Phages have limited effect on the organoleptic properties (such as pH, colour, odour and taste)[24–26] of food. In comparison, preservatives are limited in their addition in high doses as they may affect the taste, odour and texture of foods[27]. Bacteria can form biofilms that are difficult to penetrate with antimicrobial preservatives, whereas phages show anti-biofilm activity[28]. Phages have specific receptors such that certain phage strains will be species or even strain specific for infection. Phage products can be designed to not harm beneficial bacteria in food, such as the starter cultures used in cheese production[29]. Lastly, phage application is not limited to preservatives and can be applied throughout the food supply chain.

An ideal phage preparation for use in the food industry would depend on the intended application. However, there are a few commonalities:

- Safe for human ingestion
    - Lytic phage(s)
    - Appropriate host range
    - Low immune response
- Effective
    - High titer
    - Large burst size and latent period
    - Compatible with intended food process/product

1.2.1. Safe for human ingestion

Phages are expected to be present in all fresh foods[12], and they contribute to the complex microbiome that varies from person to person. A high percentage of these are temperate phages that have integrated into their host bacterium[17]. As temperate phages may be integrated into their host, there is a risk associated with these phages as they may led to the transfer of genes between bacteria. This transfer can include the transfer of virulence factors, causing a previously safe bacteria to become harmful. For this reason, the use of lytic phages is preferred for phage therapy and commercial products.

The chosen lytic phages must have a host range that can only target the harmful pathogen(s). Infection of commensal bacteria may upset the delicate balance of the human microbiome. A recent systemic review concluded that the use of phage for bacterial decolonisation is safe and well tolerated[30]. Recently, a *Klebsiella pneumoniae* phage cocktail was also found to be safe and tolerable when ingested by human subjects[31]. However, even with species-specific phage preparations, there may be some effects on the abundance of other species within the microbiom[32,33]. Febvre et al.[33] investigated the effects of commercial *E. coli* phages given to adults over 28 days as a supplement. Phage treatment successfully reduced *E. coli* populations and the subjects found the product tolerable[34]. However, while global changes were not seen, several other bacteria species increased or decreased with the anti-*E. coli* phage treatment[33]. The authors did note that despite these changes, global microbiota was maintained[33], in contrast with antibiotics which are expected to disrupt the microbiota.

While phage is generally considered safe for human use, there is some concern in the field over widespread use given that that phage interacts with human cells[35]. General statements about the effect of phage on human cells cannot be made. Just as phage target specific bacteria, the effect of phage on human cells can be dependent on the phage proteins and cell type, with anti-inflammatory and inflammatory observed in different contexts[36]. To minimize inflammatory response, the proteins that phages encode may be screened against known food allergens through computational methods[37] based on World Health Organization guidelines[38]. Phages are able to be cleared by the immune system through the phagocytosis[35].

1.2.2. Efficacy

The efficacy of phage can be dependent on the phage itself, the food type, temperature, and contamination level[39]. Unlike many bacteria, phages are not motile. Commercial phage preparations thus take a "strength in numbers" approach, with a high titer applied. The higher relative amount of phage to bacterial concentrations, referred to as multiplicity of infection (MOI), can improve the rate of bacterial control[40]. Another factor to consider in a phage preparation is the latent period and burst size. Latent period is the time it takes between phage adsorption and the lysis and subsequent release of replicated phage. Burst size refers to the number of replicated phage released upon lysis. A small latent period means that more viral replications can be completed in a given amount of time. This might be more useful for foods where there is a time sensitive factor. A large burst size would also help quickly increase the relative concentration of phage against bacteria for faster intervention.

Effectivity of a phage product is not solely reliant on the phage itself but is also dependent on the environment. Phages may have different efficacy when held at colder temperatures[28]. Temperature-dependent efficacy is relevant for refrigerated foods and processing plants where temperatures are regularly kept to 2-8 °C. Another factor to consider is that a given phage may not be effective within a certain food matrix[39]. For instance, phage K was able to reduce bacterial contamination in pasteurized milk but was unable to reduce contamination in unpasteurized milk[41]. Similarly, Oliveria et al.[42] showed that phage P100 is effective in reducing bacteria in melons and pears, but not apples or apple juice. These factors must be considered when designing commercial phage products for food safety applications.

1.3. Bacteriophage application in food

A list of studies investigating the direct application of phage in food is given in the review article by Vikram et al.[12]. Phages can be applied in four areas of the food supply chain: as preharvest therapy, sanitation, control, or preservation (Figure 2). Preharvest treatment includes the use of phage in crops to improve production yield by preventing bacterial spoilage of produce[43]. Another way that phages can be applied preharvest is to live animals. In this application, phages may be used to reduce bacterial burden of animals prior to slaughter by either applying phage on the hides of animals[44] or introducing phage into animal water or solid feed[45] to reduce bacterial colonization of their digestive system.

Post-harvest, phage preparations can also be used as a supplement to hygienic practices in processing facilities, such as meat packaging plants. In these cases, generally phage products are diluted in water to prepare a spray that can be used to sanitize stainless steel food surfaces[46]. Phage products can also be used during processing to control the growth of pathogenic bacteria, such as the inclusion of phage during cheesemaking[29].

Lastly, phage can be applied post-production to food as a preservative. Phage can be applied as a spray directly onto the food or included as a package coating. The inclusion of phage at this step acts as a failsafe in the event of sterilization failure during processing, as well as an additional fail safe against post-processing contamination. The addition of a fail safe type of product is beneficial as it may be difficult to detect bacterial contamination in a commercial product. The use of an

active preservative like phage is especially useful for ready-to-eat foods, such as salads and deli meats. These foods do not undergo an additional step of bacterial removal/elimination prior to ingestion, e.g. washing or cooking, making these types of foods of particular concern for outbreaks. However, implementation of phage products is not necessarily optimized for existing food safety procedures[12] (e.g. incompatibility with harsh sanitation chemicals, sensitivity to heat treatment).

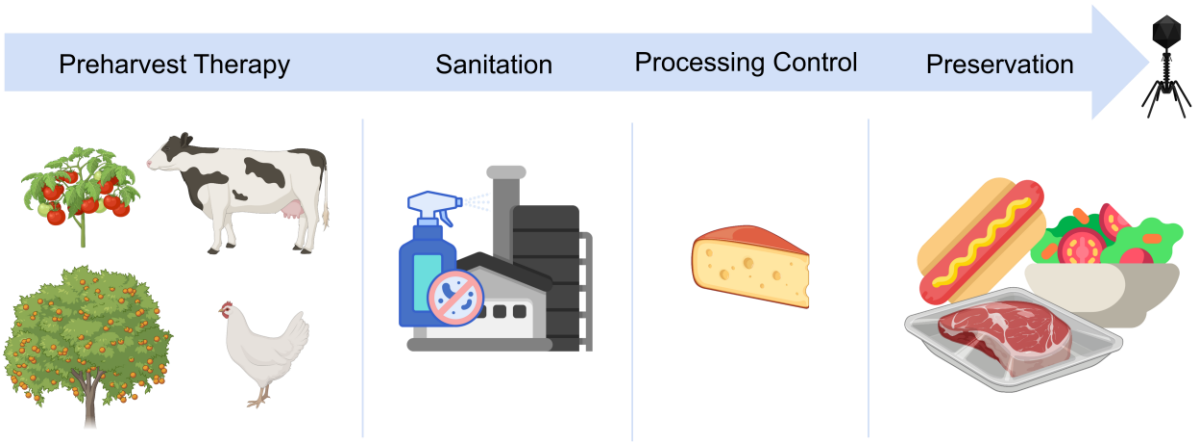

Figure 2 Areas of bacteriophage application throughout the food supply chain for control of foodborne pathogens. Figure based on the concept given by Sillankorva et al.[47]. Figure prepared using icons from Biorender.com and icons by Freepik and Flat Icons from flaticon.com

The field of phage application has progressed such that, globally, there are at least 44 commercially available phage products (Table 1). The company with the most commercial phage products is Phagelux (4 products), which is based in China, in combination with its USA-based subsidiary Omnilytics (9 products) (Table 1). The next most prolific commercial phage companies are Intralytics (9 products), which is based in the United States, and Elivava Biopreparations (6 products), which is based in Georgia.

The majority of the commercial products are designed for application within the food supply chain. The exceptions are PreforPro and the products by Elivava Biopreparations. The PreforPro product is designed as a supplement for human ingestion as a method of digestive system biocontrol[33] and is available for purchase online. The phage products by Elivava Biopreparations may be used as an over the counter pharmaceutical product[48] in Eastern Europe.

Table 1 Commercially available bacteriophage products. Table significantly updated from Liu et al.[28]. *No peer-reviewed literature available but product is available for purchase through company website as of March 14 2024.

Product	Phage(s)	Host	Application	Company	Ref.
AgriPhage – Spot and Speck	Not available	*Xanthomonas campestris* pv. *vesicatoria*	Prevention and treatment of bacterial spot and bacterial speck	OmniLytics	[49]
AgriPhage – Tomato Canker	Not available	*Clavibacter michiganensis* pv. *michiganensis*	Prevention and treatment of tomato bacterial canker	OmniLytics	[43]
AgriPhage – Fire Blight	Not available	*Erwinia amylovora*	Prevention and treatment of fire blight on apple and pear trees	OmniLytics	N.A.*
AgriPhage – Citrus Canker	Not available	*Xanthomonas citri* pv. *citri*	Prevention and treatment of citrus bacterial canker	OmniLytics	N.A.*
AgriPhage – Nut and Stone Fruit Peach Spot	Not available	*Xanthomonas arboricola* pv. *pruni*	Prevention and treatment of peach bacterial spot	OmniLytics	N.A.*
AgriPhage – Nut and Stone Fruit Cherry Canker	Not available	*Pseudomonas syringae* pv. *syringae*	Prevention and treatment of cherry bacterial canker	OmniLytics	N.A.*
AgriPhage – Nut and Stone Fruit Almond Blast, Walnut Blight, & Hazelnut Blight	Not available	*Xanthomonas arboricola* pv. *pruni* *Xanthomonas arboricola* pv. *Juglandis* *Pseudomonas syringae* pv. *syringae.*	Prevention and treatment of relevant bacterial infections in nut trees	OmniLytics	N.A.*
Finalyse SAL	BP-63 LVR16A	*Salmonella*	Reduction of contamination in poultry processing	OmniLytics	N.A.*
Finalyse Hide Wash	Not available	*E. coli*	Treatment of contamination on livestock hides prior to slaughter	OmniLytics	[44]
Biolyse – PB	Not available	soft-rot *Enterobacteriacea*	Elimination of bacteria causing bacterial soft rot	APS Biocontrol	N.A.*
Bafasal	PCM F/00069 PCM F/00070 PCM F/00071 PCM F/00097	*Salmonella*	Addition to feed to reduce contamination in poultry prior to slaughter	Proteon Pharmaceuticals SA	[50]
Bafacol	Not available	*E. coli*	Addition to feed to reduce contamination in poultry prior to slaughter	Proteon Pharmaceuticals SA	N.A.*
Bafador	50AhydR13 PP 60AhydR15 PP 25AhydR2PP	*Pseudomonas* *Aeromonas*	Addition to water tank for health of fish	Proteon Pharmaceuticals SA	[51]

	22PfluR64 PP 67PfluR64 PP 71PfluR64 PP 98PfluR60 PP				
Secure Shield E1	6 of the following: DSM 103290 DSM 104013 DSM 104014 DSM 104015 DSM 104016 DSM 104018 DSM 104019 DSM 104020 DSM 104021 DSM 104022 DSM 104023	*E. coli*	Treatment of beef carcasses	FINK TEC GmbH	N.A.*
BIOTECTOR – Poultry	Not available	*E. coli* *C. perfringens* *Salmonella*	Addition to feed to reduce contamination in chicken prior to slaughter	CJ CheilJedang	N.A.*
BIOTECTOR – Swine	Not available	*E. coli*	Addition to feed to reduce contamination in pigs prior to slaughter	CJ CheilJedang	N.A.*
Lunin	Not available	*Staphylococcus* *E. coli* *P. aeruginosa*	Pathogen control in chicken farms	Phagelux	N.A.*
Luxon	Not available	*Vibrio parahaemolyticus*	Prevent early death of shrimps	Phagelux	N.A.*
Lumon	Not available	*Staphylococcus* *E. coli* *P. aeruginosa* *Salmonella* *K. pneumoniae*	Pathogen control in cattle farms	Phagelux	N.A.*
Luzon	Not available	*Staphylococcus* *E. coli* *P. aeruginosa* *Salmonella*	Pathogen control in pig farms	Phagelux	N.A.*
Shijunsha	Not available	*Staphylococcus* *E. coli* *P. aeruginosa* *Salmonella*	Pathogen control in poultry breeding environment	Phagelux	N.A.*

Inspektor	L8 SAEN098P03 SAEN098P01	*Salmonella*	Administration on poultry farms throughout rearing process to reduce contamination	PhageLab	[52]
Fórmida	Not available	*E. coli*	Administration on poultry farms throughout rearing process to reduce contamination	PhageLab	N.A.*
Phagein	Not available	*E. coli* *Salmonella*	Administration in calves to prevent enteric disease	PhageLab	N.A.*
ListShield	LIST-1 LIST-2 LIST-3 LIST-4 LIST-36 LIST-38	*L. monocytogenes*	Treatment of contaminated foods and sanitation of food processing facilities	Intralytix	[25]
SalmoFresh	SPT-1 SBA-178 SBA-1781 SIT-128 SSE-121 SDT-15	*Salmonella*	Treatment of contaminated foods and sanitation of food processing facilities	Intralytix	[20]
ShigaShield	SHSML-52-1 SHFML-11 SHSML-45 SHFML-26 SHBML-50-1	*Shigella*	Treatment of contaminated foods and sanitation of food processing facilities	Intralytix	[53]
EcoShield PX	ECML-117 ECML-359 ECML363	*E. coli*	Treatment of contaminated foods	Intralytix	[54]
CampyShield	J350 J375 J386	*Campylobacter*	Treatment of contaminated meat and sanitation of meat processing facilities	Intralytix	N.A.*
SalmoLyse	SPT-1 SBA-178 SBA-1781 SIT-128 SSE-121 SDT-15	*Salmonella*	Treatment of contamination in pet food	Intralytix	[55]

ListPhage	List-1 List-2 List-3 List-4 List-36 List-38	*L. monocytogenes*	Treatment of contamination in pet food	Intralytix	N.A.*
Ecolicide	ECML-117 and/or ECML-134	*E. coli*	Treatment of contamination in pet food	Intralytix	N.A.*
Ecolicide PX	ECML-117 ECML-359 ECKL-363	*E. coli*	Treatment of contamination on livestock hides prior to slaughter	Intralytix	N.A.*
PhageGuard L (Listex)	P100	*L. monocytogenes*	Treatment of contaminated foods and sanitation of food processing facilities	Micreos Food Safety	[26]
PhageGuard S (Salmonelex)	Fo1a S16	*Salmonella*	Treatment for meat processing facilities	Micreos Food Safety	[56]
PhageGuard E	EP75 EP335	*E. coli*	Treatment for hide processors	Micreos Food Safety	[23]
Pyo Bacteriophage	Not available	*S. aureus* *S. pygoenes* *S. sanguis* *S. salivarius* *E. coli* *P. aeruginosa* *P. mirabilis* *P. vulgaris*	Treatment of foodborne pathogens	Elivava BioPreparations	[57]
Intesti Bacteriophage	Not available	*Shigella* *Salmonella* *E. coli* *P. mirabilis* *P. vulgaris* *S. aureus* *P. aeruginosa* *Enterococcus*	Prophylaxis and treatment of pathogens	Elivava BioPreparations	[58]
SES Bacteriophage	Not available	*S. aureus* *S. epidermidis* *S. pyogenes* *S. sanguis* *S. salivarius* *S. agalactiae*	Prophylaxis and treatment of pathogens	Elivava BioPreparations	[59]

		E. coli			
Staphylococcal Bacteriophage	Sb-1	*S. aureus*	Prophylaxis and treatment of pathogens	Elivava BioPreparations	[57]
Enko Bacteriophage	Not available	*Shigella* *Salmonella* *E. coli* *S. aureus* *S. epidermidis*	Prophylaxis and treatment of pathogens	Elivava BioPreparations	[59]
Fersisi Bacteriophage	Not available	*S. aureus* *S. epidermidis* *S. pyogenes* *S. sanguis* *S. salivarius* *S. agalactiae*	Prophylaxis and treatment of pathogens	Elivava BioPreparations	[58]
PreforPro	LH01-*Myoviridae* LL5-*Siphoviridae* T4D-*Myoviridate* LL12-*Myoviridae*	*E. coli*	Ingestion by humans as a probiotic	Deerland Enzymes	[33]
Erwiphage PLUS	PhiEaH2 PhiEaH1	*Erwinia amylovora*	Treat and prevent fireblight in apple crops	Erwiphage	[60,61]

1.4. Strategies for enhancing bacteriophage application in food

The incompatibility of phage with certain environmental stressors and food matrices provides a barrier to application[39]. An excellent, comprehensive review by Liu et al.[28] details how environmental challenges and delivery challenges obstruct phage use throughout the food supply chain. Two possible methods of overcoming these challenges 1) adaptational evolution and 2) protective matrices.

1.4.1. Adaptive evolution

Lytic, wild-type phages are generally considered for commercial use. Improvement of inherent phage stability could be achieved through genetic modification. However, genetic engineering of phage changes the classification to a genetically modified organism (GMO). This approach is avoided as GMOs have different safety regulations adding another hurdle in achieving phage approval. Instead, adaptational evolution, also referred to as experimental evolution, can be used to prepare phage populations that are resistant to a given stress. In this approach, a wild type phage population is exposed to a given stress. The surviving phage are then propagated to obtain a new population. This process is repeated to obtain a population that is more resistant than its ancestral counterpart through enforced selection.

The adaptational evolution approach been successfully implemented by Tom et al.[62] for improved survival of phage T7 to UV exposure. The authors motivation was to prepare a phage population that could be used to prevent bacterial disease in crops. In Chapter 2, three of the four examples of environmental challenges given by Liu et al.[28] are considered for improved phage application: low pH, high temperature, and desiccation.

Adaptational evolution is limited in its commercial potential as individual phage strains need to be subjected to the conditioning process to obtain a resistant phage population, and the adaptational evolution process is highly condition specific[63]. Adaptational evolution also does not improve phage applicability when the issue is related to the phage format – for instance the incompatibility of liquid phage with powdered foods, such as milk powders. Instead, protective matrices for phage can be used to overcome these challenges[28].

1.4.2. Phage fortification and gut delivery through encapsulation within protective matrices

There has been some work done on the use of edible coatings to maintain an active concentration of preservatives over the course of a food's shelf life[27]. A similar approach can be used to prepare phage for different food applications (Table 2). Liu et al.'s[28] review also discusses how the use of protective matrices for phage can be used to overcome both the environmental challenges and delivery challenges for application in the food supply chain. Briefly, there are four main methods for encapsulation of phage for food applications: vacuum/air drying, gelation, freeze drying (also referred to as lyophilization), and spray drying (Table 2). Reviews on these methods can be found elsewhere[28,64]. The excipient choice is dependent on the final application and compatibility with the food matrix. Shannon et al.[64] provides a review of excipients used in preparing phage within a dry format, including phages designed for inhalable delivery. Generally, there are two primary

reasons to design a protective matrix for phage: 1) delivery to the gut, and 2) stabilization for long term storage (Table 2).

Stabilization of a phage for long term room temperature storage reduces the cost of maintaining refrigerated phage. These dry formats can also be simpler to ship as they do not require refrigeration and have less weight than their liquid counterpart. Stabilization within a dry format can also prolong a high titer of phage. Generally, a polysaccharide or disaccharide is used to stabilize the phage. Mechanisms of stabilization with sugars is discussed elswewhere[65]. For phage products designed for delivery to the gut, the protective matrix must be designed to withstand passage through the stomach. Eudragit can be used as a pH-sensitive coating on encapsulated phage[66,67] wherein the particles remain intact within the acidic stomach environment and dissolve in the basic intestinal tract.

Encapsulation of phage within protective matrices can also protect against specific stressors found in food processing. An example would be thermal treatments, such as pasteurization, that are used to eliminate pathogens in milk, liquid egg products, juices, and canned goods[68]. However, many phages of foodborne pathogens cannot withstand the temperatures used in these thermal treatments[28]. Chapter 3 investigates a protective matrix for commercial phage that can withstand pasteurization. The excipient mixture was designed to minimize phage mobility during the heat exposure within an aqueous environment.

Conversion to a dry format through encapsulation can be completed when integration of a liquid phage product is not feasible. For instance, Leung et al. [69] and Kamali et al. [70] encapsulated phages into film preparations that were suitable for use with meat packaging. Similarly, Chapter 4 investigates a phage powder preparation suitable for direct inclusion with powdered infant formula. The phage powder was designed for long term stability. The safety of the excipient system was also considered given that the target population was infants. Given the vulnerability of the population, care was taken to only consider stabilizing excipients that are already present in infant formula.

Table 2 Summary of studies investigating the encapsulation of phages for food industry application

Encapsulation Method	Bacteriophage	Excipient system	Application	Ref.
Freeze drying	-Anti-*cronobacter* SG01	Collagen peptide-trehalose	Direct inclusion with powdered infant formula for long term dry storage	[71]
Air drying	-Anti-*listeria* P100 -Anti-*E. coli* AG10 -Anti-*salmonella* CG4	Pullulan-trehalose	Long term dry storage, anti-bacterial food packaging for ready-to-eat meat	[69]
Vacuum drying	-Anti-*listeria* P100	Pullulan-trehalose	Long term dry storage	[72]
Spray drying	-Anti-*campylobacter* CP30A	Trileucine-trehalose Pullulan-trehalose Leucine-trehalose	Long term dry storage	[73]
Air drying	-Anti-*listeria* A511	Whey protein concentrate-pullulan-Poly (lactic) acid	Anti-bacterial food packaging for raw chicken	[70]
Spray drying	Anti-*E. coli* cocktail -ΦJLA23 -ΦKP26 -ΦC119 -ΦE142	Modified starch-maltodextrin-SM buffer	Protection against hot and freezing temperatures, protection against UV exposure. Reduction of bacteria accomplished when tested on tomatoes.	[37]
Gelation	Anti-*salmonella* cocktail -Felix O1 -14 isolated wildtype phages (PEW 1-14)	Alginate-calcium-zinc	Microencapsulation of phage for addition to animal water or feed. Reduction of *Salmonella* found when administering phage to pigs prior to introduction to a *Salmonella* positive environment mimicking a slaughterhouse	[74]
Spray drying	Anti-*salmonella* cocktail -SPFM10 -SPFM14	Trehalose-mannitol-leucine-Euradagit S100	Pelleted powders to be mixed with animal feed to reduce pork contamination. Prophylactic administration to piglets significantly reduced gut *salmonella* colonization	[66]
Spray drying	-Anti-*pseudomonas* PEV20	Leucine-lactose	Tableted powders for oral delivery to target intestinal bacterial infections	[75]
Gelation	Anti-*salmonella* cocktail -UAB_Phi20 -UAB_Phi78 -UAB_Phi87	Alginate-$CaCO_3$	Microcapsules for oral delivery to target intestine of chickens. Protection against stomach acid.	[76]
Spray drying Freeze drying Electrospraying	-Anti-*salmonella* Felix O1	Whey protein isolate-inulin-gum arabic-tween 80	Tableted powders for oral delivery to target intestinal bacterial infections, long term dry storage	[77]

Spray drying	-Anti-*salmonella* Felix O1	Eudragit S100-trehalose	Tableted powders for oral delivery to target intestinal bacterial infections	[67]
Freeze drying	Anti-*salmonella* cocktail: -SLP004 -SLP005 -SLP050	Whey protein isolate-trehalose	Long term refrigerated storage, easy addition to foods	[78]
Spray drying	-Anti-*E. coli* K5	Alginate-calcium-milk protein	Long term dry refrigerated storage, inclusion in dry and semi-solid products – specifically in cereal products. Unstable in milk and milk products.	[79]
Freeze drying	-Anti-*E. coli* T7	Polyvinylpyrrolidone-trehalose	Colorimetric detection of *E. coli* in food matrices	[80]
Freeze drying	-Anti-*staphylococcus* Team1	Maltodextrin-glycerol	Concentration of phage, long term dry storage	[81]
Freeze drying	-Anti-*salmonella* SPT 015	Whey protein isolate-trehalose	Protection against temperature and pH exposure	[82]
Freeze drying Spray drying	Anti-*staphylococcus* cocktail -phiIPLA35 -phiIPLA88 -phiIPLA-RODI -phiIPLA-C1C	Trehalose-skim milk	Long term dry storage	[83]
Air drying of microspheres	-Anti-*staphylococcus* Phage K	Maltose	Oral delivery to target intestinal bacterial infections	[84]

1.5. Conclusion

Phages are an effective tool that can be applied to food safety to improve public health. The ability to select phages that eliminate pathogenic bacteria but remain harmless to beneficial bacteria is not shared by traditional food sterilization procedures, including chemical sanitizers, thermal treatments, and preservatives. Their lack of affect on nutritional and organoleptic is also useful for food applications. However, phage sensitivity to certain food properties and food processes, such as exposure to high heat, low pH, UV, or their inability as liquid preparations to be used with dry/powdered foods, limits their widespread use.

The remainder of this work summarizes the efforts to overcome barriers to phage application in the food supply chain through two strategies: adaptational evolution and encapsulation within protective matrices. Chapter 2 investigates adaptational evolution as a method of preparing resistant populations of a commercially available phage product. Chapter 3 investigates the preparation of a protective matrix for protection of phage during pasteurization. Chapter 4 investigates the preparation of a protective matrix for the inclusion of phage in powdered infant formula. The work in this thesis is comprised of published manuscripts and manuscripts in preparation for submission.

1.6. Research Objectives

The following objectives were pursued in this thesis:

Chapter 2

Adapational evolution as applied to a commercial phage product to obtain populations with improved survival to generic stressors for intended application in the food production industry: desiccation, heat, and low pH exposure. The adapted phage populations were expected to undergo shifts in genome with each subsequent generation upon repeated stress. The adapted phage populations with improved survival are expected to translate to improved application within realistic food processing conditions.

Chapter 3

Developing of a food-safe matrix that could be used to protect bacteriophage against heat treatment within an aqueous environment. The food-safe matrix needed to be designed to compose only of sugars to allow application in milk. The encapsulated phage were expected to be protected from significant loss during pasteurization within milk through the designed stabilization matrix.

Chapter 4

Developing an infant-safe powder that could be used to encapsulate phage to allow for direct inclusion with powdered infant formula. The powder needed to be designed using only ingredients found in powdered infant formula, and be compatible with the production, composition, pH, and storage of powdered infant formula.

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Chapter 2: Stress exposure of evolved bacteriophages under laboratory versus food processing conditions highlights challenges in translatability

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Preface

In this chapter, we consider the limitations of bacteriophages (phages) in food applications and the possibility of improving applicability through selection. Phages may be useful in reducing bacterial contamination of food, however, phages are sensitive to many environments commonly found in food processing. We explore the use of adaptational evolution, wherein phage populations are subjected to stresses and only surviving phages are propagated, to create stress-resistant populations of the phage. The later populations showed clear resilience to a given stress as compared to their ancestors when tested under laboratory conditions. However, we found that this improvement in resilience under laboratory conditions did not translate to success under real-world conditions. This chapter establishes that while phages can be selected for certain phenotypic traits, this will not necessarily lead to applicability in the real world. We point out that, unlike laboratory conditions, realistic conditions are much more complex with many possible stress factors. The results of this chapter motivated the remainder of this thesis. Rather than pursuing methods to directly improve stability of phage populations, we focused on creating protective food safe matrices. These temporary matrices would allow phages to be applied in environments wherein unprotected phages would otherwise degrade.

This chapter is a reformatted version of the published manuscript, with minor text changes due to external examiner feedback. I was responsible for experimental design, data collection, and data analysis in the quantification of bacteriophage infectivity and comparison of application performance sections, and I contributed to data analysis of the gene sequencing section. I was also responsible for the manuscript composition. Alexandra Szewczyk was responsible for the experimental design, data collection, and data analysis for bacteriophage selection, reversion-testing, and DNA extraction and contributed to manuscript edits. Jake Szamosi was responsible for experimental design, data collection, and data analysis for the gene sequencing section and contributed to manuscript edits. Vincent Leung assisted with concept development, experimental design, and contributed to manuscript edits. Carlos Filipe and Zeinab Hosseinidoust were supervisory authors who were involved in concept formation, discussion of results, and manuscript edits.

Citation

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Abstract

Bacterial viruses, or bacteriophages, are highly potent, target-specific antimicrobials. Bacteriophages can be safely applied along the food production chain to aid control of foodborne pathogens. However, bacteriophages are often sensitive to the environments encountered in food matrices and under processing conditions, thus limiting their applicability. We sought to address this challenge by exposing commercially available Listeria monocytogenes bacteriophage, P100, to three stress conditions: desiccation, elevated temperature, and low pH, to select for stress-resistant bacteriophages. The stressed bacteriophage populations lost up to 5.1 log10 in infectivity; however, the surviving subpopulation retained their stress-resistant phenotype through five passages with a maximum of 2.0 log10 loss in infectivity when exposed to the same stressor. Sequencing identified key mutation regions but did not reveal a clear mechanism of resistance. The stress-selected bacteriophage populations effectively suppressed L. monocytogenes growth at a modest multiplicity of infection of 0.35-0.43, indicating no trade-off in lytic ability in return for improved survivability. The stressed subpopulations were tested for survival on food grade stainless steel, milk pasteurization, and within acidic beverages. Interestingly, air drying on stainless steel and pasteurization in milk led to significantly less stress and titer loss in bacteriophage compared to similar stress under model lab conditions. This led to a diminished benefit for stress-selection, thus highlighting a major challenge in real-life translatability of bacteriophage adaptational evolution.

Keywords: virus; adaptational evolution; food safety; stability; bacteriophage application; resistance

2.1. Introduction

Listeriosis is an infection caused by exposure to the foodborne pathogen, *Listeria monocytogenes*. Although *L. monocytogenes* has a relatively low incidence rate, its high mortality rate (20-30%) [1,2] and its implication in numerous worldwide outbreaks [3,4]. makes it a pathogen of significant concern. Listeriosis may be caused by ingesting contaminated foods that do not require cooking prior to consumption, such as milk/milk products, fruit and vegetables, and ready-to-eat meats. Foods at high contamination risk are subjected to processing procedures to prevent the transmission of foodborne pathogens, with subsequent refrigeration being strongly recommended to slow bacterial growth. However, *Listeria* has been shown to be resistant to, or capable of adapting to, several food-preservation methods (e.g., osmotic stress from high salt concentrations [5–7], desiccation [8,9], low pH [5,6], UV light [5,7], and heating [7]), and is able to grow under refrigerated temperatures [10].

The use of bacteriophages, viruses that infect bacteria, has shown promise as a method of preventing/tackling bacterial contamination in the food production chain [11–14]. Bacteriophage, or phage for short, treatments are highly specific in targeting their host bacteria [15], thus minimizing their effect on the natural human biome. Phage preparations lack a specific taste or smell [16], and, because of their high specificity, may be designed so as to not interfere with beneficial bacteria in food products. Therefore, phage biocontrol can be used at every stage of the food production chain, from farm to market to consumer, without interfering with food quality or palatability. Whether applied as a prophylactic or as a disinfectant, phages will be exposed to harsh environmental conditions through physicochemical conditions in the food matrix, the food processing procedure, or during storage. Conditions such as low pH (common in fruits and fruit juice) [17], elevated temperatures (even at a relatively low 65 °C) [18], and desiccation [19] have all been demonstrated to reduce the concentration of viable phage (and hence antimicrobial activity) significantly and drastically. We have previously reported an effective method of stabilizing phages against desiccation in solid form [19,20]. This method, however, cannot be generalized to offer protection against other environmental stressors. In this work, we investigate adaptational evolution as a method to select phage populations resistant to a given stressor. The subjection of phage preparations to cycles of a certain stress condition has been effectively used to generate phage populations that are more resistant to UV light [21] and heat treatment [22,23] compared to the ancestral population. Selection cycles conducted on bacterial cultures infected with phage have resulted in the selection of phages with improved lytic ability such that amplification was observed at temperatures that would have been inhibitory to the wild type [24]. However, many of these studies have been conducted under idealized laboratory conditions and their results have not been confirmed under realistic environments.

Phages have proven to be an effective intervention strategy for controlling *L. monocytogenes*. In 2006, Listshield, a phage cocktail designed to control *L. monocytogenes* [25], became the first phage-based commercial food-safety product to receive FDA approval [26]. Since then, multiple phage products have been commercialized for biocontrol in food. Listex is of these commercial products [27–29]. Listex, unlike many other commercial products that are formulated as cocktails, consists of P100 phage as its sole strain. In this study, *Listeria* phage P100 was exposed to three common environmental stressors—vacuum desiccation, heating, and low pH—to select for phages with

strong resistance to these stressors. We investigated the reversion rates of stress-selected populations, their genomic sequencing, and their biocontrol ability. Finally, the reversion-tested phage populations were tested under three food applications that exposed phage to the investigated stressor and would thus benefit from increased phage survivability. The survival of the reversion-tested phages was compared to that of the P100 stock under these realistic conditions.

2.2. Materials and Methods

2.2.1. Bacteria and Bacteriophage Strains

The commercially available *Listeria* phage, Listex P100, was purchased from Micreos BV (The Hague, Netherlands). The stock Listex P100 titre contained 2×10^{11} plaque-forming units (PFU) per mL as reported by the manufacturer and verified prior to experimentation.

Listeria monocytogenes serotype 1/2a (ATCC Number: BAA-2659), which was used as the propagation phage host, and *Listeria monocytogenes* serotype 4b, which was supplied by the Canadian Food Inspection Agency, were both used to construct lytic curves. The genome for the latter has been sequenced and deposited to the NCBI under Ascension number JAJOHJ000000000.

2.2.2. Chemicals

The tryptic soy broth (TSB), agar, Tris base, and agarose used in this research were purchased from Fisher Bioreagents (Ottawa, ON, Canada), while gelatin was obtained from Sigma Aldrich (Oakville, ON, Canada). Yeast extract (YE), magnesium sulfate, calcium chloride, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from VWR (Mississauga, ON, Canada). TSB (30 g/L) or TSB+0.6% (w/v) YE was used as the growth media. Agar plates were prepared using 30 g/L and 15 g/L. Calcium-magnesium (CM) buffer with a pH of 7.5 was prepared from 5 mM CaCl2•2H2O, 20 mM MgSO4•7H2O, 6 mM Tris-Cl (pH 7.5), 0.05 g/L gelatin. The buffer was prepared with MilliQ water and the media were prepared with deionized water. All buffers and media were steam-sterilized prior to use.

2.2.3. Bacteria culture, bacteriophage propagation, and quantification

All bacterial *L. monocytogenes* strains were grown in TSB or TSB+0.6% YE. The cultures were prepared by inoculating from frozen stocks and incubating overnight in a shaking incubator at 37 °C and 180 RPM, and the resultant bacterial concentrations were determined using the standard colony count method. Briefly, we plate bacterial suspension on agar plates , incubate overnight at 30 °C, and count the number of colonies that show up on the plate to determine the number of cell-forming units (CFU).

Phage propagation was performed by growing the phage suspension after exposure on a standard plaque overlay [30] assay using *L. monocytogenes* serotype 1/2a as the phage host. Briefly, 100 µL of a given overnight bacterial culture was added to a tube containing 3 mL of soft agar (30 g/L TSB, 5 g/L agarose), and mixed via vortexing. Next, 100 µL of the serially diluted phage was added to the tube and the infected culture was vortexed once more before being poured onto a TSB-agar plate. The soft agar plates were incubated overnight at 30 °C. The resulting plaques were isolated by removing the plaque-containing soft agar layer from the overlay plate with an L-shaped cell spreader and relocating it to a 50 mL culture tube, followed by the addition of 1 mL of CM buffer to elute the phage from the soft agar layer. Chloroform (3 µL) was then added to disrupt the

bacterial cell membranes and release any remaining encapsulated phage particles. The phage-containing soft agar was left to elute for 15 min at room temperature before the phage–containing liquid portion was separated from the solids via centrifugation at 7000×g for 5 min. The phage suspension was quantified by serially diluting the phage as necessary in CM buffer and plating. The phage samples were quantified using the previously described standard plaque overlay method, where number of plaque forming units (PFU) on each plate was determined after overnight incubation at 30 °C. Exposure trials were then repeated with the newly selected and propagated phage population.

2.2.4. Bacteriophage selection under stress and reversion-testing

Listex P100 phage from a single stock ($2x10^{11}$ PFU/ml) was exposed to one of three environmental stresses—desiccation, elevated temperature, and low pH. The selected phages were then propagated non-selectively and quantified before undergoing the subsequent stress cycle, as illustrated in Figure 1. In total, five cycles of selection were completed, with one cycle consisting of exposure, selection, and propagation. Random samples of the selected phages underwent an additional five unstressed cycles of propagation to assess reversion to previous levels of resistance. The surviving phages were propagated by growing the phage suspension after exposure on a standard plaque overlay assay [30] using *L. monocytogenes* serotype 1/2a as the phage host followed by quantification using the standard PFU count method. Experiments were performed in triplicate to confirm that a selected improvement in survivability could be replicated.

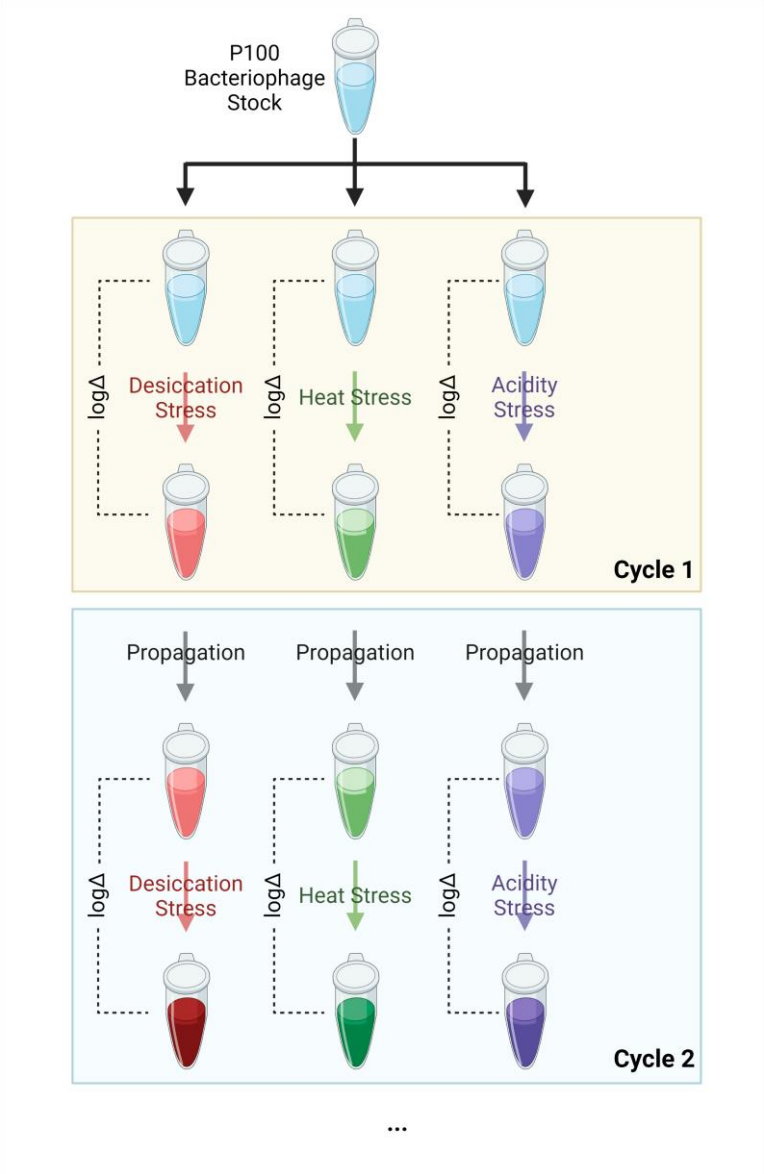

Figure 1. Schematic illustrating the stress-propagation cycle conducted on the P100 bacteriophage samples. The samples underwent five selection cycles to select for bacteriophages that were resistant to either desiccation, heat, or acidic (low pH) conditions. Each color (red, green, and purple) represents a certain collection of random mutations in phage that provide resistance to a certain stressor. Color intensity represents the process of theoretical enrichment of stress resistant phage from the stock of wild type phage which contains a mixture of random mutations. All experiments were completed in three biological replicates. Figure created with Biorender.com.

Vacuum desiccation

A 50 µL aliquot of the stock was added to a microtube and placed in a vacuum desiccator, which was then run continuously for 4 h. Following desiccation, the remaining film in the microtube was re-suspended in 500 µL of CM buffer.

Elevated temperature

A 100 µL aliquot of the stock phage aliquots was placed in a microtube heater heated to 60°C for 1 h, following which the phage suspension was cooled.

Low pH

1 M HCl and water was added to 100 µL stock phage to a final concentration of 2 mM HCl and final volume of 1 mL, yielding a pH of 2.65 based on molar concentration. The suspension was then incubated at room temperature for 1 h before being neutralized with equimolar quantities of 1 M NaOH.

The phage samples that were subjected to 5 cycles of desiccation stress, high temperature stress, and low pH stress are subsequently referred to S5D, S5T, and S5pH, respectively. After the fifth selection cycle for each stress condition, the sample was plated using a soft agar overlay and re-propagated five times in the absence of the stressor. These samples were then subjected to the same stress conditions given above and titer loss was quantified to determine whether the phages had reverted to their previously susceptible state. These reversion-tested phage samples are henceforth referred to as R5D, R5T, and R5pH, which correspond to the desiccation, high-temperature, and low-pH stress conditions, respectively. All experiments were performed in triplicate and in succession to obtain biological triplicate experiments. Phage titer before and after stress exposure was reported. Magnitude of improvement of phage survivability was calculated by calculating the titer reduction on exposure to a given stress. This titer reduction was then averaged across the replicates.

2.2.5. Bacteria DNA Extraction and Sequencing

The DNA from the *Listeria* serotype 4b sample of unknown strain was extracted using a DNA elution kit (GenElute Bacterial Genomic DNA Kit; Sigma Aldrich; Oakville, ON, Canada) in accordance with the manufacturer's protocol. Analysis of the eluted DNA sample with a plate reader (Synergy Neo2; Biotek; Winooski, VT, USA) determined that the *L. monocytogenes* serotype 4b sample contained DNA concentration of 30 ng/uL. The complete genome was sequenced using Illumina MiSeq™ System Next Generation Sequencing (NGS), with a genome coverage of 15×. The genome was assembled using the SPAdes genome assembler [31] and then uploaded to the NCBI under Ascension number JAJOHJ000000000.

2.2.6. Bacteriophage DNA Extraction and Sequencing

A modified phenol-chloroform extraction method [32] was used to isolate phage DNA from the fifth selection cycles and the final reversion cycles. The phage samples were first filtered using a 0.22 µm syringe filter and 500 µL of the filtered sample was used for DNA extraction. To remove residual bacterial DNA, 0.5 µL of 0.5 mg/mL DNase, 0.5 µL of 12.5 mg/mL RNase, and 5 µL of 1 M MgSO4 were added to the samples and allowed to incubate at 37°C for 1 hr. The DNase and RNase were deactivated by heating the samples in a water bath for 10 min at 65°C. Proteinase K (10 µL of 10 mg/mL), $CaCl_2$ (15 µL of 0.1 M), and SDS (120 µL of 10 % w/v) were then added to the phage samples and left overnight on a 55°C heating block to break down the phage capsid. One volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added to the treated-phage samples, vortexed briefly, and then centrifuged at 22,000×g for 10 min. Following centrifugation, 400 µL of the aqueous layer was extracted in 100 µL increments to ensure that the organic layer was left undisturbed. Next, chloroform was added to the phenol-chloroform aqueous extraction at a 2:1 volumetric ratio. The resultant solution was then vortexed and centrifuged as previously described. Once again, 300 µL of the aqueous layer was extracted in 100 µL increments following

centrifugation. 3 µL of 3 M ammonium acetate and a 2:1 volumetric ratio of undiluted anhydrous ethanol, which had been stored at -20 °C, were then added to the second extraction and left to sit for 1 h in a freezer set to -20 °C. Following incubation, the solution was centrifuged at 22,000×g for 10 min at 4 °C. The supernatant was then discarded, and the pellet was resuspended in 70% ethanol and stored at -4 °C. After brief vortexing, the solution was centrifuged for a final time and the pellet was isolated. The microtube was then left open in a biosafety cabinet for about 1 h to evaporate any residual ethanol. The remaining DNA pellet was then resuspended in 50 µL of buffer.

In total, seven DNA samples were isolated: the ancestral P100 stock, S5D, S5T, S5pH, R5D, R5T, and R5pH. The DNA from biological replicates were mixed for sequencing. The DNA samples were submitted to the McMaster Mobix Lab for sequencing and genomic analysis. Sequencing was performed using Illumina MiSeq™ System Next Generation Sequencing (NGS), with a read length of 2×250 bp and a total coverage of 5% to 0.7% per sample. The genome was aligned, and mutations were identified using the *breseq* genomic analysis pipeline [33]. The identified mutations were then compared to a reference P100 genome obtained from NCBI Accession NC_007610.1 [34]. Fixation mutations were excluded from analysis.

The relevant gene products (gp39, gp40, gp102, gp108) were entered into the NCBI BLASTp system [35] to determine analogous proteins. Only matches with Expect (E) values less than 2.00×10^{-16} and % identities of greater than 80% were included. Additionally, there were two critical mutations at positions 95862 and 95893 that fell within the noncoding region from position 95,836-95,947. This region was cross-referenced in the BLASTn system through Geneious Prime (San Diego, CA, USA) to search for similar regions in other phages.

2.2.7. Quantification of bacteriophage infectivity

The ability of the reversion tested P100 phage samples to infect *L. monocytogenes* was compared to the ancestral P100 stock through evaluation of plaque morphology and kill curves. These experiments were performed on P100 stock and each of the three biological replicates of the R5D, R5H, and R5pH samples, for a total of ten phage samples.

Efficiency of plaquing and plaque morphology

An efficiency of plaquing assay was conducted on the *L. monocytogenes* serotype 1/2a and 4b samples using standard soft-agar overlay [30] to assess whether the reversion-tested phages exhibited reduced strain infectivity as compared to the ancestral P100 stock. The plaque count exhibited on *L. monocytogenes* serotype 4b hosts was normalized for each phage sample by the plaque count exhibited on the *L. monocytogenes* serotype 1/2a host to determine if host infectivity changed as a result of the selection process. All experiments were completed in triplicate. Plates were imaged and plaque morphology was compared using ImageJ (U.S. National Institutes of Health; Bethesda, MD, USA).

Kill curves

A growth kinetic profile of *L. monocytogenes* infected with the phages was conducted to compare the lytic ability of the reversion-tested phages to the ancestral P100 stock. Lytic curves were obtained for both the *L. monocytogenes* 1/2a and 4b serotypes. The kill curves were constructed

using a plate reader (Synergy Neo2; Biotek; Winooski, VT, USA) to measure bacterial concentration, terms of the culture optical density (OD_{600}), over time. Subcultures of the *L. monocytogenes* ½a and 4b serotypes were first grown to an approximate OD_{600} of 0.4-0.6m,, such that the bacteria were in exponential growth phase. Bacterial subculture was diluted in TSB media and added to a flat bottom 96-well plate (353072; Corning; Corning, NY, USA). Initial OD_{600} was approximately 0.2, corresponding to $3.1\pm0.3x10^8$ CFU/mL and $2.8\pm0.1x10^8$ CFU/mL for the 1/2a and 4b serotypes, respectively. Phages were subsequently added to the wells at a multiplicity of infection (MOI) of 0.37 and 0.41 for the 1/2a and 4b serotypes, respectively, with three wells of bacterial subculture left uninfected as a control. The optical density was recorded over 14 h to provide a relatively short-term comparison of stock P100 performance against the reversion-tested phages. Readings occurred every 10 min where incubation temperature was set to 30 °C with continuous agitation of the well plate. The generation time of the cultures was determined by fitting an exponential trendline to the exponential growth sections of each curve. The fit, as measured by the coefficient of determination R^2, of these exponential trendlines ranged from 0.94-0.99.

2.2.8. Comparison of application performance

Bacteriophage survival on stainless steel surfaces

The P100 stock and desiccation-selected R5D samples were tested under conditions that mimic the sanitation of food-contact surfaces to determine potential differences in survival. P100 and R5D were diluted to $1x10^9$ PFU/ml in media (TSB+ 0.6% YE) and 100 µL of each sample was separately applied to a 2 cm x 2 cm sheet of food-grade 316 stainless steel (88885K71; McMaster-Carr, Chicago, IL, USA). Three biological replicates were prepared for both the P100 stock and the R5D samples. The phage treated stainless steel sheets were then left to air dry for 24 h. Recovery of the phage from the stainless steel was based on procedures given elsewhere [36,37]. Briefly, the dried, phage-treated stainless-steel sheets were transferred to a sterile conical tube containing 10 mL of CM buffer and a small amount of sterile glass beads. The conical tube was then vortexed for exactly 2 min. The resulting suspension was then serially diluted. Enumeration of surviving P100 was completed using a small scale soft agar overlay method [38], wherein 20 µL of the diluted suspension was plated onto a *L. monocytogenes* bacterial lawn and tilted to run down the length of the plate. Phage enumeration accounted for sampling dilution.

Bacteriophage survival within milk after batch pasteurization

The Canadian Food Inspection Agency's general guideline for the batch pasteurization, also known as low temperature long time pasteurization, of milk-based products below 10% fat recommends processing at 63 °C for 30 min to eliminate foodborne pathogens [39]. The ability of the reversion-tested heat-selected R5T samples and ancestral P100 samples to survive batch pasteurization was compared. All samples were first diluted to $1x10^9$ PFU/mL in TSB+0.6% YE. 100 µL of a given phage sample was subsequently added to 900 µL of commercial 2% milk (purchased from a local grocery store) within a 1.5 mL Eppendorf tube to obtain a concentration of $1x10^8$ PFU/mL. This phage concentration is recommended in pasteurized milk for the production of queso fresco cheese [40]. The phage-containing milk samples were then placed within a water bath (76308-896; VWR, Mississauga, ON, Canada) set to 63 °C. A water bath was used instead of a dry bath to simulate relatively fast heating time. After 30 min the samples were immediately transferred to an ice bath to mimic the rapid cooling step of pasteurization. After 15

min the phage samples were then serially diluted and plated using the small-scale soft agar overlay method.

Bacteriophage survival in apple juice

Survival of P100 stock and the acid-stressed R5pH samples was tested in commercially available apple juice (purchased from a local grocery store). The pH of the apple juice prior to experiments was measured to be 3.5 (SevenExcellence pH Meter; Mettler Toledo, Port Melbourne, Australia). Phage samples were first diluted to $1x10^9$ PFU/ml within TSB+0.6% YE media to minimize the influence of any buffers or different volumes. 100 µL of these diluted samples were then added to 900 µl of commercial apple juice to obtain an initial concentration of $1x10^8$ PFU/ml. Concentration of the phage within the apple juice was then measured after 1 h and after 6 h using the aforementioned soft agar overlay method. Experiments were repeated thrice to obtain biological triplicates.

2.2.9. Statistical Analysis

All experiments were performed in triplicate. Statistical analysis was performed using a two-tailed paired t-test through GraphPad Prism 9 (GraphPad Software; San Diego, CA, USA), where viral and bacterial concentration was log-transformed prior to analysis.

2.1. Results and Discussion

2.3.1. Enhanced stress resistance and low reversion rates for selected bacteriophage

Listex P100 phage was selected for survival when exposed to one of three environmental stresses – desiccation, elevated temperature, and low pH. The selection process consisted of exposing phage from a single stock to a given stress and subsequently propagating the selected phage.

Figure 2 compares the number of infective phage virions in the P100 stock, stress-selected phage samples, and reversion-tested phage samples before and after exposure to the different stress conditions: desiccation (Figure 2A), elevated temperature (Figure 2B), and low pH (Figure 2C). Infective phage concentration was measured in terms of PFU/ml, where the differences between the concentrations before and after exposure indicate the sample's resistance to the given stress, with greater reduction indicating lower resistance.

The trend in phage survivability over the selection cycles differed between the stress conditions. The phage samples selected for desiccation resistance (Figure S1) showed significant improvement in survivability to desiccation exposure after only one cycle of selection, with subsequent cycles of selection showing little to no further improvement. On the other hand, the phage samples selected for elevated temperature resistance (Figure S2) showed that the concentration of surviving phage after temperature exposure appeared to increase with every successive selection cycle. However, the trend of improved survivability appears to plateau with further selection cycles. Like the samples selected for desiccation resistance, the sample selected for low pH resistance (Figure S3) demonstrated significant improvement in survivability to low pH exposure after only one cycle (0 $\log_{10}$ loss) that was maintained over the successive cycles of selection. The first cycle likely

resulted in the inactivation of phages with detrimental mutations for survival against a given stress in all cases.

The ancestral P100 sample, which did not undergo selection beforehand, was clearly the most susceptible to the different stress conditions, with reductions in plaque forming units of 4.7, 5.1, and 2.9 $\log_{10}$ following exposure to the desiccation, high-temperature, and low-pH stress conditions, respectively. P100 has been previously reported to experience significant titer loss when exposed to a pH of 3 (>5 $\log_{10}$) [17], to a temperature of 65 °C (4 $\log_{10}$), which is within pasteurization temperature ranges [18], or to desiccation [19]. Five successive stress cycles were performed to isolate and select P100 phages resistant to either desiccation, elevated temperatures, or low pH. The phages selected via this approach exhibited greater survivability in comparison to the ancestral P100 stock under the same stress conditions (Figure 2). The S5D, S5T, and S5pH samples, which previously underwent 5 cycles of selection for resistance against a given stress, experienced titer reductions of only 1.8, 1.8, and 0 $\log_{10}$, respectively.

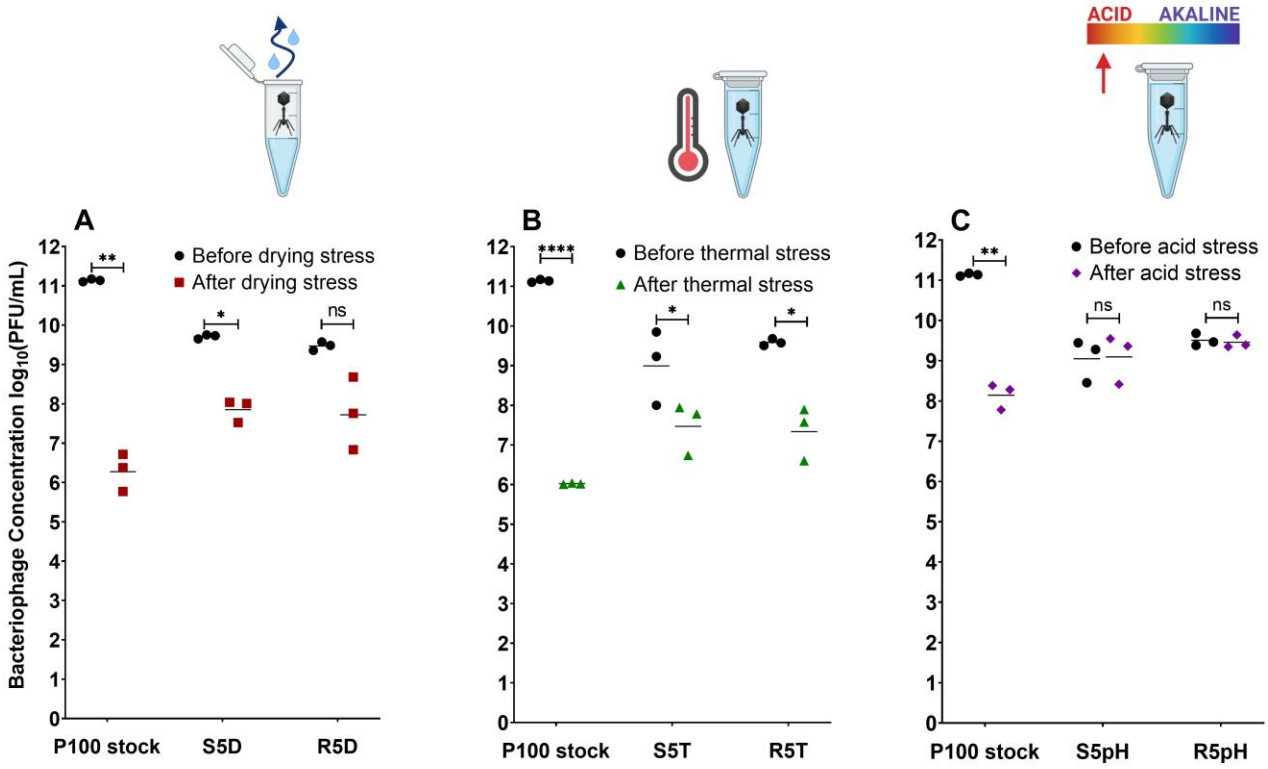

Figure 2. Measured concentrations of viable phage in the P100 stock, a sample that has undergone 5 cycles of selection for a given stress, and a sample that has undergone 5 cycles of selection followed by 5 cycles of propagation without exposure to stress before and after exposure to: A) desiccation for 4 h; B) temperatures of 60 °C of 1 hr; and C) estimated pH of 2.65 for 1 hr. Results are shown as the individual results of triplicate experiment, where the geometric mean has been marked. Phage concentration prior to exposure presented in black and concentration after exposure presented in colour. Significant differences in phage concentration before and after exposure to a given stress are marked as * - $p \leq 0.05$, ** - $p \leq 0.01$, and **** - $p \leq 0.0001$. Nomenclature: n.s.: not significant; S5D: P100 sample that underwent 5 cycles of desiccation selection; S5T: P100 sample that underwent 5 cycles of elevated temperature selection; S5pH: P100 sample that underwent 5 cycles of low-pH selection; R5D: S5D sample that was propagated 5 times in the absence of stress; R5T: S5T sample that was propagated 5 times in the absence of stress; R5pH: S5pH sample that was propagated 5 times in the absence of stress. Icons from Biorender.com.

The selected R5D, R5T, and R5pH phage samples, which are the later generations of the selected samples that have been grown in the absence of stress for 5 cycles, experienced titer reductions of 1.2, 2.0, and 0 $\log_{10}$, respectively. These reversion-tested samples exhibited similar infectivity to the selected population, suggesting that the reversion-tested populations did not suffer complete reversion in potential beneficial mutations that made them stress-resistant even in the absence of the stress condition. In summary, all stress-selected and reversion-tested samples showed viable phage retention upon exposure to a given stress that was greater compared to the ancestral P100 stock. Direct comparisons to calculate the difference in magnitude of retention between the selection cycles cannot be made as the concentrations prior to exposure differed between samples. However, in all cases the titer endpoint of the ancestral phage was at least an order of magnitude lower than the stress-selected and reversion-tested phage populations. This result occurred despite the ancestral populations starting with a higher initial concentration, suggesting that the stress selected, and reversion selected samples have improved resistance to the relevant stress.

The plaque assay method determines concentration of phage in terms of plaque forming units, and thus our data clearly demonstrated that stress-selected phage populations retain a significantly higher concentration of phage that survive and are infective against *L. monocytogenes* following stress exposure. The greatest improvement in survivability was observed among the stress-selected and reversion-tested low-pH populations (Figure 2C), which showed no statistically significant reduction in titer before and after the reversion test (p>0.05). Conversely, a decrease in viable phage titer was still observed in all samples exposed to desiccation (Figure 2A) or elevated temperatures (Figure 2B). Regardless, improvement in titer retention ensures that there is a larger amount of phage available to control a bacterial contamination. Notably, the potentially elevated P100 concentrations in food products due to increased survivability of the resistant populations is not cause for concern, as P100 only infects bacteria and does not transduce bacterial DNA [41]. Furthermore, an oral toxicity study in rats did not reveal any negative effects resulting from the ingestion of P100 [27].

2.3.2. Genomic sequences of stress-selected samples and reversion-tested samples

The genomes of the ancestral P100 stock, the stress-selected phages, and the reversion-tested phages were sequenced and aligned prior to comparison with a P100 reference genome [34]. The ancestral P100 stock was included in this analysis to account for potential mutations that occurred due to a bottlenecking effect, wherein genetic drift occurs due to the severe reduction in population resulting in decreased genetic diversity. A total of 28 mutations as compared to the refence genome were determined to be fixation mutations and were excluded from further analysis. Figure 3 shows the comparison of the P100 stock and both the stress-selected and reversion-tested samples with respect to the location, type, and frequency of the observed mutations. Mutation frequency is defined here as the percentage that a given mutation occurs within the sample data, where samples consist of DNA picked from three biological replicates. The location and type of mutation are shown in the left axis, where "-" refers to a deletion, "+" refers to an insertion, and "r" refers to a repeated segment. The hypothetical gene protein corresponding to each mutation is annotated on the right axis, according to information published for the P100 genome [27]. At this point the functions for many P100 gene proteins are unknown.

Both the stress-selected phage samples and the reversion-tested samples exhibited comparably improved resistance to a given stress. Therefore, mutations occurring with high frequencies in both the stress-selected and the reversion-tested samples, where the mutations also occur in low frequencies in the ancestral P100 stock, may be responsible for the increased resistance to a given stress. Sequencing was completed on samples that were picked from the three biological replicates. The biological replicates display similar levels of resistance to a given stress phenotypically. However, as they evolve independently of each other, the biological replicates may have selected for different mutations over the selection process, leading to different mechanisms of resistance between the replicates.

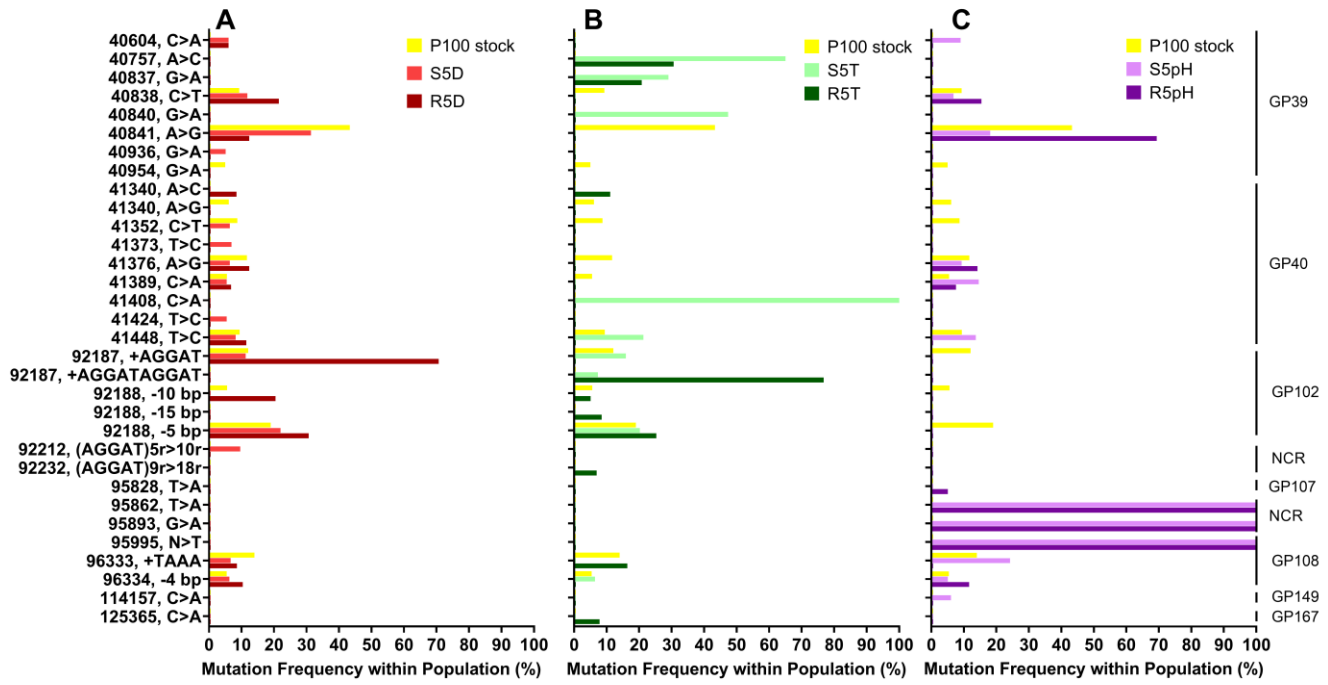

Figure 3. Mutation frequency for the P100 stock (yellow) and A) the desiccation-selected S5D (light red), R5D (dark red) samples, B) the heat-selected S5T (light green), R5T (dark green) samples, and C) the acid-selected S5pH (light purple), and R5pH (dark purple) samples as compared to a reference P100 genome [34]. The location and type of mutation are shown in the left axis, where "-" refers to a deletion, "+" refers to an insertion, and "r" refers to a repeated segment. The hypothetical gene protein corresponding to each mutation is annotated on the far right. Abbreviations: GP: gene product; NCR: non-coding region. Nomenclature: S5D: P100 sample that underwent 5 cycles of desiccation selection; R5D: S5D sample that was propagated 5 times in the absence of stress, S5T: P100 sample that underwent 5 cycles of high-temperature selection; R5T: S5T sample that was propagated 5 times in the absence of stress, S5pH: P100 sample that underwent 5 cycles of 5 of low-pH selection; R5pH: S5pH sample that was propagated 5 times in the absence of stress.

There are no mutations with similarly high levels of frequency in both the samples that had undergone 5 cycles of desiccation selection and 5 cycles of reversion (*i.e.*, S5D and R5D), as shown in Figure 3A. The most significant mutation affected gp102, specifically the insertion of an AGGAT sequence at location 92187. This mutation was present at a frequency of 71% in the reversion-tested sample, R5D, but was present in lower frequencies (10%) for the S5D sample. The higher frequency of this mutation in the later R5D generation might be attributable to a bottlenecking effect. Given that both the S5D and R5D samples demonstrated similar levels of resistance desiccation (<2 $\log_{10}$ loss), we would expect that mutations responsible for desiccation resistance in the stress-selected phages (S5D) will be preserved in the reversion-tested phages (R5D). Therefore, no mutations highly correlated to desiccation resistance were clearly identified. The lack of genotype change that correlates with phenotype change may instead point to an increase in phenotypic plasticity, that is, the ability of a genotype to exhibit different phenotypic responses under different environmental conditions. Recently, Schaum, et al. [42] demonstrated that when the diatom *Thalassiosira pseudonana* was exposed to thermally fluctuating environment every 3-4 generations had higher plasticity than those grown within stable environments. The authors suggest that the evolution of phenotypic plasticity was responsible for the higher thermal tolerance of the exposed samples as compared to samples grown in the stable environment.

The four highest frequency mutations in the S5T sample were point mutations that affected the gp39 and gp40 gene proteins: position 40757 (65%), position 40837 (29%), position 40840 (47%) and position 41408 (100%) (Figure 3B). Only two of these mutations are present in the later R5T generation: position 40757 (31%) and 40837 (21%). The highest occurring mutation in the R5T generation is an insertion of an AGGATAGGAT sequence affecting gp102 (77%), however, it is present at lower frequencies in the heat resistant S5T sample (7%). There are no singular mutations that occur at very high frequencies in both the S5T and R5T samples. It is possible that the different biological replicates of these samples exposed to high temperatures may have developed distinct mutations affecting the same gene proteins that improved their survivability.

Unlike the samples that underwent desiccation and high temperature selection, there are mutations that occur with 100% frequency in all the samples that underwent low pH selection (Figure 3C). Both the stress-selected S5pH and the reversion-tested R5pH sample exhibited mutations affecting gp108 at position 95995, as well as at positions 95862 and 98593—which both fall within a non-coding region from position 95836 to 95947—with 100% frequency. Critically, none of these mutations appeared at significant frequencies in the stock sample (≤0.5%). Furthermore, the S5pH and R5pH samples were the only samples to demonstrate no statistically significant loss after exposure to their respective stress. This genomic analysis suggests that there is a clear mechanism for stable low pH resistance that each of the biological replicates selected for during the selection process.

Based on the mutation frequency, a few key locations within the genome appear to have been affected by the selection process for the desiccation selected phages (gp102), the elevated temperature selected phages (gp39 and gp40), and the low pH selected phages (gp108 and a non-coding region extending from position 95,836 to position 95,947). Since the functions of these regions have not been determined for the P100 phage, we searched for highly similar regions in other phages. These phages, the relevant regions, and their functions, where previously published, are given in Table S1. The results of these comparisons suggested that the mutations affecting the heat-stressed samples may impact a receptor-binding protein/tail-fiber protein (gp39) and/or an assembly chaperone (gp40). The mutation affecting the pH-stressed sample may impact an anti-CRISPR endonuclease (gp108), in addition to the noncoding region. The function of the gp102 region that may be affecting desiccation resistance has not been identified at this time.

Gp39, which we assume to be a receptor-binding/tail-fiber protein based on analogous proteins, has previously been predicted to have a melting temperature of 55-65 °C [18]. Therefore, mutations in this protein that lead to an increase in melting temperature would consequently result in a phage population that is resistant to heat stress. Kering et al. [22] also studied the adaptation evolution of phages with improved thermal stability by subjecting various populations to heating at 60 °C for one h. Their findings showed that the only heat-resistant mutations in phages CX5-1 and P-PSG-11-1 were point mutations that affected tail-tubular proteins. Additionally, mutations affecting a receptor binding protein/tail-fiber protein and an assembly chaperone, the expected function of Gp40, were previously found in *Listeria* phages that were successfully adapted through laboratory co-evolution to infect generally phage-resistant rhamnose-deficient *L. monocytogenes*, thus improving host range [43].

The genomic correlations in this study are inconclusive; therefore, further work is required to identify and characterize the mechanisms of resistance. The benefits of mutations are very clear with respect to survival, but the disadvantages are not necessarily evident in the growth phase of propagation. Thus, an efficiency of plaquing assay and lytic curves were used to assess reproduction ability.

2.3.3. Antibacterial potency preserved in reversion-tested bacteriophage

The lytic behaviors of the P100 stock and the reversion-tested selected phage samples were assessed via a comparison of plaque morphology, and the construction of a kill curve for two representative strains of *Listeria* serotypes 1/2a and 4b. The serotypes 1/2a and 4b are responsible for the significant majority of the clinical cases of listeriosis [44–46].

The *L. monocytogenes* serotype 1/2a strain used in this study was obtained commercially. However, the *L. monocytogenes* serotype 4b strain was gifted. This bacterial strain was then sequenced in an attempt to characterize it. Multilocus Sequencing (MLST) [47] determined that the sequence type of *L. monocytogenes* serotype 4b was 2. The assembled genome was also entered into the NCBI BLASTn system[35] to identify any similar strains. The results of this analysis revealed that the *L. monocytogenes* serotype 4b sample genome possessed strong similarities to *L. monocytogenes* strains 02-6679 (NCBI Ascension number NZ_CP008821.1) and 02-6680 (NCBI Ascension number NZ_CP007462.1), strains that have been isolated from stool and cheese, respectively.

Plaque morphology of the *L. monocytogenes* serotype 1/2a and 4b samples infected with the P100 stock and the reversion-tested phage samples is shown in Figure 4. Overall, plaque morphology is consistent between the reversion-tested phage strains and the ancestral P100 stock for both tested *Listeria* strains. All plaques are rounded, completely clear, exhibit well-defined edges, and are approximately the same size for a given *Listeria* strain. The heat selected R5T sample is an exception as its plaque size appears slightly larger than all other samples. The results of an efficiency of plaquing assay are given in Table S2, where *L. monocytogenes* serotype 1/2a was used as the reference strain. Infection of *L. monocytogenes* 1/2a was treated as the reference given that the virion count for each sample was unknown. Infectivity of the *L. monocytogenes* serotype 4b sample with the ancestral P100 stock was approximately half that of the infectivity of the *L. monocytogenes* serotype 1/2a sample. Infectivity of the *L. monocytogenes* 4b sample with the reversion-tested phage samples relative to *L. monocytogenes* 1/2a was determined to not be significantly different than that of the P100 stock. The maintenance of plaque morphology in the reversion-tested population suggests no trade-off in lytic ability.

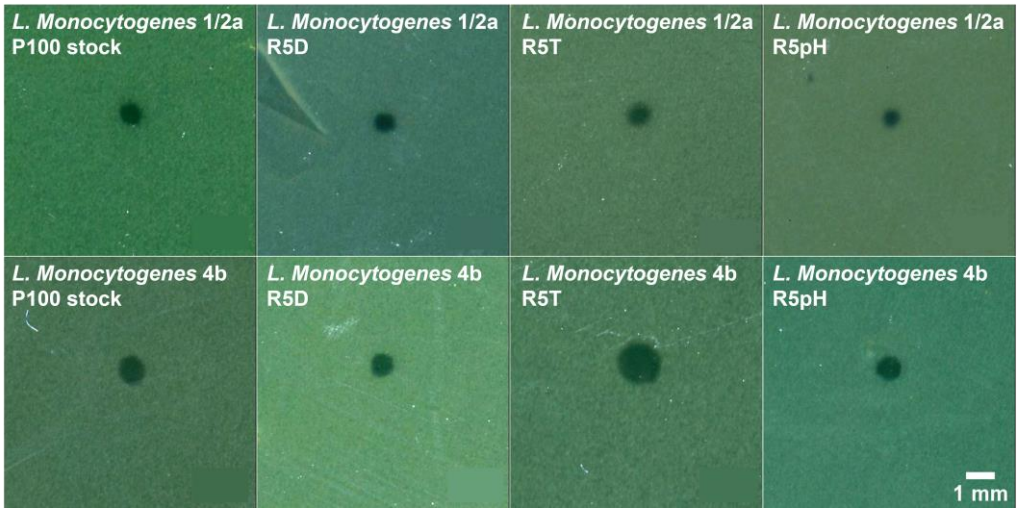

Figure 4 Plaque morphology of the different P100 bacteriophage samples infecting the *L. monocytogenes* serotype 1/2a (top row) and 4b (bottom row) bacteria samples, where the plaques represent a clearing in the bacterial lawn due to bacteriophage infection. Plaques shown here are representative for each imaged soft agar overlay plate. All plaques are completely clear, round, and display defined edges. Plaque size appears to be similar for infection of a given *L. monocytogenes* strain. Nomenclature: R5D: P100 sample that underwent 5 cycles of desiccation selection followed by 5 propagation cycles in the absence of stress; R5T: P100 sample that underwent 5 cycles of high temperature selection followed by 5 propagation cycles in the absence of stress, R5pH: P100 sample that underwent 5 cycles of low pH selection followed by 5 propagation cycles in the absence of stress.

Figure 5A compares the change in optical density (OD_{600}) of a *L. monocytogenes* serotype 1/2a bacterial suspension over 14 h when left uninfected and when infected with the different P100 samples OD_{600} is proportional to the concentration of the bacterial suspension. Figure 5B more closely compares the lytic behavior of the P100 ancestral stock and the reversion-tested P100 phages against *Listeria monocytogenes* serotype 1/2a in terms of the change in OD_{600} over time. The uninfected *L. monocytogenes* serotype 1/2a suspension replicates to a very high concentration over time while infection with the P100 stock effectively eliminates the bacteria (Figure 5A). As presented in Figure 5B, the similar lytic trend of the ancestral P100 stock and the reversion-tested phage samples upon infecting the *L. monocytogenes* serotype 1/2a sample demonstrates their strong ability both to suppress the growth of and kill the bacteria in the sample. Furthermore, the maintained low OD_{600} after the elimination of the bacteria indicates that there was no phage-resistant bacterial growth in any of the infected samples over the 14-h monitoring period.

Further analysis of the lytic curves with respect to the maximum OD_{600} (indicative of the maximum bacterial concentration) and the generation time of each infected *L. monocytogenes* serotype 1/2a sample is provided in Figure 5C. All of the lytic curves for the reversion-tested phages showed statistically insignificant maximum OD_{600} values and generation time compared to the ancestral P100 sample.

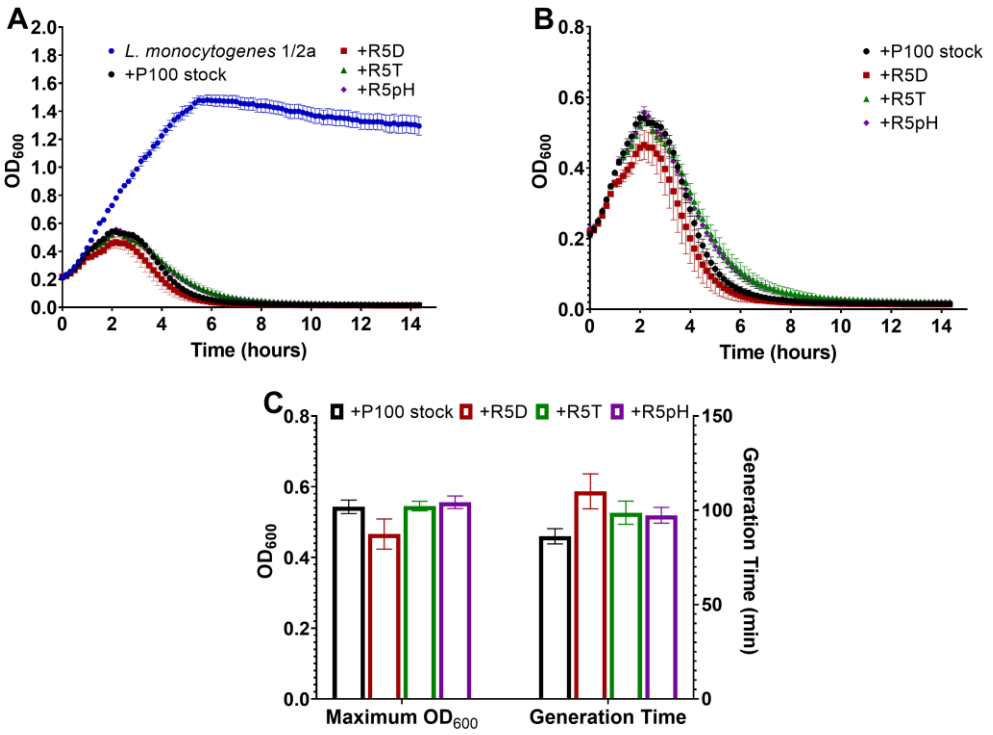

Figure 5. A) Growth curve of a *L. monocytogenes* serotype 1/2a sample left uninfected as compared to infection with P100 stock and reversion-tested P100 samples selected for resistance to desiccation (R5D), resistance to high-temperature exposure (R5T), and resistance to low-pH exposure (R5pH). B) Same growth curve of an *L. monocytogenes* serotype 1/2a sample infected with P100 stock and reversion-tested P100 samples R5D, R5T, R5pH shown at a higher OD_{600} resolution to compare the different growth curve trends. C) Maximum OD_{600} reading and generation time (min) for *L. monocytogenes* serotype 1/2a samples infected with different P100 bacteriophage samples. Three biological replicates were tested for the different engineered P100 samples, with all experiments being performed in triplicate. Results are shown as average of the biological replicates ± standard deviation for the reversion-tested phage and the average of the technical replicates ± standard deviation for the P100 stock. Differences in maximum OD_{600} and generation time between the P100 ancestral stock and the reversion-tested samples was determined to be statistically insignificant. Nomenclature: R5D: P100 that sample underwent 5 cycles of desiccation selection followed by 5 propagation cycles in the absence of stress; R5T: P100 sample that underwent 5 cycles of high-temperature selection followed by 5 propagation cycles in the absence of stress; R5pH: P100 sample that underwent 5 cycles of low-pH selection followed by 5 propagation cycles in the absence of stress.

Growth kinetics of *L. monocytogenes* serotype 4b left uninfected as compared to when infected with the P100 samples is presented in Figure 6A. Similarly, Figure 6B shows the lytic trend difference between the P100 ancestral stock and the reversion-tested P100 phages against *Listeria monocytogenes* serotype 4b. Comparison with the uninfected bacterial control (Figure 6A) clearly demonstrates that the ancestral P100 stock and all of the reversion-tested P100 phages were able to eliminate *L. monocytogenes* serotype 4b (Figure 6B). Like the lytic curves for the *L. monocytogenes* serotype 1/2a samples, there was no evidence of bacteria-resistant regrowth over the 14-h experiment.

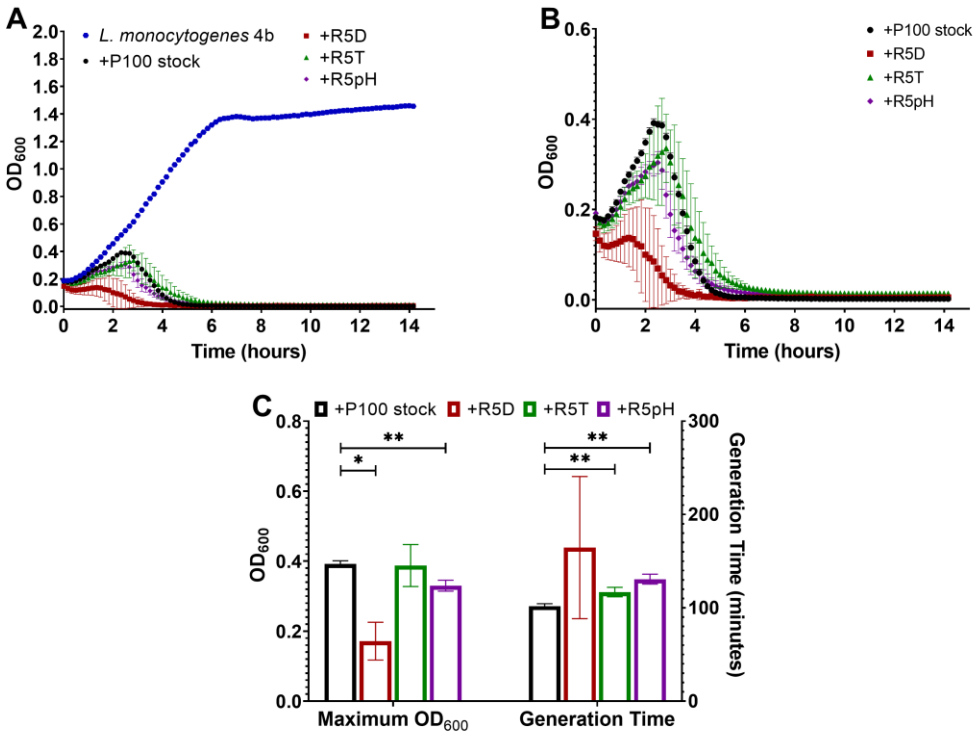

Figure 6. A) Growth curve of an *L. monocytogenes* serotype 4b sample left uninfected as compared to infection with P100 stock and reversion-tested P100 samples selected for resistance to desiccation (R5D), resistance to high-temperature exposure (R5T), and resistance to low-pH exposure (R5pH). B) Same growth curve of an *L. monocytogenes* serotype 1/2a sample infected with P100 stock and reversion-tested P100 samples R5D, R5T, R5pH shown at a higher OD_{600} resolution to compare the different growth curve trends. C) Maximum OD_{600} readings and generation times in min for *L. monocytogenes* serotype 4b samples infected with different P100 bacteriophage samples. Three biological replicates were tested for the different engineered P100 samples, with all experiments being performed in triplicate. Results are shown as average of the biological replicates ± standard deviation for the reversion-tested phage, except for the R5D generation time result, and the average of the technical replicates ± standard deviation for the P100 stock. The generation time for one of the R5D biological replicates could not be calculated as there was no measured bacteria growth after infection, thus the results for this sample are given as the average ± standard deviation of two biological replicates. Statistically significant differences compared to the P100 stock are marked as follows: * - $p \leq 0.05$, and ** - $p \leq 0.01$. Nomenclature: R5D: P100 sample that underwent 5 cycles of desiccation selection followed by 5 propagation cycles in the absence of stress; R5T: P100 sample that underwent 5 cycles of high-temperature selection followed by 5 propagation cycles in the absence of stress; R5pH: P100 sample that underwent 5 cycles of low-pH selection followed by 5 propagation cycles in the absence of stress.

Unlike the *L. monocytogenes* serotype 1/2a experiments, differences in lytic behavior were observed among the different P100 samples that were used to infect *L. monocytogenes* serotype 4b. Although the general lysis trend was similar (limited exponential growth followed by a fast drop in OD), the reversion-tested phages appeared to exhibit better performance than the ancestral P100 stock against *L. monocytogenes* serotype 4b. This improved performance is clearly demonstrated in Figure 6C, which provides the maximum OD_{600} values and generation times for *L. monocytogenes* serotype 4b infected with the different phage samples. The phage samples exposed to desiccation stress (R5D) and low pH stress (R5pH) had a significantly lower maximum OD_{600} than the P100 stock, indicating that these samples are more efficient at eliminating the *L. monocytogenes* serotype 4b bacteria compared to the ancestral phage. No statistically significant differences in maximum OD_{600} were observed between the P100 stock and the heat-stressed samples (R5T). The samples exposed to high-temperature stress (R5T) and low pH stress (R5pH) had significantly higher generation times than the ancestral P100. The average generation rate of the desiccation-stressed samples (R5D) was not statistically significantly different from those of the P100 stock. However, one of the R5D samples had to be excluded from the average generation time calculation as its lytic curve showed bacterial death occurred at a higher rate than bacterial growth almost immediately after infection.

The similarity in lytic trend and maximum OD_{600} between the P100 stock and the reversion-tested phage samples suggest equal biocontrol of the *L. monocytogenes* 1/2a strain. Whereas the infection of the serotype 1/2a sample with the different phage samples exhibited a similar trend in lytic behavior, infections of the serotype 4b sample yielded greater differences between the ancestral phage and the reversion-tested phages. All reversion-tested phages showed either reduced maximum OD_{600} or higher generation times than the ancestral phage. This result suggests that the reversion-tested phages offer improved reproduction and lysis ability against the serotype 4b sample. Overall, these results show that the reversion-tested phages performed similarly or better than the P100 stock in lysing both tested strains of *L. monocytogenes*. Previously, Kashiwagi et al. [48] found that the ancestral phage demonstrated higher thermal stability than reversion-tested phages that were replicated in a bacterial culture held at the inhibitory temperature of the ancestral strain. Nonetheless, our results are consistent with those of other studies [22,23,49] that show no trade-off in lytic ability for phages selected for greater survival. Instead, our results suggest that improved survival under stressed conditions also leads to similar or improved lysis under unstressed conditions.

2.3.4. Comparison of infectivity for P100 stock and reversion-tested bacteriophages on food preparation surfaces and in food matrices

The survival of the ancestral phage and the reversion-tested phage populations was tested in three food matrices/preparation surfaces representing the selection stresses encountered in this study: desiccation, elevated temperature, and acidic conditions. The results of these application experiments are given in Figure 7.

P100 has been approved for use as a food-contact surface cleaning solution to prevent and treat the proliferation of *L. monocytogenes*. Its application sheet notes that the application can work for up to 24 h and recommends that the stock $2x10^{11}$ PFU/ml phage stock must be diluted to 1% ($2x10^{9}$

PFU/ml) prior to spraying onto the relevant surface[50]. The survival of P100 and reversion-tested R5D at an initial concentration of $1x10^{11}$ PFU/ml was evaluated after 24 h of drying on food-grade stainless steel surfaces under ambient conditions. There is evidence in the literature that application of P100 on surfaces within food processing environments can reduce the incidence of *Listeria*[12]. Increased long-term survival of phage on these surfaces will ensure a higher amount of phage is available to act against *Listeria* contamination. We observed that both ancestral and reversion-tested phage populations underwent ~2.5 $\log_{10}$ loss (Figure 7A) when air-dried on stainless steel. Leung et al.[19] also reported ~2 $\log_{10}$ titer reduction when the same volume (100µL) of P100 was air-dried for 24 h in a well plate under ambient conditions, with complete loss of infectivity after 48 h[19].

Our results in Figure 2 show that the R5D sample had a greater resistance to vacuum drying within a tube for 4 h (~2 $\log_{10}$ loss) as compared to the P100 stock sample (~7 $\log_{10}$ loss). Vacuum drying of the phages within an enclosed space was chosen as the method of desiccation for adaptational evolution. Stabilization of P100 phages within a sugar matrix was previously accomplished via vacuum drying[20]. Adaptation to vacuum stress was tested in this study as a method to improve inherent stability and thus reduce processing loss. Vacuum drying also reduced the possibility of contamination during stress cycle exposure as compared to open ambient air drying. However, air drying over a flat surface, the more realistic real-life application scenario tested here, may result in a different level of stress as compared to vacuum desiccation within a tube or air drying within a well plate [19], based on available surface area and drying medium. The mechanistic differences in the drying method may explain why the R5D samples showed improved resistance to vacuum drying compared to the ancestral samples but did not show improved survival to air drying.

Next, we evaluated the survival of P100 stock and the heat-selected R5T sample in commercial milk under conditions that mimic batch pasteurization (exposure to 63 °C for 30 min). *L. monocytogenes* has been implicated in outbreaks linked to products made with pasteurized milk [51]. Post-processing contamination may be limited by the addition of P100 to milk. However, government agencies restrict the addition of ingredients to milk post-pasteurization [52]. Our results show that the P100 stock underwent ~4 $\log_{10}$ loss whereas the R5T sample underwent ~3 $\log_{10}$ loss when pasteurized (Figure 7B). The difference in survivability between these two samples was determined to be statistically insignificant. Similar results were achieved by Ahmadi et al. [18], which reported that the commercial preparation of P100 underwent ~4 $\log_{10}$ loss when held at 65 °C in a circulating water bath for 20 min. We hypothesize that the choice of medium may have affected the selection process. In our phage adaptational evolution experiments, the P100 ancestral stock was selected under successive heat exposure cycles using TSB media as a medium. This may have resulted in phage populations optimized for heat shock survival in TSB only. Previously, Komora et al. [53] demonstrated that different food matrices can have protective or harmful effects on the survival of P100 when exposed to high pressure. Therefore, the lack of specificity toward a given food matrices in phage adaptational evolution experimental design may have impaired the R5T sample's ability to survive batch pasteurization (63 °C for 30 min) in milk.

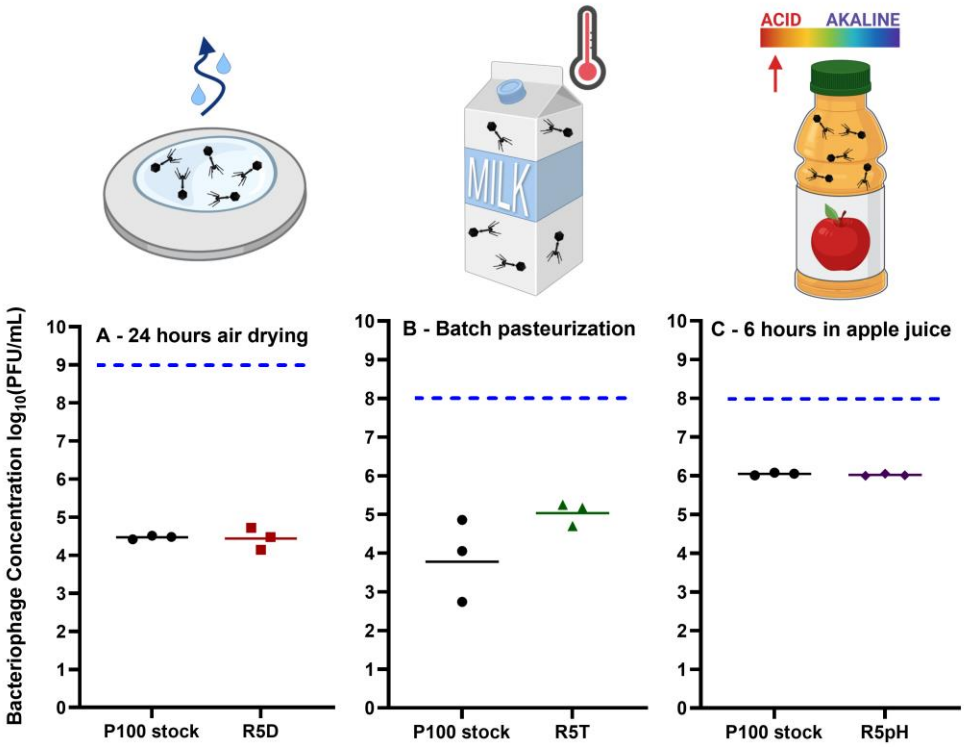

Figure 7. Remaining bacteriophage concentration (PFU/ml), of P100 stock and the reversion-tested bacteriophage samples R5D, R5T or R5pH after exposure to A) 24 h of air drying on a food-grade stainless-steel sheet, B) 63 °C for 30 min within milk (batch pasteurization), and C) apple juice for 6 h. Triplicate results are shown, where the geometric mean is marked as a horizontal line. Black circles represent the P100 ancestral stock, red squares represent the R5D sample, green triangles represent the R5T sample, and purple diamonds represent the R5pH sample. The horizontal dotted blue line represents the initial concentration of the P100 and reversion-tested samples. This starting concentration was selected based on available application sheets for P100, that is, a concentration of $1x10^9$ PFU/ml on food preparation surfaces and $1x10^8$ PFU/ml within liquid food matrices. Nomenclature: R5D: S5D sample that was propagated 5 times in the absence of stress; R5T: S5T sample that was propagated 5 times in the absence of stress; R5pH: S5pH sample that was propagated 5 times in the absence of stress. Icons from Biorender.com

Finally, we evaluated the survivability of reversion-tested phage in an acidic beverage. Many fruits, such as lemons, blueberries, and apples, are acidic, with citric fruits having the lowest pH. A study conducted by the Canadian Food Inspection Agency found that apples and melons have demonstrated higher incidence of *L. monocytogenes* contamination than other fruits in Canadian retail markets [54]. However, phage P100 has been found to be effective at reducing contamination in melons, but not apples [55]. Therefore, apple juice was chosen as a medium to test the applicability of the phage populations selected for increased survival under low pH conditions. Apple juice was chosen instead of apple slices to prevent influence of desiccation on a solid food matrix on phage survival. Both the P100 stock and R5pH samples underwent statistically insignificant levels of loss after 1 h (Figure S4) and approximately 2 $\log_{10}$ loss after 6 h within commercial apple juice (Figure 7C). There was no significant difference in survival between the two samples for any given timepoint. This degree of loss was much less than ~3 $\log_{10}$ loss experienced by the P100 ancestral stock after exposure to a pH of 2.65 (Figure 2). The improved survivability of P100 stock after 1 h within apple juice (pH 3.5) may be due to the reduced acidity as compared to the selection environment (pH 2.65). Previously, Fister et al.'s [17] found that the titer of P100 was stable at pH 4

for 24 h but experienced greater than 5 $\log_{10}$ loss after only one h exposure to pH 3 or lower. Their result suggests P100's lethal acidity is somewhere between pH 3 and 4.

The P100 phage has previously been theorized to have limited applicability in certain fruits and fruit juices due to low pH [53,55]. Oliveira et al. [55] tested the ability of P100 to eliminate *L. monocytogenes* in melon (5.77 pH), pear (4.61 pH) and apple juice (3.7 pH). Their results found the P100 was stable in both melon and pear juice when held at 10 °C for 8 days whereas P100 in apple juice underwent 7 $\log_{10}$ loss under the same conditions. Our work showed that both the P100 stock and the R5pH sample had similar titer loss patterns within a commercial apple juice at approximately 3.5 pH. Under experimental adaptational evolution (Figure 2), the R5pH sample had shown greater survivability within a 2.65 pH environment for 1 h than the P100 stock. Given these results, it was expected that the R5pH sample would also show improved survivability as compared to P100 when held within acidic apple juice. However, the acidity of the apple juice may not be the only factor affecting phage titer loss. Leverentz et al. [56] included magnesium chloride in the application of a phage cocktail to reduce contamination of *L. monocytogenes* on apples. The magnesium chloride was theorized to improve phage recovery due to neutralization of the acidic environment. However, they found that the addition of magnesium chloride had no effect on phage effectiveness. The results shown in our study further imply that P100 inactivation in apples may be due to factors other than pH, such as the presence of tannins, which have also been linked to antiviral effects [57]. Further work is needed to determine the factors affecting phage loss on apples in order to select against them. Our study indicates that while selecting for phages resistant to a given stress under laboratory conditions is promising, their improved survival does not necessarily translate to realistic food applications. This further highlights the importance of mechanistic understanding of phage infectivity under stress conditions.

2.4. Conclusions

L. monocytogenes is an especially pernicious food-borne pathogen due to its ability to survive and grow despite the application of common bacterial-control methods, such as refrigeration, desiccation, heating, and exposure to acidic environments. Commercial phage P100 is able to successfully infect and lyse *L. monocytogenes*. However, P100's sensitivity to desiccation, high heat, and low pH results in significant loss of infectivity when exposed to these stressors. Using directed evolution, batches of P100 phage was selected for resistance to key stressors common in food processing and/or food matrices, namely, desiccation, elevated temperatures, and acidic environments. Our stress-selected phages demonstrated improved survival up to several orders of magnitude greater than the ancestral population under stress and retained their stress-resistant phenotype even after several passages in the absence of the stressors. Full characterization of the possible mutations responsible for these selected phages' increased resistance to each stress was hindered by the lack of information on the proteome of phage P100, a limitation that is a major bottleneck in the field of phage biology.

Despite promising results under a laboratory setting, the same benefit over ancestral phage was not translated when the reversion-tested phages were used under more realistic conditions. Specifically, the selected phages did not demonstrate significantly increased survivability over the ancestral phages when dried on food-grade stainless steel, nor when batch pasteurized within milk.

This may be due to all the complexities of the stresses encountered within a realistic environment, such as the physical and chemical components of the matrix, that are not replicated during the selection process. The acid resistant phages also exhibited the same survivability as ancestral phage within apple juice, which may be due to the presence of tannins within some fruits. Even though evolutionary adaptation has garnered more attention in recent years as the means to increase efficacy of phage, our work highlights the challenges affecting the applicability of this method in real life, complex, heterogeneous environments such as food matrices or even within the human body. Therefore, we conclude that investigation into evolutionary adaptation to improve phage efficacy requires more mechanistic investigation.

Supplementary Materials: Supplementary information is available. Full dataset and analysis have been uploaded to Zenodo: 10.5281/zenodo.7246639

Author Contributions: M.G.: formal analysis, investigation, methodology, visualization, writing – original draft. A.S.: investigation, methodology, writing – review and editing. J.Sz.: formal analysis, software, visualization, writing – review and editing. V.L.: methodology, writing – review and editing. C.F.: methodology, project administration, supervision, writing – review and editing. Z.H.: methodology, project administration, supervision, writing – review and editing.

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Data Availability Statement: The data presented in this study available from a repository: 10.5281/zenodo.7246639.

Conflicts of Interest: The authors declare no conflict of interest.

2.5. Supplementary Material

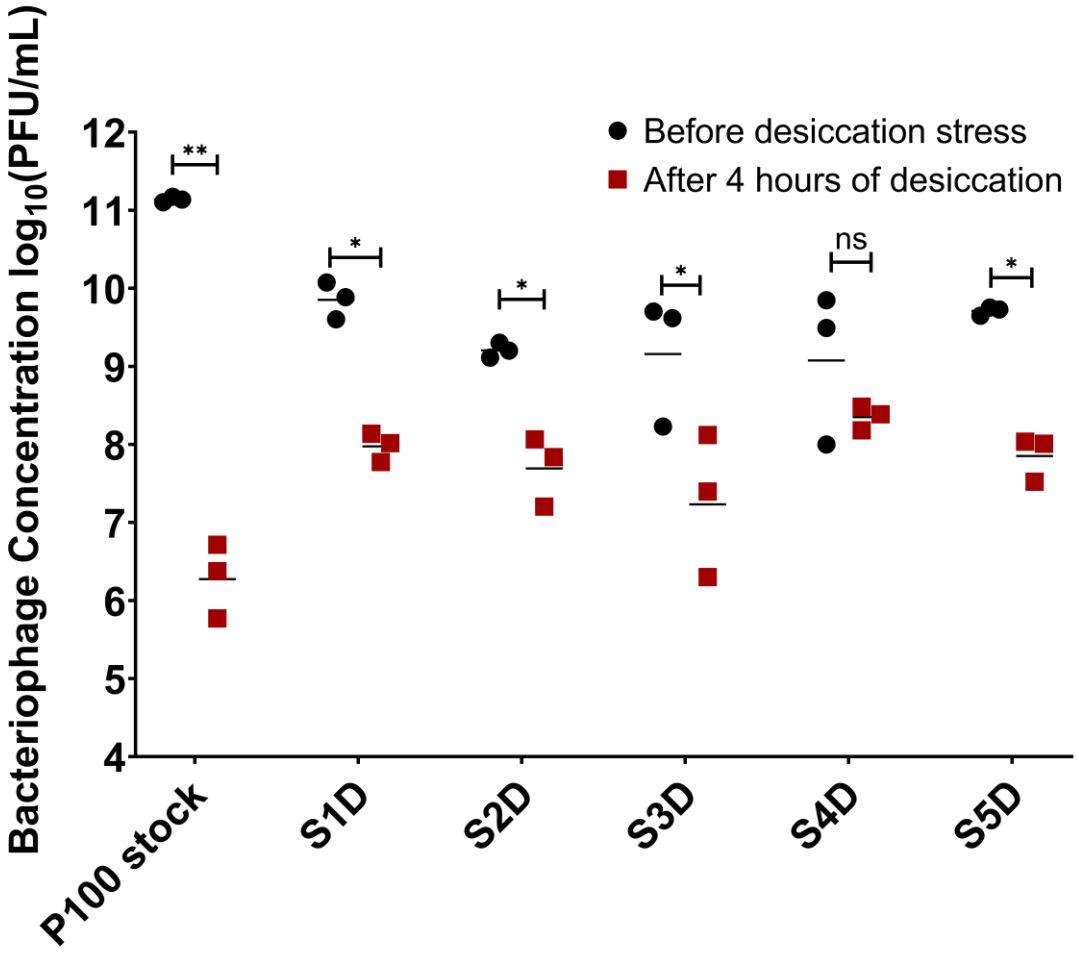

Figure S1. Measured concentrations of viable bacteriophage in the P100 stock as compared to samples that have undergone 1-5 cycles of selection before and after exposure to desiccation for 4 hrs. Results are shown as the individual results of triplicate experiment, where the geometric mean has been marked. Bacteriophage concentration prior to exposure presented in black and concentration after exposure presented in red. Significant differences in bacteriophage concentration before and after exposure to desiccation are marked as * - $p \leq 0.05$ and ** - $p \leq 0.01$. Nomenclature: n.s.: not significant; S1D: P100 sample that underwent 1 cycle of desiccation selection; S2D: P100 sample that underwent 2 cycles of desiccation selection; S3D: P100 sample that underwent 3 cycles of desiccation selection; S4D: P100 sample that underwent 4 cycles of desiccation selection S5D: P100 sample that underwent 5 cycles of desiccation selection.

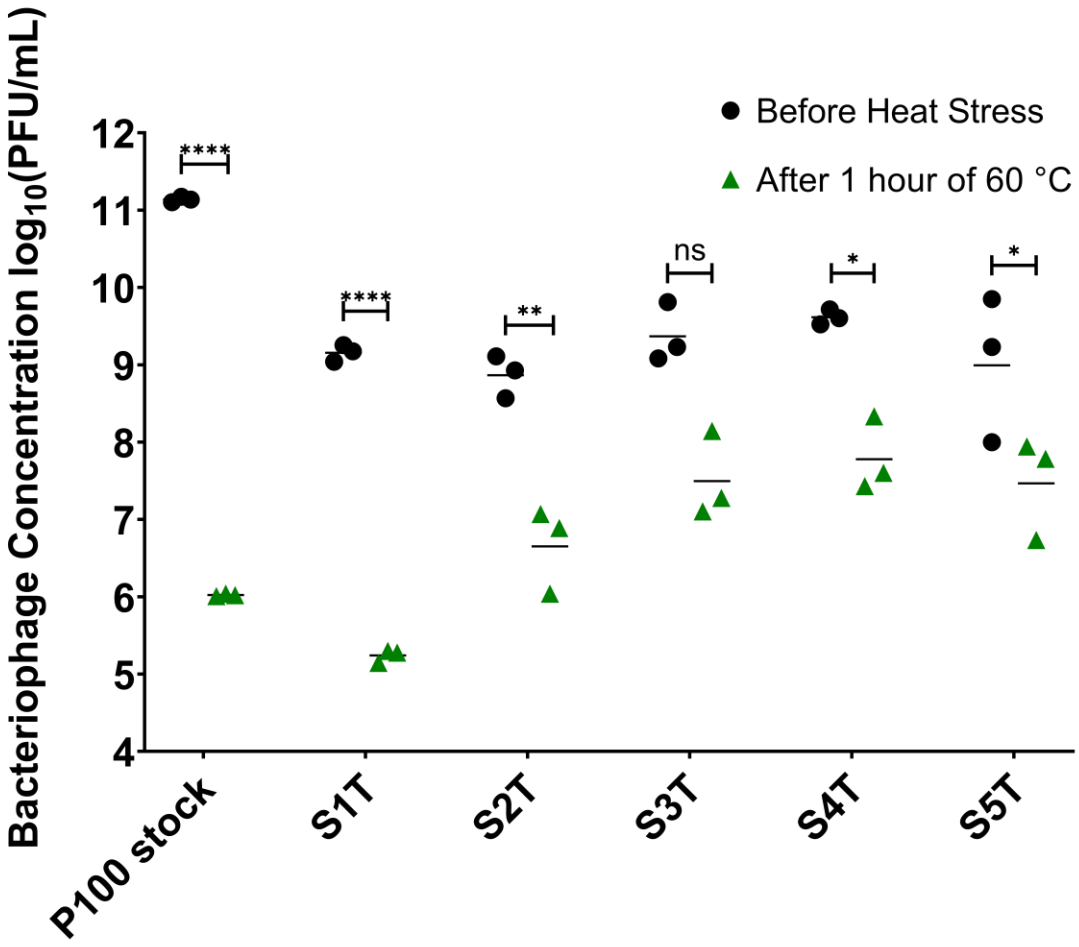

Figure S2. Measured concentrations of viable bacteriophage in the P100 stock as compared to samples that have undergone 1-5 cycles of selection before and after exposure to temperatures of 60 °C of 1 hr. Results are shown as the individual results of triplicate experiment, where the geometric mean has been marked. Bacteriophage concentration prior to exposure presented in black and concentration after exposure presented in green. Significant differences in bacteriophage concentration before and after exposure to 60 °C are marked as * - $p \leq 0.05$, ** - $p \leq 0.01$, and **** - $p \leq 0.0001$. Nomenclature: n.s.: not significant; S1T: P100 sample that underwent 1 cycle of elevated temperature selection; S2T: P100 sample that underwent 2 cycles of elevated temperature selection; S3T: P100 sample that underwent 3 cycles of elevated temperature selection; S4T: P100 sample that underwent 4 cycles of elevated temperature selection S5T: P100 sample that underwent 5 cycles of elevated temperature selection.

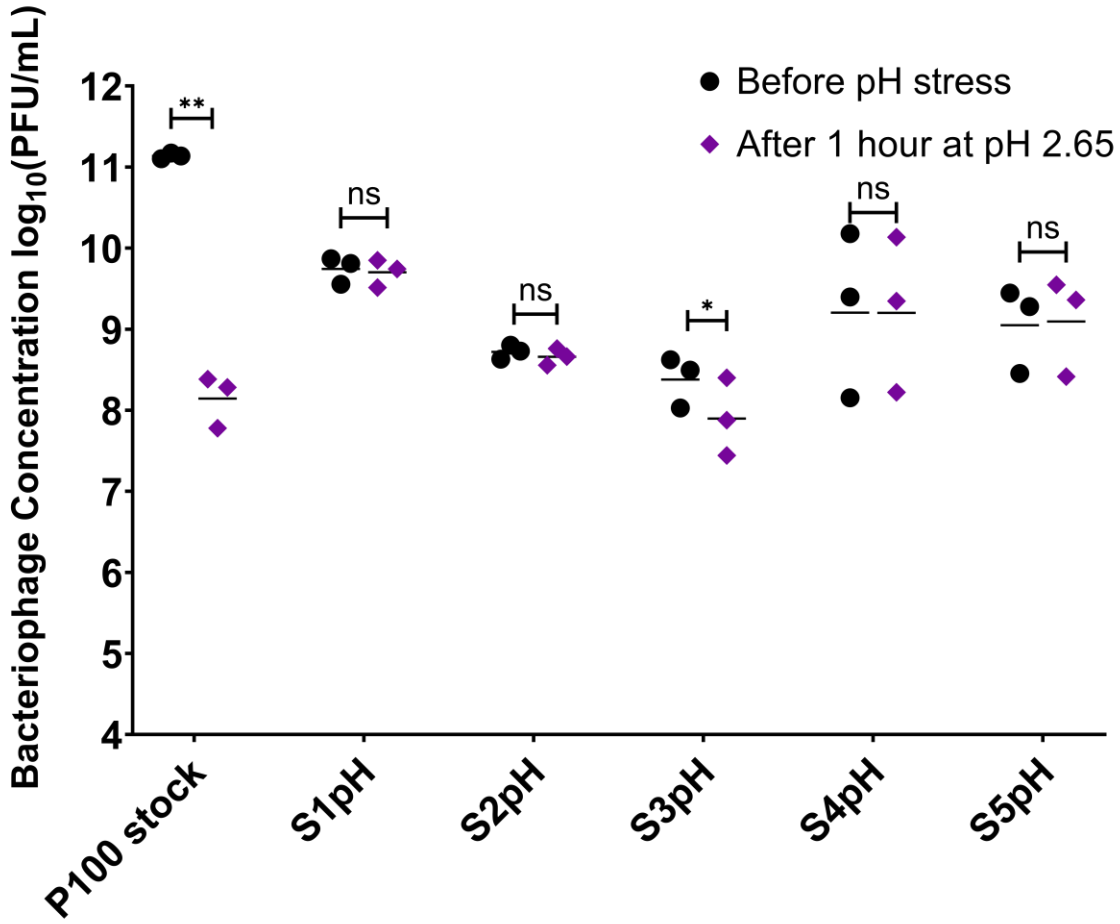

Figure S3. Measured concentrations of viable bacteriophage in the P100 stock as compared to samples that have undergone 1-5 cycles of selection before and after exposure to pH of 2.65 for 1 hr. Results are shown as the individual results of triplicate experiment, where the geometric mean has been marked. Bacteriophage concentration prior to exposure presented in black and concentration after exposure presented in purple. Significant differences in bacteriophage concentration before and after exposure to 60 °C are marked as * - $p \leq 0.05$ and ** - $p \leq 0.01$. Nomenclature: n.s.: not significant; S1D: P100 sample that underwent 1 cycle of low-pH selection; S2D: P100 sample that underwent 2 cycles of low-pH selection; S3D: P100 sample that underwent 3 cycles of low-pH selection; S4D: P100 sample that underwent 4 cycles of low-pH selection S5D: P100 sample that underwent 5 cycles of low-pH selection.

Table S1 Analogues to key P100 genomic regions affected by selection found in other listeria bacteriophages. Protein functions are given if previously determined. Abbreviations: GP: gene product; HP: hypothetical protein, NCR: non-coding region

Bacteriophage	P100 regions					Reference
	GP39	GP40	GP102	GP108	NCR Position 95,836-95,947	
20422-1				HP		[58]
List-36	HP	HP	HP	HP		Rajanna, C., et al. (2014) Direct Submission
LMSP-25	HP	HP	HP	HP		Woolston, J., et al. (2014) Direct Submission
LMTA-148	HP	HP	HP		NCR	Woolston, J., et al. (2014) Direct Submission
LMTA-34	HP	HP	HP	HP	NCR	Woolston, J., et al. (2014) Direct Submission
LMTA-57		HP	HP	HP	NCR	Woolston, J., et al. (2014) Direct Submission
LMTA-94	HP			HP	NCR	Woolston, J., et al. (2014) Direct Submission
LP-039					NCR	[59]
LP-048	HP	HP	HP		NCR	[60]
LP-064		HP			NCR	[60]
LP-066					NCR	[59]
LP-083-2	HP	HP			NCR	[60]
LP-124					NCR	[60]
LP-125	HP	HP	HP	HP	NCR	[60]
LP-Mix_6.1		HP			NCR	[43]
LP-Mix_6.2	HP	HP			NCR	[43]
vB_Lino_VEfB7	Receptor binding protein	Tail fiber assembly chaperone	HP	AntiCRISPR endonuclease		[61]
vB_Liva_VAfA18	Tail fiber protein		Tail fiber assembly chaperone	HP	AntiCRISPR endonuclease	[61]
vB_LmoM_AG20	HP	HP	HP		NCR	Anany, H., et al. (2012) Direct Submission
A511	Receptor binding protein	Assembly chaperone	HP	HP	NCR	[62]
WIL-1					NCR	Silva-Castro, G.A., et al. (2014) Direct Submission
P100plus	Receptor binding protein	HP		HP	NCR	Dunne, M., et al. (2020), Direct Submission
P200	Receptor binding protein	Assembly chaperone			NCR	Dunne, M., et al. (2020), Direct Submission

P61	HP	HP	HP	HP	NCR	[63]

Table S2 Efficiency of plaquing for the different P100 bacteriophage samples infecting the *L. monocytogenes* serotype 1/2a and 4b samples. *L. monocytogenes* serotype 1/2a was used as the reference bacterial strain. Statistical analysis showed no significant difference in the infectivity of the *L. monocytogenes* 4b sample with the conditioned bacteriophage strains as compared to the ancestral P100 stock. Three biological replicates were tested for the different engineered P100 samples, with all experiments being performed in triplicate. Results are shown as average of the biological replicates ± standard deviation for the conditioned phage and the average of the technical replicates ± standard deviation for the P100 stock. Nomenclature: R5D: P100 sample that underwent 5 cycles of desiccation selection followed by 5 propagation cycles in the absence of stress; R5T: P100 sample that underwent 5 cycles of high temperature selection followed by 5 propagation cycles in the absence of stress, R5pH: P100 sample that underwent 5 cycles of low pH selection followed by 5 propagation cycles in the absence of stress.

Bacteriophage Sample	***L. monocytogenes* 1/2a**	***L. monocytogenes* 4b**
P100 stock	1	0.51 ± 0.21
R5D	1	0.70 ± 0.22
R5T	1	0.72 ± 0.09
R5pH	1	0.49 ± 0.06

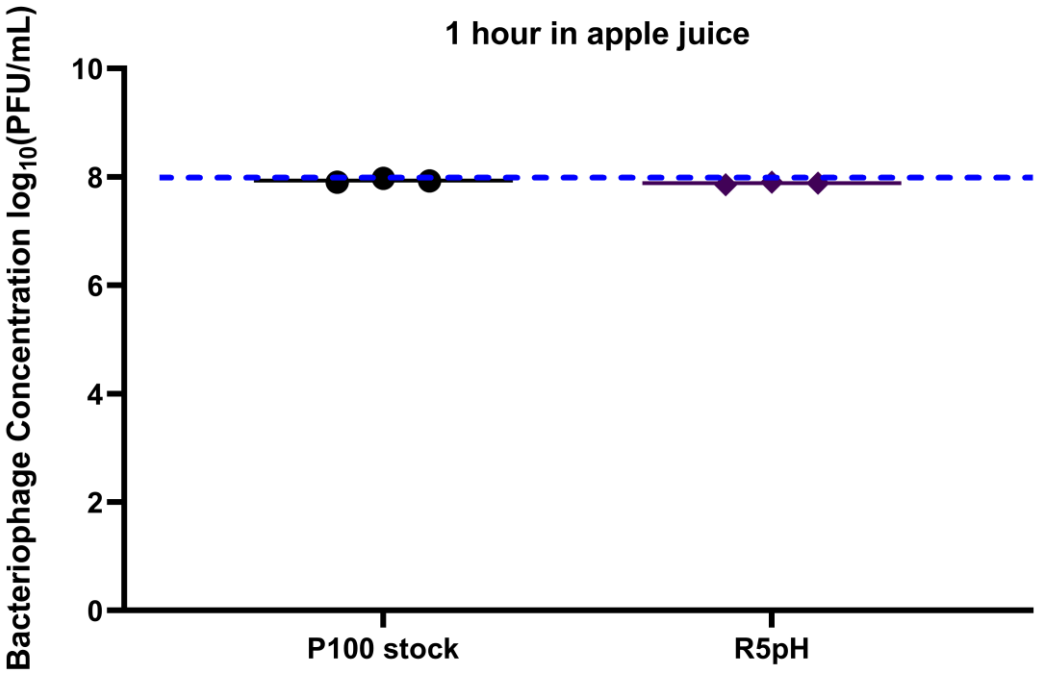

Figure S4. Remaining bacteriophage concentration (PFU/ml), of P100 stock and the reversion-tested phage samples R5pH after exposure to apple juice for 1 h. Triplicate results are shown, where the geometric mean is marked as a horizontal line. Black circles represent the P100 ancestral stock and purple diamonds represent the R5pH sample. The horizontal dotted blue line represents the initial concentration of $1x10^8$ PFU/ml for the P100 and R5pH samples. Nomenclature: R5pH: S5pH sample that was propagated 5 times in the absence of stress.

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Chapter 3: Structured pullulan-trehalose particles for stabilization of P100 bacteriophage during milk pasteurization exposure enables intervention of post-processing *Listeria monocytogenes* contamination

Preface

In this chapter, we focus on a novel application of the pullulan-trehalose stabilizing system for short-term high temperature exposure under aqueous conditions. Pullulan-trehalose has been previously shown to be a potential stabilizing system for various biologics (e.g. vaccines, phages) for long term storage at room temperature within a dry format. Our work focuses on designing a particle with pullulan at the exterior and trehalose at the interior such that the particle can remain intact in water-based solutions for short periods of time. During this time, encapsulated biologics may be protected from high temperature exposure while in a liquid. We apply this technology to encapsulating phages for survival of the pasteurization process. Many phages would be unable to survive the direct high heat exposure during pasteurization. The protective powder we have designed is capable of withstanding dissolution for the short pasteurization time, and importantly, it is composed of food safe ingredients. The results we have presented here provides a novel application for a studied system. The ability to add phages to food products, even liquid products, prior to heat treatment acts as an additional protection against post-processing contamination.

I was responsible for concept formation and development, experimental design, data collection, data analysis, and manuscript composition. Varsha Singh provided training on the spray dryer and was responsible for data analysis of the differential scanning calorimetry results. Neil Lin assisted with data collection. Lei Tian assisted with data visualization. Logan Groves assisted with material resourcing. Michael Thompson was a supervisory author who provided equipment resources and manuscript edits. Carlos Filipe and Zeinab Hosseinidoust were supervisory authors involved in discussion of results, experimental design, and manuscript edits.

Citation

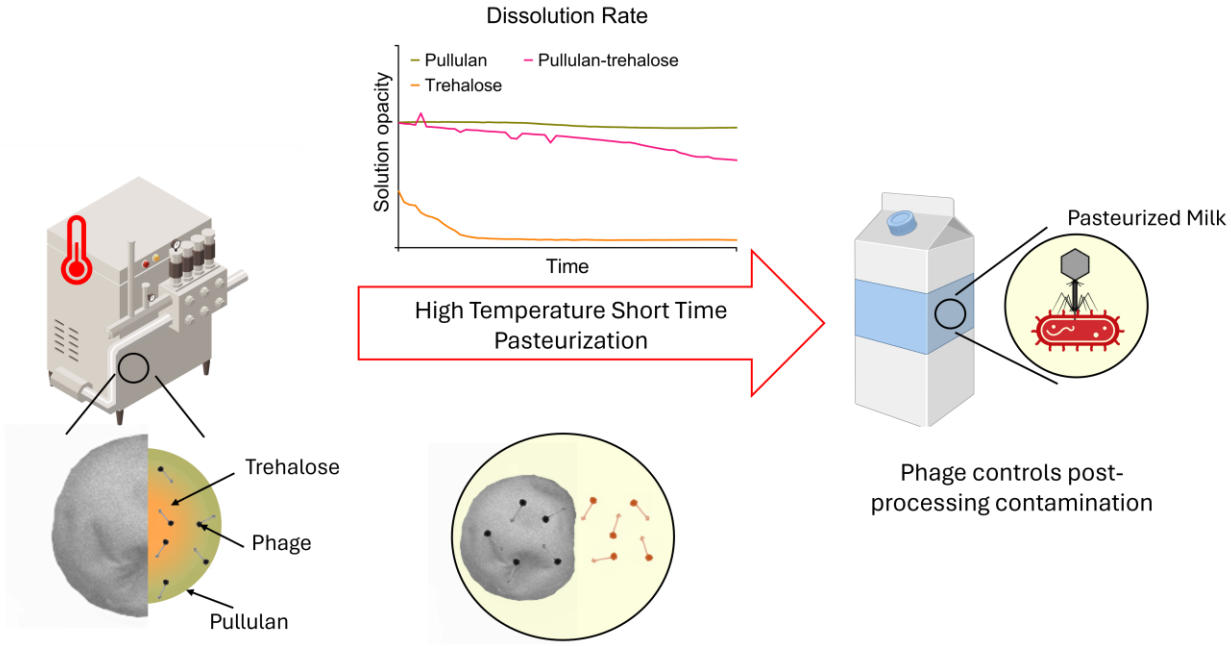
Manuscript prepared for submission. Investigation into encapsulation of Felix O1 with this system is detailed in Appendix A.

Engineered food-safe sugar particles enables bacteriophage intervention of post-pasteurization contamination. **Gomez, M.**, Singh, V., Lin, N., Tian, L., Groves, L., Thompson, M., Hosseinidoust, Z., Filipe, C.

Abstract

Bacteriophages are a promising intervention method against bacterial contamination of pasteurized dairy products as they do not affect palatability and offer targeted protection. However, there are outstanding barriers to the incorporation of bacteriophages in the dairy product production pipeline, including pasteurization sensitivity and shelf stability. Furthermore, government regulations limit inclusion of additives post-pasteurization. Combining materials technology and particle engineering, slow-dissolving, protective microparticles composed of food-safe sugars pullulan and trehalose were produced via spray drying. This protective encapsulating system was used with commercial P100 bacteriophage as a model. Particles were designed to have pullulan accumulate at the particle exterior for delayed dissolution, with an inverse radial distribution as compared to trehalose. Dissolution experiments and particle morphology confirmed the presence of pullulan at the particle exterior. The pullulan-trehalose system preserved bacteriophage activity in different dairy milks throughout simulated pasteurization (72 °C for 15 s). A significant activity loss of >4.5 logs (<0.003% survival) was observed otherwise. The stored powder experienced 1.3 log loss over 7 months of storage under ambient conditions (22 °C), with pasteurization protection maintained after storage. A challenge study with *Listeria monocytogenes* in commercial milk formed that the added bacteriophage powder post-pasteurization effectively controlled the bacterial growth over one week of refrigerated storage.

Keywords: Thermal stabilization, pasteurization, spray drying, trehalose, pullulan, food-safe



3.1. Introduction

The risk of food-borne pathogens has been mitigated through established food processing techniques, e.g., pasteurization, irradiation, and high-pressure processing. However, there are limited methods of detection and very few interventions for handling post-processing detection of contamination. Outbreaks have been linked to pasteurized milk products[1] - including milk[2], chocolate milk[3], soft cheese[4], and ice cream[5]. The responsible pathogen in many outbreaks linked to milk is *Listeria monocytogenes*[1]. The Centers for Disease Control and Prevention (CDC) has reported that *L. monocytogenes* has the highest hospitalization rate at 90.5% and the second highest fatality rate of foodborne pathogens at 21%[6]. In addition to the human cost of outbreaks, there is also an environmental and economic cost. Necessary recalls are not only costly and contribute to food waste, but also lead to the erosion of public trust. The development of easy-to-implement secondary protection methods are therefore highly sought by the food industry to reduce or eliminate the downstream contamination of pasteurized milk.

A potential method of bacterial pathogenic control is bacteriophages. Bacteriophages, also referred to as phages, are viruses that infect bacteria[7–9]. Lytic phages replicate within the target bacteria, eventually lysing the host. This releases the replicated phage into the surroundings, now capable of infecting even more bacteria. Their replication mechanism highlights the self-amplifying feature of phages, whereas their high host specificity makes them an attractive alternative to antibiotics as phages are unlikely to harm the natural human biome. Furthermore, their high specificity ensures that targeted phage interventions will not interfere with the bacterial cultures utilized in further dairy processing, such as cheese production.

There are several bacteriophage commercial products that have been approved in various countries as food processing aids. One such product is PhageGuard L (previously known as Listex), which is comprised of the anti-*Listeria* bacteriophage P100. P100 has demonstrated a clear ability to eliminate or reduce *Listeria* growth in many ready-to-eat foods[10,11]. P100 has been shown to be stable in refrigerated milk, with no change in phage titer when stored for 28 days within whole milk at 4 °C[12]. However, government regulations limit the addition of ingredients post-pasteurization and P100 has seen significantly decreased bioactivity (0.0001% survival rate)[13] when exposed to conditions similar to high temperature short term (HTST) pasteurization (15 s at 72 °C)[14].

A temporary food-safe protection system to enable survival in a high-temperature aqueous environment before eventual dissolution and release of the phages can be engineered to address this issue. A promising candidate is a system comprised of the sugars pullulan and trehalose. Both sugars have been granted “Generally Recognized as Safe” (GRAS) designation by the US Food and Drug Administration[15,16] and are regularly used as food ingredients/additives. The pullulan-trehalose system has been investigated as a preservation system with various applications. Pullulan-trehalose powders[17,18] or films[19,20] have been shown to stabilize bacteriophage and vaccines in a dry format intended for room temperature storage. Pullulan-trehalose has also been used to prepare dissolving microneedle vaccines that are stable when stored at 37 °C for one month[21]. These systems focus on long term stability at elevated temperature within a dry format. Design for short-term aqueous stability is a novel application of the pullulan-trehalose system.

To this end, the aim of this study was to engineer a pullulan-trehalose powder that could encapsulate phage P100 and protect the phage from degradation during pasteurization. Powder was produced via spray drying, where processing loss, batch reproducibility, dissolution pattern,

and morphology of the powder was investigated. To assess protective ability, phage encapsulated powder was added to commercial dairy milk and subjected to simulated pasteurization. Ability of the phage powder to reduce contamination in milk post-pasteurization was evaluated through *L. monocytogenes* challenge study over refrigerated storage.

3.2. Materials and Methods

3.2.1. Chemicals

Trehalose dihydrate (CAS#: 6138-23-4), gelatin (CAS# 9000-70-8), agarose (CAS#: 9012-36-6), Palcam selective agar (PALCALM) (75977) and its supplement (02336) were purchased from Sigma Aldrich (Oakville, ON, Canada). Yeast extract (YE) (97064-372), magnesium sulfate (CAS#: 7487-88-9), calcium chloride (CAS#: 10043-52-4), hydrochloric acid (CAS#: 7647-01-0) and sodium hydroxide (CAS#: 1310-73-2) were purchased from VWR (Mississauga, ON, Canada). Tris base (CAS#: 77-86-1), sodium chloride (CAS#: 7647-14-5), tryptic soy broth (DF0370-17-3), agar (CAS# 9002-18-0), Oxford selective agar (OXA) (OXCM0856B) and its supplement (OXSR0140E) were purchased from Fisher Scientific (Ottawa, ON, Canada). Food-grade pullulan was gifted by the Hayashibara company (Okayama, Japan).

All chemical preparations used deionized water as the solvent. Media and buffers were sterilized via an autoclave. TSB+0.6% (w/v) YE was used as the growth media for *listeria*. Agar plates were prepared using 30 g/L TSB and 15 g/L agar. PALCAM and OXA selective agar plates were prepared according to the manufacturer's instructions. Soft agar for the overlay assay were prepared using 30 g/L TSB and 5 g/L agarose. Preparation of calcium chloride-magnesium sulfate (CM) buffer for dilution of *listeria* phage is described elsewhere[20,22].

3.2.2. Bacteria and Bacteriophage

The primary model phage used for this study was commercially available anti-*listeria* phage P100 (PhageGuard Listex; Micreos Food Safety; Wageningen, The Netherlands). Concentration of the stock P100 solution was confirmed to be approximately 1.5×10^{11} plaque forming units (PFU)/ml. The host for this phage was *Listeria monocytogenes* serotype 1/2a (ATCC Number: BAA-2659). The secondary phage model used to assess versatility of the encapsulation system was anti-*salmonella* phage Felix O1 (DSM Number: DSM 18524), where the host was *Salmonella enterica* serovar Typhimurium (ATCC Number: 700720).

All interactions requiring exposure of bacteria or phage was conducted within a biological safety cabinet to maintain sterility. *S. enterica* and *L. monocytogenes* cultures in TSB or TSB+0.6% YE media, respectively, were grown within a shaking incubator set to at 37 °C and 180 RPM for up to 18 h. Bacteria cultures were subsequently stored in the refrigerator for up to 5 days.

3.2.3. Bacteriophage propagation

Propagation of Felix O1 was completed by adding 100 µL of overnight *S. enterica* culture and 100 µL of stock Felix O1 at 1×10^{5} PFU/ml to 30 mL of TSB and incubating at 37 °C and 180 RPM for approximately 24 h. 10 µL of chloroform was added to the resulting suspension and allowed to sit for 15 min before undergoing centrifugation at 7000 rcf for 15 min. The Felix O1 supernatant

was then collected and filtered using a 0.22 µm filter. P100 used in this study was diluted directly from the commercial stock, additional propagation was unnecessary.

3.2.4. Bacteriophage quantification

Concentration of phage within liquid samples was quantified using a standard plaque overlay assay [23] within a biological safety cabinet. Plaques are clearings formed on a bacterial lawn as a result of virus lysis. Briefly, the phage sample was first serially diluted in CM buffer. Then, 100 uL of an overnight bacterial culture of the appropriate host was added to a tube containing 3.5 mL of soft agar then vortexed to mix. The mixed culture was then poured onto a TSB agar plate and allowed to dry. 10 µL of the diluted phage samples were then added on top of the bacterial layer of the agar plate to produce a spot test[24] or 20 µL was added and allowed to run down the length of the plate to produce a tear drop test [25]. The P100 and Felix O1 plates were incubated overnight at 30 °C or 37 °C, respectively, before the plaques were counted. Phage concentration was calculated as the number of PFU per mL of the original sample.

3.2.5. Transmission electron microscopy

The bacteriophage P100 was imaged through transmission electron microscopy. A sample taken directly from the PhageGuard Listex stock bottle was added to a copper-palladium grid. The sample was then negatively stained using 1% uranyl acetate and subsequently imaged (1200EX TEMSCAN; JEOL; Peabody, MA, USA.

3.2.6. Film production

Film production of P100 containing pullulan-trehalose films was based on the optimized stabilizing excipient system of Leung et al's[26] work. Briefly, a mixture of 10% pullulan+0.5M trehalose was prepared and allowed to dissolve on a stir plate overnight. The resultant solution was then filter-sterilized using a 0.2 um filter (SF0.22PES; FroggaBio; Concord, ON, Canada) and P100 was added to a final concentration of 1×10^9 PFU/ml. 200 µL of the P100+10% pullulan+0.5M trehalose solution was added to a microcentrifuge tube.

An additional 10% pullulan only solution was prepared and filter sterilized. 100 uL of the pullulan-only solution was added on top of the phage containing 10% pullulan+0.5M trehalose layer. As the addition of pullulan at high concentrations results in very viscous solutions, mixing of the layers was delayed. Due to the multilayered nature of the sample, an initial feedstock sample of the phage concentration was not taken. Instead, initial phage concentration was assumed to be the added concentration of 1×10^9 PFU/ml.

Desiccation of the phage suspensions into a film followed a similar procedure as Leung et al[20]. The open tube, multilayered phage suspensions were arranged on a tube rack and placed within a desiccator connected to a vacuum pump. The suspensions were then vacuum dried for approximately 1 day (22 h) to produce a phage film. The phage film was reconstituted with 1 mL of deionized water with 4 min of continuous vortexing. The reconstituted phage film was then measured for phage concentration, where sampling dilution was accounted for. The loss of phage due to vacuum desiccation was calculated by subtracting the log-transformed values of the assumed initial concentration (1×10^9 PFU/ml) from the P100 concentration recovered from reconstituted films.

Characterization of the phage films, including phage enumeration and pasteurization survival testing, was completed within 1 day of completed drying in order to minimize the effect of storage on the phage films. All experiments were conducted in biological triplicate with distinct samples.

3.2.7. Powder production

Three different excipient systems were investigated for their ability to stabilize phage during spray drying: 1) pullulan alone, 2) trehalose alone, and 3) a mixture of 30% pullulan and 70% trehalose by mass fraction. All investigated systems were formulated to a concentration of 100 mg/ml. Representative phage-free formulations were also spray dried for scanning electron microscopy to compare powder properties.

Feedstock preparation began with trehalose or pullulan powders weighed out via a mass balance. These powders were added to deionized water and allowed to dissolve for at least 1 h (trehalose-only formulations) or overnight (formulations containing pullulan) on a stir plate. Formulations were then volume adjusted with deionized water to the correct concentration. Formulations that were intended for phage-free spray drying were then filtered using a 0.45 µm syringe filter (SF0.45PES; FroggaBio; Concord, ON, Canada) to minimize clogging of the spray dryer atomizer. Formulations that were intended for phage encapsulation were filtered using a 0.22 µm filter to sterilize the solution.

Commercial P100 phage or propagated Felix O1 was then added directly to achieve a concentration of approximately 1×10^9 PFU/ml or 1×10^8 PFU/mL, respectively, in all phage-containing formulations. Solutions were then vortexed to mix the phage. In the case of the 10% w/v pullulan formulation, the solution was hand shaken for several minutes due to its high viscosity. 1 mL of each formulation was removed prior to spray drying as an initial feedstock measurement. All phage containing formulations were stored in the refrigerator prior to spray drying and were held within an ice bath during spray drying to minimize loss due to room temperature exposure.

Spray drying was conducted using a laboratory scale spray dryer (B-290; BÜCHI Corporation; New Castle, DE, USA) placed within a biological safety cabinet. Use of the biological cabinet was necessary to prevent phage aerosol exposure to the user during spray drying. Spray drying was conducted using an inlet temperature was set to 110 °C and the aspirator rate was set to 75% (approximately 30 m^3/h gas flow rate [27]), the pump rate was set to 10%, (feed flow rate of approximately 3 mL/min [27]), and the atomizing gas column height was set to approximately 35 mm (approximately 538 standard L/h [27]). These drying and atomizing conditions were predicted by a model to result in an outlet condition of 59 °C and 10% RH [28].

Powder yield was determined through Eq. 1, where C_f refers to the concentration of the feedstock, V is the volume of feedstock spray dried, and m_c is the mass of powder collected after spray drying. The powder mass collected from the spray dryer included material transfer loss.

$$Yield = \frac{C_f * V - m_c}{C_f * V} \times 100\% \qquad \text{Eq. 1}$$

Phage concentration within the powder was determined by reconstituting 100 ± 5 mg of powder with 1 mL of CM buffer, to the initial feedstock concentration of 100 mg/ml. Phage quantification, as described above, was then performed on the rehydrated powder. CM buffer was used as the rehydration solvent to maintain stability of the rehydrated powder under refrigerated storage. Phage loss due to processing was determined by subtracting the log values of the reconstituted phage concentration from the feedstock phage concentration.

Phage enumeration and pasteurization survival experiments on the spray dried powder was completed either the same day or the day after spray drying to minimize effect of storage time on the results. Similarly, all scanning electron microscopy was completed within two days of spray drying. All powders were immediately stored post-spray drying within glass vials sealed with parafilm. The vials were placed within moisture-resistant aluminum bags (2178K19; McMaster-Carr; Elmhurst, IL, USA) filled with desiccant.

3.2.8. Kill curves

Growth kinetic profiles of different *L. monocytogenes* samples were conducted to confirm that the presence of trehalose or pullulan does not hinder P100 lytic ability. The negative control sample consisted of *L. monocytogenes* at 10^5 CFU/ml in TSB-YE. The positive control sample consisted of 10^5 CFU/ml *L. monocytogenes* with 10^7 PFU/ml of P100 stock in TSB-YE. Four samples containing either trehalose or pullulan was prepared: 0.5% pullulan, 0.5% trehalose, 0.35% trehalose + 0.15% pullulan, and 7% trehalose + 3% pullulan. The powders were dissolved in TSB+YE then filter-sterilized using a 0.2 um filter prior to addition of P100 (10^7 PFU/ml) and L. monocytogenes (10^5 CFU/ml). Volume of P100 and *L. monocytogenes* added was consistent for all samples. 200 µL of each sample was added to a 96 well plate (10861-564; VWR; Mississauga, ON, CA) and the absorbance at 600 nm was read (Synergy Neo2; Biotek; Winooski, VT, USA) every 15 min for 24 h at an incubation temperature of 30 °C. All absorbance values were normalized by subtracting the value of TSB-YE, which was measured to be 0.107. All experiments were repeated in triplicate in separate wells.

3.2.9. Powder dissolution

Powder dissolution over time was assessed using a microplate reader (Synergy Neo2; Biotek; Winooski, VT, USA). An analytical balance (XS105; Mettler Toledo; Mississauga ON Canada) was used to measure out 20 ± 1 mg of either spray dried trehalose, spray dried pullulan, or spray dried pullulan-trehalose. The powder was carefully transferred to a single well of a 96 well plate (07-000-166; Fisher Scientific; Ottawa, ON, Canada) such that the powder was evenly distributed across the well. The microplate reader was set to an incubation temperature of 65 °C. 200 µl of deionized water was added to the powder via pipette immediately preceding absorbance readings. Absorbance was read at 600 nm every 5 s over 60 min. Control readings of all formulations was completed on the solid powder absorbance over time and the dissolved powder absorbance over time. All experiments were completed in triplicate on separate samples.

3.2.10. Scanning electron microscopy

Microscopy was conducted on the spray dried trehalose, spray dried pullulan, and spray dried pullulan-trehalose samples to compare particle morphology. Powder samples were mounted onto carbon tape covered aluminum stubs (16111; Ted Pella; Redding, CA, USA). All samples were

then sputter coated with gold to a thickness of approximately 15 nm (S150B; Edwards High Vacuum International; Crawley, UK or Q300 D Plus; Quorum; Laughton, UK). To minimize moisture exposure during transportation, samples were held within a stub holder box that was placed into an aluminum bag filled with desiccant. Images were taken using an electron microscope (TESCAN VEGA LSU; TESCAN; Brno-Kohoutovice, Czech Republic). Accelerating voltage was set to 5-10 kV and images were taken at a magnification of ×1000-7000. Images were adjusted for brightness and contrast; no other image processing was performed. Size distribution of the particles was determined using the image platform Fiji [29]. For each formulation, 193-386 particles within a SEM image were sized, where Feret's diameter was given as particle.

3.2.11. Modulated differential scanning calorimetry (MDSC)

Heat flow curves were obtained for spray dried phage-free samples of pullulan, trehalose, and pullulan-trehalose through DSC (Q200; TA Instruments; New Castle, DE, USA). MDSC was run for all samples using the procedure and analysis described by Manser et al.[30].

3.2.12. Pasteurization survival

Canadian guidelines for high temperature short time (HTST) pasteurization of milk products with less than 10% fat requires holding milk at 72 °C for 15 s[14]. After pasteurization, milk is immediately cooled to prevent loss of nutrients. The required holding time given by Health Canada does not account for the heating time, which is estimated to be 55 s for industry HTST pasteurization [31,32].

The phage films and phage powders were subjected to heating conditions designed to simulate industry HTST pasteurization. The phage concentration was then assessed post-concentration to evaluate the effectiveness of the film or powder protection system against pasteurization. First, a water bath (768308-896; VWR; Mississauga, ON, CA) was preheated to 73-75 °C. 1 mL of commercially available dairy milk was then added to microcentrifuge tubes containing either phage film or phage powder. The tube was placed within a floating rack that was subsequently placed within the preheated water bath for 2-2.5 min. Preliminary work found that this timeframe accounted for both heating time for 1 mL of milk within a microcentrifuge tube and the 15 s of mandated holding time. Immediately after, the samples were plunged into an ice bath to simulate the rapid cooling step of pasteurization. After at least 15 min of cooling within the ice bath, the samples were then vortexed for at least 4 min to fully dissolve the matrix. Phage quantification, as described above, was then conducted on the pasteurized samples.

The pullulan-trehalose phage films were evaluated for pasteurization survival in 1 mL of 2% milk purchased from a local grocery store. As a control, 1 tube of the phage film was fully dissolved in milk prior to pasteurization by vortexing continuously. Phage enumeration was completed using the tear drop test, with plating completed in technical duplicate. Pasteurization survival experiments were tested in biological triplicate on both the intact and the dissolved film samples.

The spray dried formulations were also evaluated for pasteurization survival in milk and water. 100 ± 5 mg of powder was massed out into a microcentrifuge tube. 1 mL of milk was then added to the tube before undergoing simulated HTST pasteurization. A control was prepared each experiment wherein the respective spray dried sample was fully dissolved in milk via vortexing

prior to exposure to pasteurization conditions. Spray dried pasteurization survival was tested in commercial skim milk, 2% milk, and 3.25% milk or water. Phage enumeration was completed using a spot test, with plating completed in technical duplicate. Pasteurization survival experiments were tested in biological triplicate, on distinct samples, on both the intact and the dissolved powder samples.

3.2.13. Storage stability

The suitability of the spray dried powder to be stored as a bulk product was assessed through a simple stability study. Briefly, the P100 containing pullulan-trehalose powder was aliquoted into a glass vial and sealed with parafilm. The glass vial was then placed into a moisture-resistant aluminum bag filled with desiccant and stored at room temperature, approximately 22 °C. At timepoint 0, 6 weeks, 10 weeks, 20, and 31 weeks of storage, 100 mg of the powder was removed from the vial and reconstituted to 100 mg/ml in CM buffer. The reconstituted powder was then measured for P100 phage concentration. For each timepoint three technical replicates were plated. The pasteurization survival of the intact powder was also assessed at the 31-week timepoint. Due to limited quantity of powder, the pasteurization survival experiments were completed in biological duplicate, where each duplicate was measured three times.

3.2.14. L. monocytogenes challenge of pasteurized phage powder in milk over 1 week

A proof-of-concept study was conducted to determine how well the pasteurized phage powder could reduce bacterial contamination within milk after 1 week of refrigerated storage. Study was designed based on Health Canada guidelines for testing *L. monocytogenes* in refrigerated foods [33]. Briefly, 100 mg of the pre-dissolved powder control and the intact phage powder samples, where P100 concentration was 3.0×10^6 PFU/ml when reconstituted, were pasteurized in 1 mL of 2% milk. *L. monocytogenes* from an overnight culture was then added to the milk samples to obtain a concentration of 1×10^3 colony forming units (CFU)/ml. The contaminated milk was then vortexed to mix, then 100 µL was removed as a timepoint 0 sample. The contaminated milk was then placed within a laboratory refrigerator (4 °C) for 1 week. The contaminated milk was tested for bacterial growth at timepoint 0, 1 day, 3 days, and 7 days of storage.

Enumeration of *L. monocytogenes* in milk was based on the Canadian government's published laboratory procedure for the enumeration of *L. monocytogenes* in foods MFLP-74. This procedure requires the use of two different types of *listeria* selective agar. First, the contaminated milk samples were serially diluted in CM buffer. 20 µL of the serially diluted sample was plated as a tear drop test onto both an OXA selective agar plate and a PALCALM selective agar plate. The plates were then incubated at 37 °C for approximately 24 h. Bacterial concentration across the two types of agar plates was averaged. Challenge experiments on the control and the intact powder sample was completed in biological triplicate.

3.2.15. Statistical Analysis

All statistical analysis comparing phage or bacteria concentration between samples was completed on the log-transformed values. Statistical analysis consisted of unpaired t-test through GraphPad Prism (GraphPad Software; San Diego, CA, USA), where statistical insignificance is defined as $p>0.05$. For results below the limit of quantification, statistical analysis was performed at the limit of quantification as a conservative estimate.

3.3. Results

3.3.1. Design and formulation of pullulan-trehalose powders for encapsulation of P100

Pullulan and trehalose (Figure 1a) were investigated as a stabilizing matrix for the commercial phage product, PhageGuard L, composed of phage P100 (Figure 1b). Kill curves of P100 infecting *L. monocytogenes* were performed in the absence or presence of pullulan, trehalose, or a pullulan-trehalose mixture (Figure 1c). Pullulan, trehalose and a mixture of 30% pullulan to 70% trehalose (by mass), was added at a concentration of 5 mg/ml. The 5 mg/ml concentration was chosen as a realistic addition to commercial products as the sugar contents of different dairy milks vary by approximately 5 mg/ml[34–38]. A high concentration of sugar (100 mg/ml) was also investigated for the 30:70 pullulan-trehalose mixture. In all cases, P100 was able to suppress the growth of *L. monocytogenes*, indicating that pullulan and trehalose did not hinder the ability of P100 to control the bacterial growth in media over the investigated timeframe.

Pullulan-trehalose structured microparticles were designed to encapsulate and stabilize P100 within a dry format. The formulation was designed to be composed primarily of trehalose (70% by mass) as the predominant stabilizer, with pullulan at the particle exterior. Particles were produced via spray drying, where a radial component distribution occurred as droplets dried into microparticles (Figure 1d). Trehalose, as a disaccharide with a low molecular weight (0.342 kDa) is expected to have a much higher diffusion coefficient than pullulan and subsequently is able to distribute towards the center of the droplet during drying. Pullulan is expected to accumulate on the surface of a pullulan-trehalose particle due to its much larger size (162 kDa[39]) and subsequently lower diffusion coefficient. The final resultant particle is expected to comprise of inverse radial distributions of trehalose and pullulan, where pullulan is concentrated at the particle exterior and trehalose concentrated at the particle center. Detailed theory and investigation of the mass distribution of pullulan and trehalose systems during the spray drying process has been completed by Carrigy et al.[40]. Resulting particles form a white, amorphous[40] powder (Figure 1e).

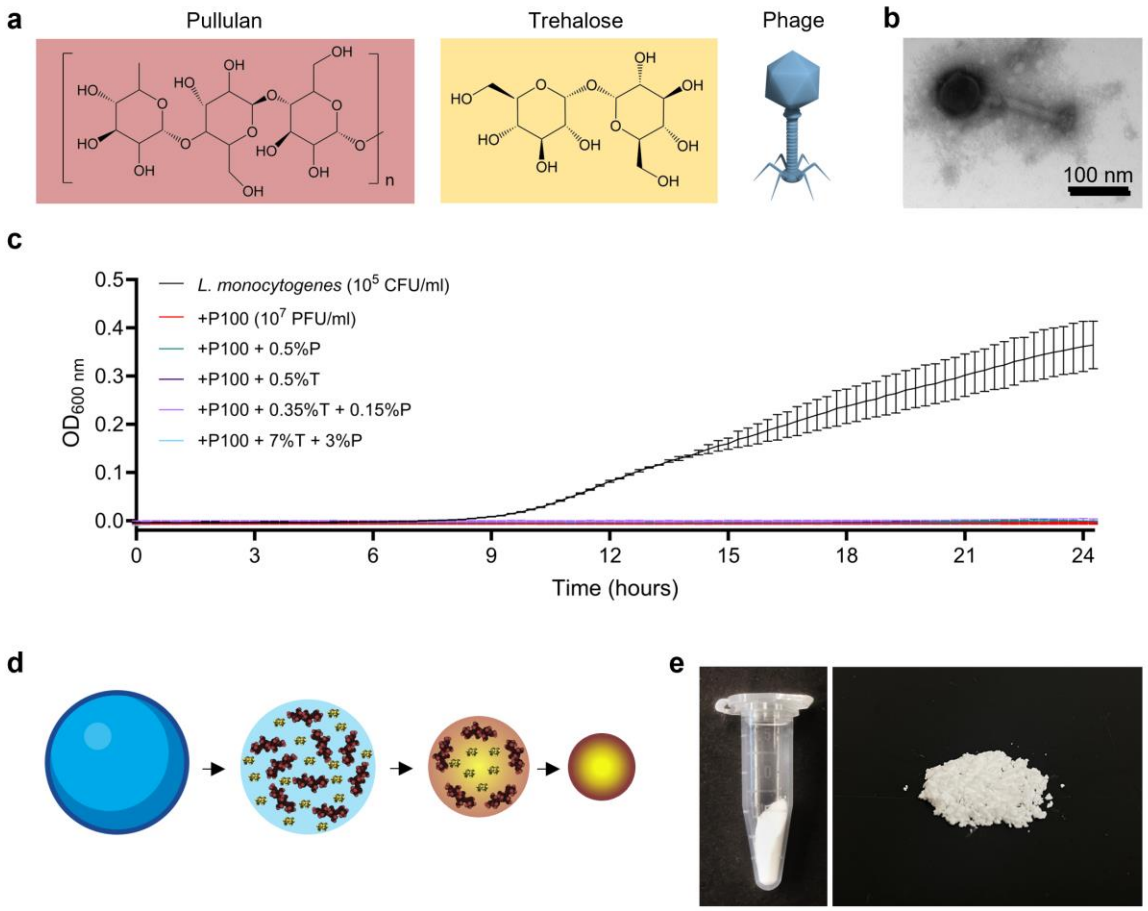


Figure 1. Layered excipient system composed of food-safe sugars can encapsulate bacteriophage P100 within a stable dry format for pasteurization protection. a. The edible sugars pullulan, a polysaccharide, and trehalose, a disaccharide, are used as the shield components to stabilize bacteriophages. **b.** Transmission electron microscopy of the anti-*listeria* bacteriophage P100. **c**. *L. monocytogenes* growth over 24 h at 30 °C, with the addition of P100 and different sugar concentrations to the TSB+YE media. P and T stands for pullulan, and trehalose, respectively, and growth was measured as absorbance at 600 nm. All percentages represent concentration as w/v. Experiments were completed in triplicate, with error bars representing standard deviation. **d.** Particle formation of the pullulan-trehalose particles from liquid droplets. As the atomized droplets experience surface recession due to evaporation, the small molecule trehalose is able to diffuse to the center of the droplet whereas larger molecule pullulan accumulates at the surface. Final microparticles have radially distributed pullulan with a higher concentration at the surface and trehalose concentrated at the particle center. **e.** Pictures of produced pullulan-trehalose powders. Pullulan-trehalose powders are white, with powders forming clumps. Chemical structures of trehalose and pullulan were made using ChemDraw, and 3D chemical models prepared using MolView.

Spray dried single excipient systems of trehalose or pullulan had a powder yield of 46% and 4%, respectively. The trehalose formulation produced highly spherical particles with either smooth particle surfaces or small indentations (Figure 2a). The pullulan particles appear bowl-shaped morphology, where particles are characterized by a large central crater, with occasional additional smaller dents appearing along the particle surface (Figure 2b). The spray-dried pullulan-trehalose powder yield ranged from 21 to 30% across three batches. The powder was composed of polydisperse irregularly shaped microparticles with indentations along the particle surface (Figure 2c). Carrigy et al.[40] note that the folding or dents on the surface, relative to a trehalose particle, is due to pullulan at the particle exterior, with increasing degree of folding occurring with higher

relative pullulan concentration. The morphology of the trehalose, pullulan, and pullulan-trehalose particles are consistent with literature[17,18,40].

Filaments of pullulan formed and deposited within the spray dryer during single excipient drying (Supplementary Figure 1). Fiber formation was not observed with the spray dried trehalose or pullulan-trehalose powders. The produced trehalose and pullulan-trehalose powders showed a logarithmic size distribution (Supplementary Figure 2 and Supplementary Table 1), consistent with the droplet production of a twin fluid atomizer[41]. The pullulan formulation produced a wider range of particle sizes, measuring as large as 88 µm, compared to spray dried trehalose and spray dried pullulan-trehalose. The filament formation and large size distribution may be due to incomplete atomization as a result of increased solution viscosity with increasing pullulan concentration[42]. Heat flow curves with respect to temperature were performed on the pullulan, trehalose, and pullulan-trehalose powders (Supplementary Figure 3). Thermal transitions determined by differential scanning calorimetry showed that the pullulan-trehalose sample possessed the individual characteristics of both pullulan and trehalose.

The dissolution pattern of each spray dried powder was assessed to evaluate the aqueous stability (Figure 2d-f). Water was added to the massed-out powders and then monitored for absorbance over time under static conditions in a plate reader set to 65 °C, where a decrease in absorbance indicated a more transparent solution. Fully dissolved powder in water was measured as a control for absorbance under the same conditions. As an additional control, solid powder was added to the wells and tested for absorbance for a measure of light blocking when no water was added. Trehalose powder dissolved almost immediately on contact with water (Figure 2d). This result is expected due to the small size and high solubility[43] of trehalose. In comparison, the pullulan powder had a significantly slower dissolution in the heated water, maintaining absorbance levels greater than or equal to the solid control for approximately 9.5 min (Figure 2e). The pullulan-trehalose powder also remained intact longer than the trehalose powder (Figure 2f), with absorbance measured greater than or equal to the solid control for approximately 2.5 min. The delayed dissolution of the pullulan-trehalose powder is due to the presence of pullulan at the exterior of the particle. The increase in absorbance measured when water was added to pullulan or pullulan-trehalose powder may be due to hydration of the polysaccharide[44,45], leading to additional light blocking.

The different formulations were then tested for their ability to encapsulate phage P100 over the course of spray drying. The phage titer loss when spray dried with trehalose alone was 3.9 log (Figure 2g), and spray dried pullulan performed significantly worse, with a 6.0 log loss in phage titer (Figure 2h). Spray dried pullulan-trehalose was able to stabilize P100 the best of the tested formulations, with an average titer loss of 2.4 log and variance of 0.3 log loss between three distinct batches (Figure 2i).

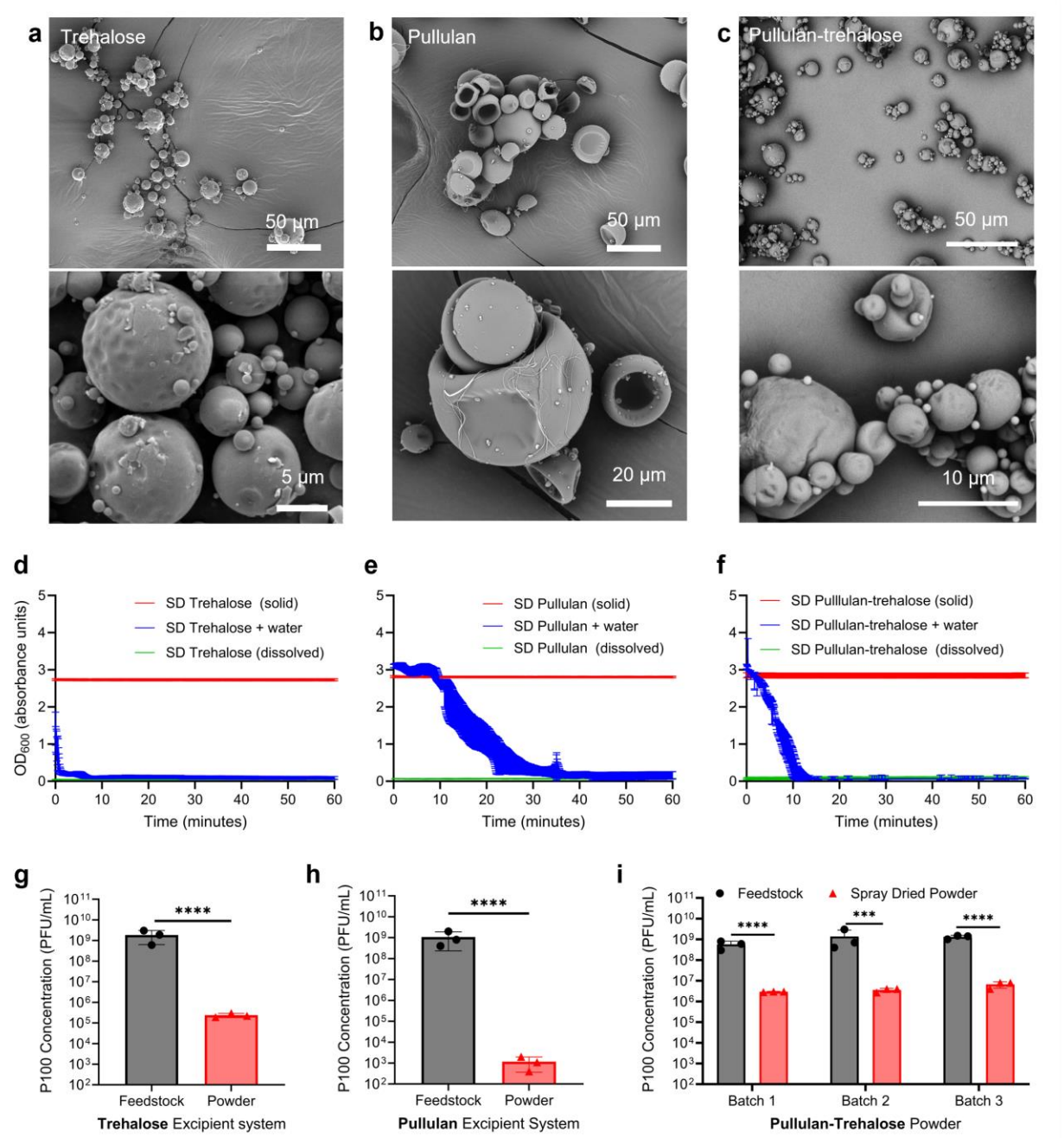


Figure 2 Spray drying of pullulan-trehalose, as compared to single excipient systems pullulan or trehalose, affects dissolution, particle morphology, and P100 phage encapsulation efficiency. a-c. Scanning electron micrographs of spray dried 100 mg/ml trehalose (**a**), spray dried 100 mg/ml pullulan (**b**), and spray dried 100 mg/ml of pullulan-trehalose formulation (30:70 mass ratio) (**c**). Scale bars are shown on the respective image. **d-f.** Dissolution of spray dried powders over 1 h when held in water under static conditions and incubated at 65 °C. For comparison, pre-dissolved powders in water were measured under the same condition. Similarly, absorbance of solid powders within the well was measured. Dissolution is measured as a function of absorbance over time, where lower absorbance indicates transparency due to dissolution. Spray dried trehalose (**d**) dissolves almost instantly with the addition of water whereas spray dried pullulan (**e**) has a significantly slower dissolution. Similarly, presence of pullulan at the exterior of the pullulan-trehalose particles slows dissolution of this formulation. **g-i.** Concentration of P100 in feedstock prior to spray drying ("Feedstock" – black circle) and rehydrated powder ("Powder" – red triangle) were each measured in triplicate and plotted when spray drying P100 with 100 mg/ml of trehalose (**g**), pullulan (**h**), or pullulan-trehalose (**i**). Spray drying of P100 with trehalose or pullulan alone led to 3.9 and 6.0 log processing loss, respectively. Spray drying of P100 with the pullulan-trehalose formulation led to an average processing loss of 2.4 log across three distinct batches. Dissolution experiments

were completed in triplicate on distinct samples. All concentration measurements were performed in triplicate on single batches of powder, error bars represent standard deviation. Standard deviation is represented by error bars. Statistical analysis was performed on the log-transformed values, where p≤0.0001 - ****. Abbreviations: SD – spray dried.

3.3.2. Pasteurization protection and shelf stability of sugar polymer encapsulated phage powders

The developed pullulan-trehalose powder was designed to protect P100 from thermal degradation when held in an aqueous medium. An application of this design is the protection of P100 during milk pasteurization. Dairy milk of different fat contents was added to the phage powder and then immediately subjected to simulated HTST pasteurization conditions (heating then holding at 72 °C for 15 s, followed by rapid cooling) (Figure 3a). Phage powder was also intentionally dissolved within milk and then subjected to pasteurization as an unprotected control. The pullulan-trehalose powder stabilized the encapsulated P100 phage during pasteurization. When protected within the pullulan-trehalose particles, P100 phage loss was insignificant during HTST pasteurization (Figure 3b). This protection was maintained for all tested dairy milks. However, P100 titer fell below the limit of detection (100 PFU/ml), a >4.5 log loss, when the phage was directly exposed to heat due to intentional powder dissolution (Figure 3b).

Pasteurization survival experiments were also completed on trehalose powder to examine the effect of particle composition on thermal protection of the phage. Pasteurization survival experiments could not be completed for the pullulan formulation due to insufficient powder yield. The titer of P100 in the trehalose powder prior to pasteurization was 4.7×10^3 PFU/mL and that fell to below the limit of detection for both pasteurized pre-dissolved powder and the intact trehalose powder (Supplementary Figure 4).

A new processing aid will need to remain stable during storage, ideally without costly refrigeration requirements. Therefore, the pullulan-trehalose P100 powder was also tested for its long-term ambient stability. Pullulan-trehalose powder was packaged for long term storage. Bulk powder was aliquoted into a glass vial and sealed with parafilm. The vial was then placed into a moisture resistant aluminum bag filled with desiccant and subsequently stored under ambient conditions. The powder underwent 1.3 log loss ($p<0.001$) over 7 months (31 weeks) of storage at 22 ^oC, where timepoint 0 is defined as the P100 titer post-powder production (Figure 3c). The 7-month stored powder experienced no loss in P100 activity when pasteurized in 2% milk, relative to the unpasteurized stored powder sample after reconstitution (Figure 3d).

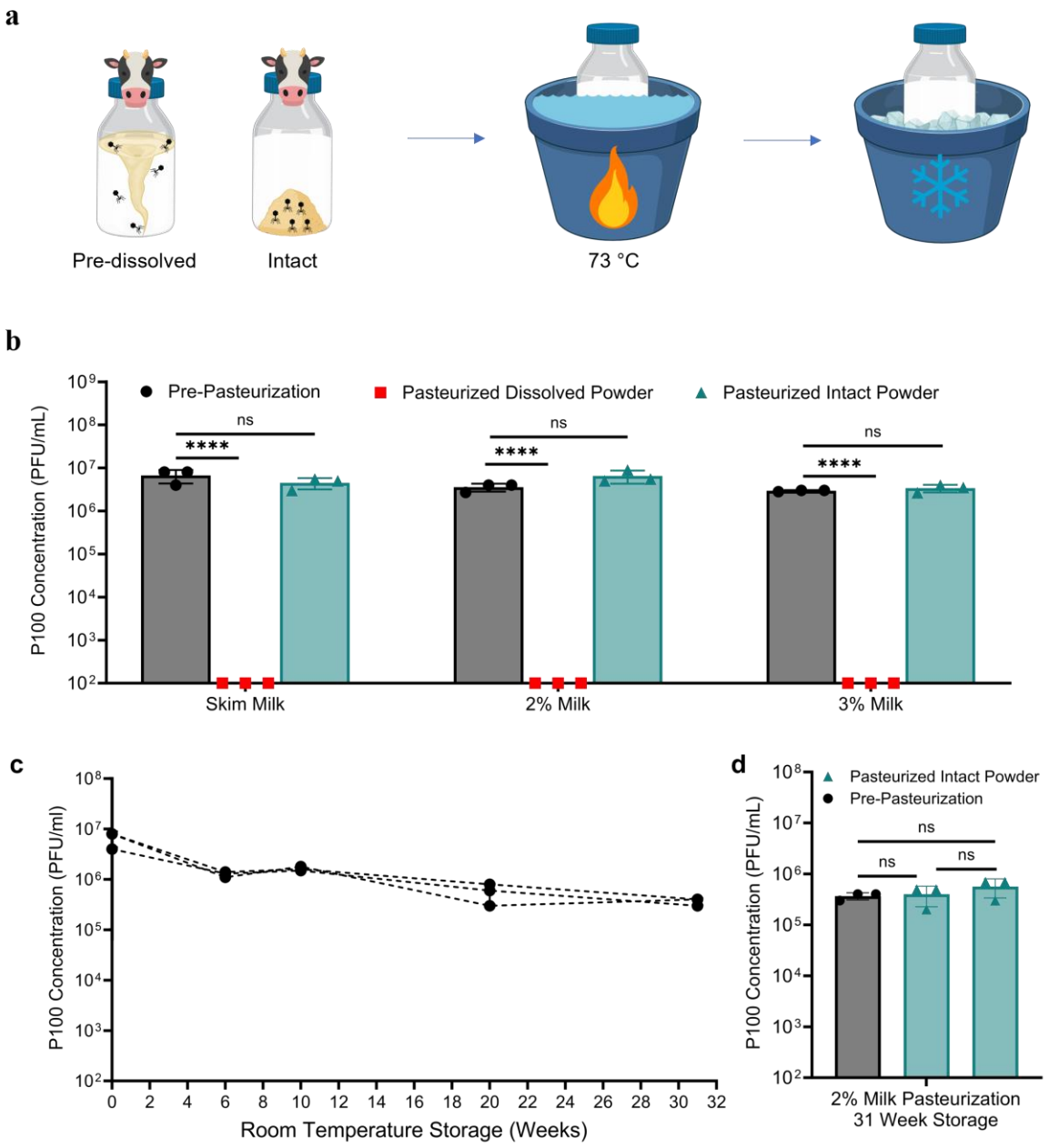


Figure 3. Phage P100 encapsulated within pullulan-trehalose powder is protected from thermal degradation by HTST pasteurization and exhibits shelf stability. a. Spray dried P100 pullulan-trehalose powder was assessed for aqueous heat stability through simulated milk pasteurization. Powder was either intentionally dissolved in milk or left intact before placing into a water bath preheated to 72-73 °C. The milk was then held in the water bath for 2.25-2.5 min to simulate high temperature short term pasteurization. The milk was then immediately transferred to an ice bath to mimic the cooling step. **b.** Comparison of phage P100 encapsulated powders when pasteurized in commercial milks with different fat content. Pre-pasteurization measurements were completed in triplicate on the relevant batch of powder. Pasteurization experiments were completed in triplicate on distinct samples for all conditions. Pasteurization in commercial skim, 2%, and 3.25% milk reduced P100 concentration of the pre-dissolved powders (black circle) to a concentration BLOQ (100 PFU/ml), significantly lower than the initial concentration (red square). The phages protected within the pullulan-trehalose system (teal triangle) underwent insignificant levels of loss when pasteurized. **c.** Concentration of the spray dried P100 pullulan-trehalose powder undergoes 1.3 log loss after 31 weeks of storage (7 months) at room temperature (~22 °C). Measurements are taken in triplicate from one batch of powder at timepoint 0, 6 weeks, 10 weeks, 20, and 31 weeks of storage. Error bars represent standard deviation for a given timepoint. **d.** Concentration of phage P100 encapsulated powders previously stored for 31 weeks at room temperature when subjected to pasteurization within 2% milk. Pre-pasteurization measurements were completed in triplicate on one sample of powder. Pasteurization experiments were completed in duplicate on

different samples (n=1, n=2), where each sample was measured three times. Statistical analysis was performed on the log-transformed values, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, $0.001 \leq p < 0.01$ - **, and $p \leq 0.0001$ - ****. For measurements BLOQ, values were analyzed at the limit of quantification for statistical analysis as a conservative estimate. Abbreviations: BP – before pasteurization, PDP – pre-dissolved, pasteurized powder, PD – pasteurized powder, BLOQ – below limit of quantification.

3.3.3. Reducing post-pasteurization Listeria monocytogenes contamination growth in milk using stabilized phage power.

The pullulan-trehalose powder retains phage activity after processing as well as pasteurization but it has not yet been established whether the remaining titer has sufficient potency to protect milk from subsequent bacterial contamination. The ability of the pullulan-trehalose phage powder to impede post-pasteurization *L. monocytogenes* contamination in commercial 2% milk was assessed over 7 days post HTST pasteurization (Figure 4). The US Department of Agriculture[46] and product packaging generally suggest consumption of commercial milk within 7 days of opening. Approximately 10^3 CFU/ml of *L. monocytogenes*, simulating a post-processing contamination based on Health Canada guidelines of 10^2-10^5 CFU/g to evaluate post-processing lethality [33], was added to milk that had been pasteurized with either pre-dissolved phage powder or intact phage powder. Commercial milk with no powder added was also inoculated with *L. monocytogenes* as an additional control.

L. monocytogenes within in the control milk sample grew from 3.1×10^3 to 9.9×10^4 CFU/ml over 7 days of refrigerated storage (Figure 4a). Similarly, the milk sample wherein added phage powder was dissolved prior to pasteurization also demonstrated *listeria* growth from 1.4×10^3 to 1.0×10^5 CFU/ml. The milk with phage powder that was protected during pasteurization within the sugar particles successfully suppressed bacterial growth, with significantly lower ($0.001 \leq p < 0.01$) *L. monocytogenes* concentration (3.8×10^3 CFU/ml) than the untreated and pasteurized, dissolved phage powder milk samples. The difference in concentration at day 0 and at day 7 was statistically insignificant for the milk sample treated with intact phage powder (Figure 4b).

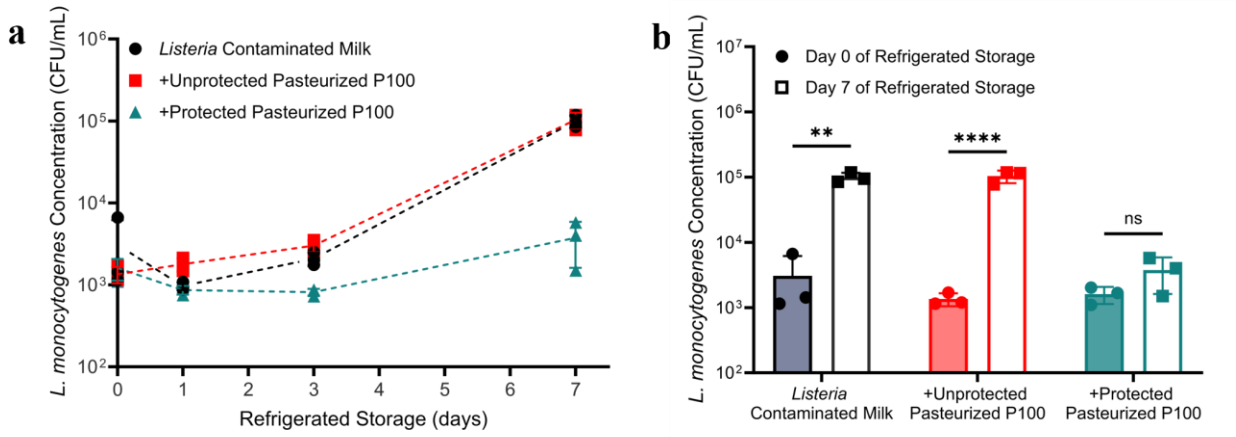


Figure 4. P100 bacteriophage powder added to milk pre-pasteurization hinders growth of a post-pasteurization *Listeria monocytogenes* contamination over one week of refrigeration (4 °C). Challenge study consisted of three arms. Approximately 1×10^3 CFU/ml of *L. monocytogenes*, simulating a post-processing contamination[33], was added to commercial milk, or to commercial milk that had been pasteurized with either pre-dissolved phage powder or intact phage powder prior to refrigeration. **a.** *L. monocytogenes* growth in untreated milk (black circle), milk with phage powder dissolved prior to pasteurization (red square) or pasteurized with intact phage powder (teal triangle) was monitored over 1 week of refrigerated storage. **b.** Comparison of bacterial enumeration at day 0 and day 7 of refrigerated storage demonstrated that the phage powder significantly reduced *L. monocytogenes* growth in milk. Results shown are the average of distinct triplicate experiments conducted in 2% milk, where error bars represent the standard deviation. Statistical analysis was performed on the log-transformed values, where $p\geq0.05$ – not significant, $0.001\leq p<0.01$ - **, and $p\leq0.0001$ - ****.

3.4. Discussion

A spray dried pullulan-trehalose system was designed to encapsulate P100 bacteriophage and protect the phage from degradation during pasteurization. The formulation was designed to have a relatively high solids content (100 mg/ml) and spray dried at a relatively high temperature (110 °C)[47] to encourage rapid particle formation. Higher solids content has been shown to reduce processing loss[48] and improve long term stability[49] of spray dried viruses, likely as there is sufficient material available to stabilize the viruses.

Trehalose, a small sugar, is known to stabilize protein-based molecules better than larger saccharides, such as pullulan[50]. Trehalose accumulation during early heat shock is critical for thermotolerance in several organisms[51–54]. In its amorphous glass form, one of trehalose's mechanisms of stabilization is through vitrificiation[55], the immobilization of a biologic leading to reduced degradation. However, trehalose is inclined for rapid dissolution (Figure 2d), making trehalose particles unsuitable for stabilizing within aqueous environments. This characteristic explains why P100 encapsulated within trehalose powder, which underwent partial dissolution during pasteurization, had a much lower P100 retention (Supplementary Figure 4) than the pullulan-trehalose powder (Figure 3).

Conversely, pullulan powders remained intact significantly longer than trehalose powder when held in heated water (Figure 2e). There are several mechanisms related to the large difference in molar volumes between pullulan (~578571 cm^3/mol)[39,56] as compared to trehalose (~224 cm^3/mol)[57] that are responsible for the difference in dissolution rate. For instance, the higher molecular weight of pullulan corresponds to higher intermolecular interactions, and thereby slower separating of pullulan from the particle surface due to interactions with water[58].

The dissolution rate of a component within water is related to diffusion. The diffusion of a component can be approximated by the Stokes-Einstein equation (Eq. 2), where *D* is the diffusion coefficient, k_B is Boltzmann's constant, *T* is the absolute temperature, η is the dynamic viscosity, and *r* is the radius of the particle. The diffusion coefficient of pullulan will be lower than trehalose due to the former's larger size. Furthermore, at the polymer-water interface, the diffusion coefficient of pullulan will be further reduced relative to trehalose due to the increased η.

$$D = \frac{k_B T}{6\pi\eta r} \quad \text{Eq. 2}$$

Pullulan has a much larger intrinsic viscosity as described by the Mark-Houwink equation (Eq. 3), where *M* is the molecular weight, and *a* and *K* are system dependent parameters. A lower diffusion coefficient will reduce the rate of diffusion, as explained by Fick's second law. Subsequently, the reduced rate of diffusion will lead to a reduced rate of dissolution.

$$[\eta] = KM^a \quad \text{Eq. 3}$$

However, pullulan alone is not a suitable stabilizer of the P100 phage. The pullulan formulation of P100 underwent a very high processing loss (>6 log) and very low powder yield. Low yield of this formulation was due to ineffective breakup of the highly viscous pullulan feed into droplets[59]. Instead, the produced streams of pullulan dried into fibers instead of droplets, leading to a low powder yield as the fibers deposit prior to collection. Results indicate that both pullulan and trehalose are necessary components for the investigated application of aqueous stability.

The designed pullulan-trehalose microparticles significantly improved the pasteurization survival of encapsulated P100. No significant loss in phage occurred post-pasteurization in any of the commercial diary milks (skim, 2%, 3.25%) (Figure 3). Protection of the phage in all cases was likely conferred due to the layered nature of the sugar microparticle. The pullulan layer at the interface delayed milk ingress into the film and subsequent dissolution of the trehalose component. As such, the predominantly trehalose stabilization matrix was maintained over the course of HTST pasteurization, protecting the phage from thermal degradation.

Spray drying has distinct advantages over other drying methods, specifically for food applications. It produces an easy-to-handle powder compatible with most industrial feeding systems. It is a simpler manufacturing process than vacuum drying and coating of thin films. It has been reported and modelled that pullulan accumulates at the particle exterior during droplet drying[40], creating a radially distribution within pullulan-trehalose particles. To achieve the same pullulan-rich interface in a film, it must be prepared using a multi-layering to-handle powder compatible with most industrial feeding systems. It is a simpler manufacturing process than vacuum drying and coating of thin films. It has been reported and modelled that pullulan accumulates at the particle exterior during droplet drying[40], creating a radially distribution within pullulan-trehalose process. For a comparison, a multi-layered pullulan-trehalose film encapsulating P100 was prepared (Supplementary Figure 5a,b), with P100 undergoing 1.8 log loss during preparation (Supplementary Figure 5c). Subjecting the reconstituted P100 phage to pasteurization in 2% milk produced an additional >4.8 log loss, with an activity below the limit of detection (50 PFU/ml) (Supplementary Figure 5d). On the other hand, subjecting the intact film to milk pasteurization before the phage was reconstituted greatly improved viral stability, with only 1.1 log loss further

after film preparation (Supplementary Figure 5e). The film was designed to be composed of a phage containing 37% pullulan and 63% trehalose layer with a 100% pullulan top layer. However, the layers may have intermixed over the course of slow vacuum drying, leading to an overall film composition of 47% pullulan to 53% trehalose. The higher relative concentration of trehalose to pullulan within the spray dried powder as compared to the film may have improved the powder's ability to protect against conformational changes during pasteurization. Results confirm that a layered pullulan-trehalose system can stabilize phage during aqueous heat exposure, however, film preparation is less advantageous than spray drying for this application.

The lowest pullulan-trehalose powder yield (21% or 3.8 g/hr) was still significantly higher yield over time than the production of films via vacuum drying over one day in this study (60.2 mg/film/day). In addition, the phage powder is compatible with current pasteurization processes wherein it may be added along with other ingredients (i.e. additional sugar for chocolate or flavored milk[14,60]) prior to pasteurization. The phage powder was stable over storage (1.3 log loss over 7 months at room temperature) and maintains pasteurization-protection, indicating potential for bulk storage. For comparison, a spray dried CP30A phage with a pullulan-trehalose stabilizing system underwent approximately 0.7 log loss/ml over one month[17] and pullulan-trehalose film encapsulated P100 underwent <2.5 log loss/film after 1 month of room temperature storage[20]. Therefore, although a film matrix greatly improved survivability of P100 when pasteurized, a powder form is more advantageous for large scale implementation. Further work is needed to determine the titer limit necessary for maintaining protection.

Critically, both trehalose and pullulan are water-soluble sugars, allowing for the gradual dissolution Figure 2f) and release of encapsulated phage into the surrounding food media. Sugars are already present in many food products, including at ~50 mg/mL in dairy milk [34–38] and so sugar-only system would have minimal effect on palatability. Furthermore, this lactose-free formulation should be compatible with pasteurized plant-based milk alternatives[61] consumed by those with lactose allergies. The challenge study demonstrated that the P100 encapsulated within pullulan-trehalose was protected from pasteurization and subsequently able to reduce the bacterial contamination within commercial refrigerated milk (Figure 4). The self-amplifying characteristic of phages offers an advantage compared to other antimicrobial interventions. However, the Canadian government requires <100 CFU/g *L. monocytogenes*[62] all ready-to-eat foods. The current phage pullulan-trehalose powder would thus be a useful layer of protection to reduce incidence of illness but insufficient to completely eliminate the bacterial threat. Based on a *L. monocytogenes* infectious dose of 10^8 CFU[63], consumption of approximately 1 L of unprotected milk from this study would infect a low-risk host, as compared to 27 L of phage-protected milk. For high-risk hosts this volume lowers to only 1-97 mL (10^5-10^7 CFU) of unprotected milk versus 27-2700 mL of phage-protected milk. Increased effectiveness of the phage intervention may be accomplished by using phages that are more infective under refrigerated temperatures[64] or increasing the relative concentration of phage to bacteria[65]. The latter may be achieved through processing optimization to reduce manufacturing loss, or spray drying robust phages, to preserve relatively higher amounts of phage per mg of powder. For instance, spray drying of the more robust phage Felix O1 under the same conditions led to a much lower processing loss of only 0.77 log (Appendix A).

3.5. Conclusion

This study details the development of a low-cost, food-safe, engineered sugar matrix for the preservation of phage during industry HTST pasteurization. The designed sugar matrix is composed of slow-dissolving pullulan in combination with glass stabilizer trehalose. The distribution of the pullulan and trehalose components was designed to prevent dissolution during pasteurization while simultaneously stabilizing the phage against thermal exposure. Encapsulation of model phages was accomplished via vacuum drying or spray drying. These sugar-encapsulated phages, in both film and powder form, exhibited drastically increased pasteurization survival within milk over their pre-dissolved counterparts. Post-pasteurization, the temporary sugar shield dissolves, and releases phages within the surrounding media for dormant protection. The powder was shown to maintain pasteurization-protective ability after seven months of room temperature storage. A contamination study demonstrated that the addition of phage powder to milk pre-pasteurization resulted in reduced bacterial growth under refrigerated storage as compared to a control.

This is the first study to develop a food-safe system for phage protection against aqueous high-temperature exposure. This is in contrast to previous work in this field that has focused primarily on encapsulating phages within dry formats for long-term room temperature stability or for oral delivery of enteric treatments. The developed phage powder is designed for easy and cost-effective integration into existing industry pasteurization procedures and is a promising method to reduce the extent and severity of outbreaks.

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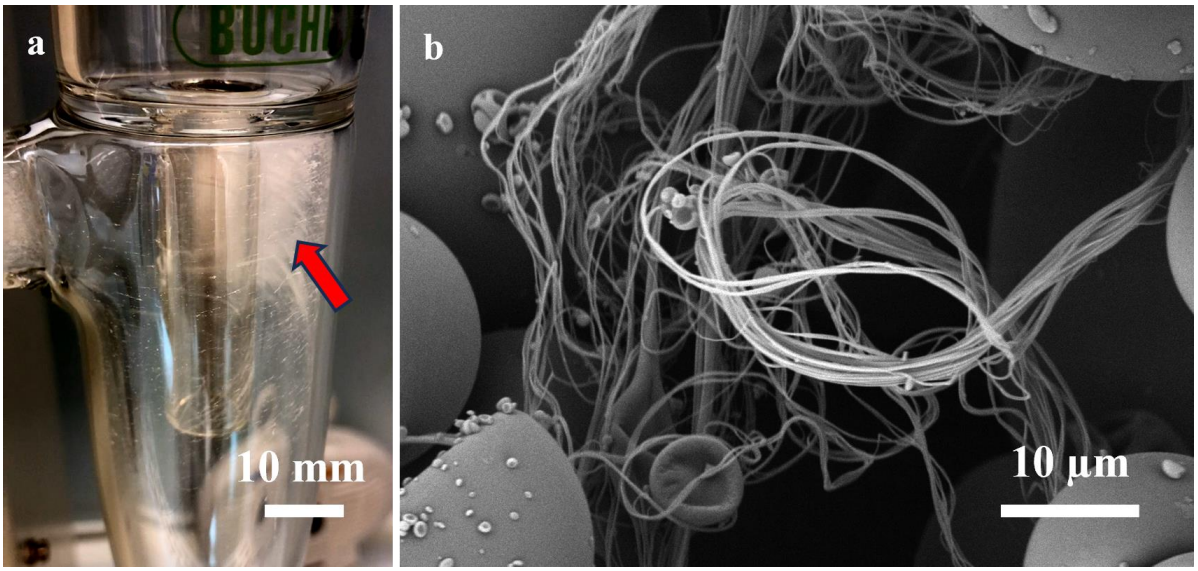
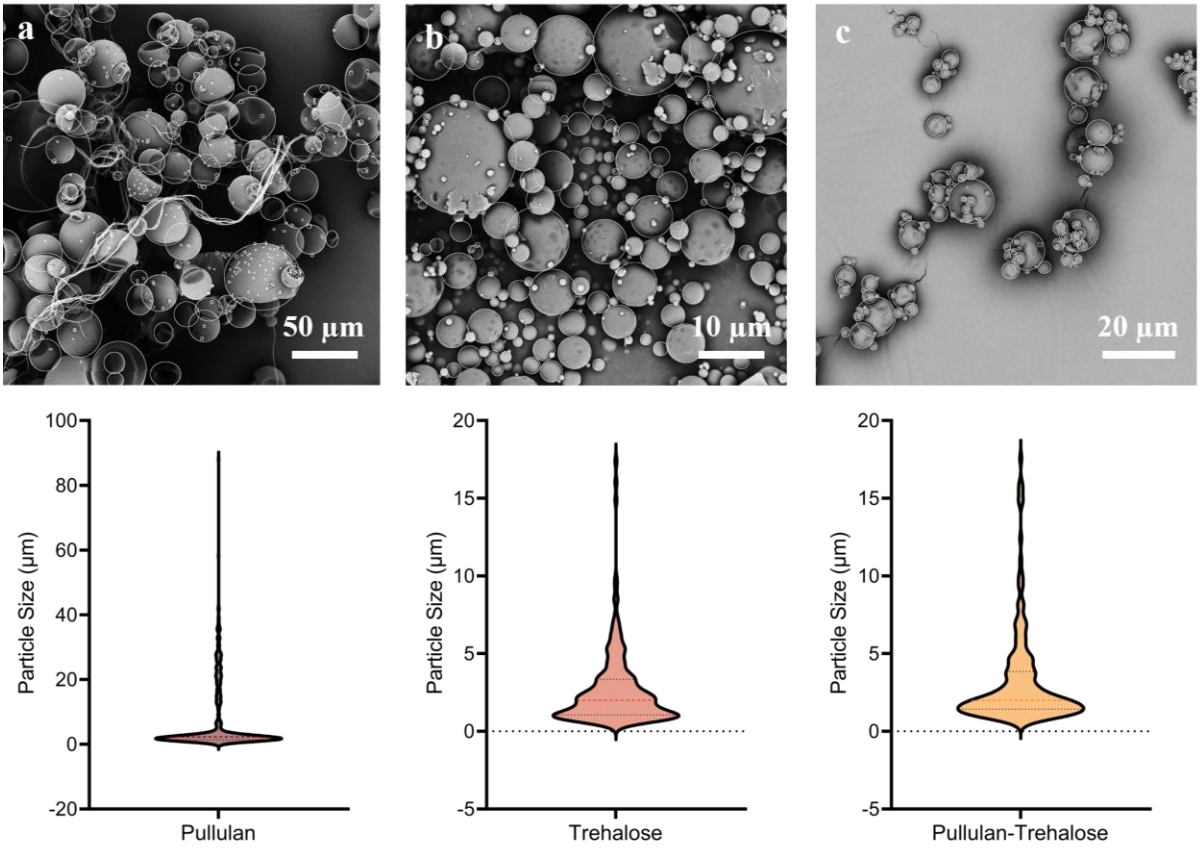
3.6. Supplementary Material



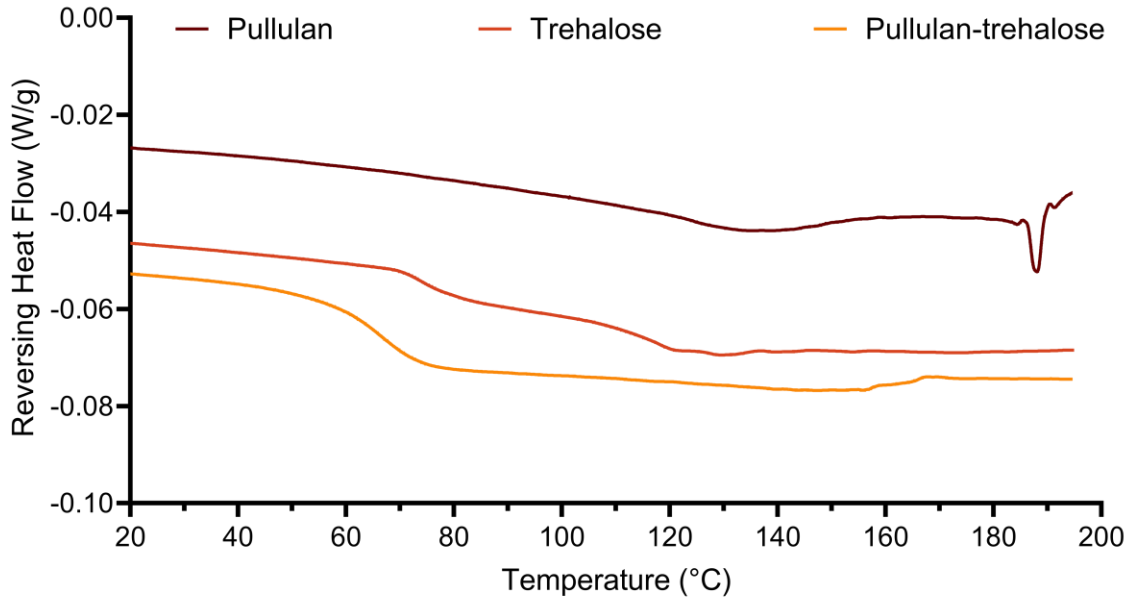
Supplementary Figure 1. Spray drying of a relatively high concentration (100 mg/ml), viscous pullulan formulation leads to the generation of pullulan fibers. a. Formation of pullulan fibers (red arrow) can be seen on a macroscopic level within the spray drying apparatus. Buildup of pullulan fibers likely contributed to a reduced yield of the pullulan formulation as compared to the trehalose and pullulan-trehalose spray dried formulations. **b.** Microscopic pullulan fibers were also present in the collected powder sample, as shown through scanning electron microscopy. Scale bars are shown on the image.



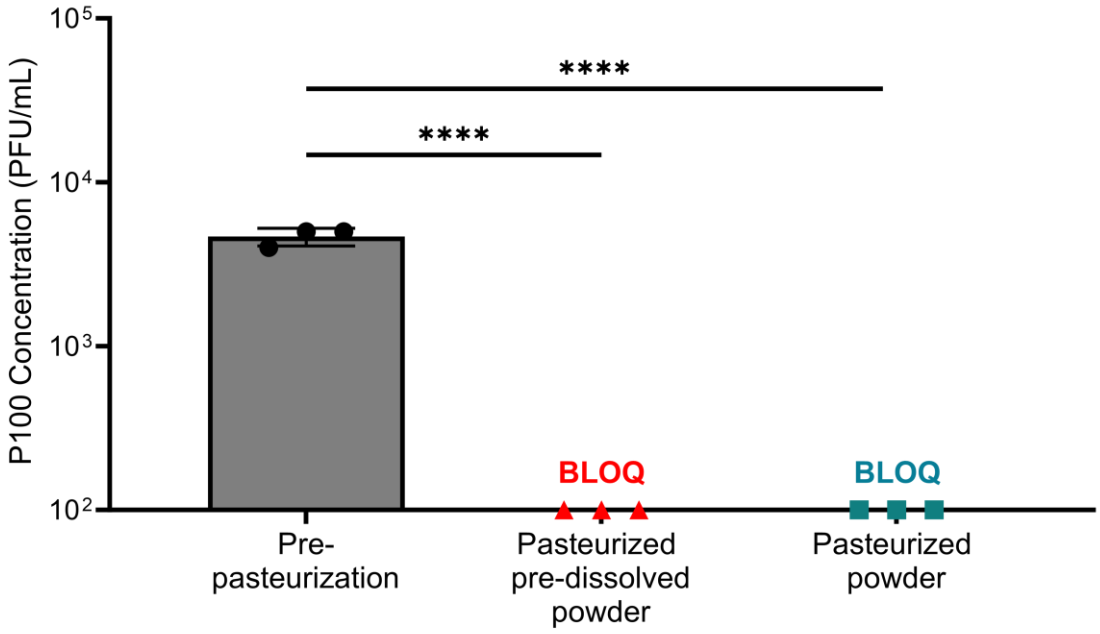
Supplementary Figure 2. Particle size distribution of the spray dried powder varied based on the excipient system. Size distribution of the **a)** pullulan, **b)** trehalose, and **c)** pullulan-trehalose spray dried formulations as measured on SEM images in ImageJ as Feret's diameter (top) and the corresponding violin plot of particle sizes (bottom). Particles size distribution of the trehalose and pullulan-trehalose samples appear to follow a lognormal distribution, as expected from the droplet generation pattern of a twin fluid atomizer. Deviation from a lognormal distribution shown in the pullulan particle size distribution is likely due to incomplete atomization due to the high viscosity of the solution.

Supplementary Table 1. Descriptive statistics of the sized SEM images shown in Supplementary Figure 2 using Graphpad's histogram analysis. Pullulan particles have a larger range and higher mean particle diameter than the trehalose and pullulan-trehalose samples.

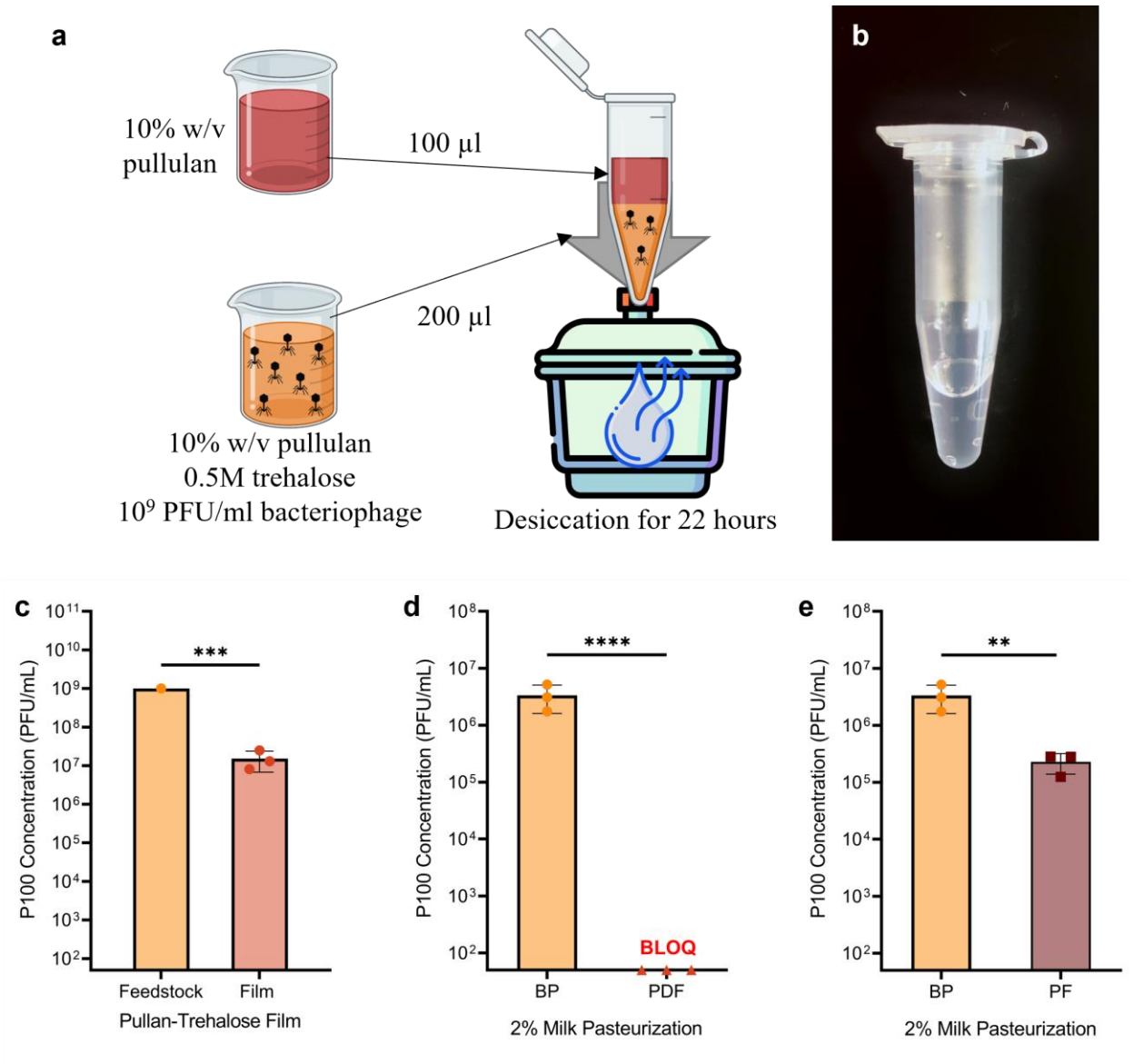
Statistics	Pullulan	Trehalose	Pullulan-Trehalose
Total number of particles	386	201	193
Minimum	0.57	0.57	0.71
25% Percentile	1.42	1.05	1.43
Median	2.27	1.99	2.00
75% Percentile	6.61	3.35	3.86
Maximum	88.07	17.39	17.56
Mean	7.29	2.67	3.24
Std. Deviation	10.80	2.46	3.126



Supplementary Figure 3. Comparison of the thermograms collected for spray dried pullulan (top, burgundy), trehalose (middle, red), and pullulan-trehalose (bottom, orange) indicate that pullulan and trehalose are present in the pullulan-trehalose powder. Similar thermal transitions within the thermograms confirm the presence of trehalose and pullulan within the pullulan-trehalose formulation.



Supplementary Figure 4. Anti-*listeria* phage P100 encapsulated within trehalose powder is not protected from thermal degradation by HTST pasteurization in 2% milk. Comparison of phage P100 concentration when pasteurized in 2% milk as a pre-dissolved powder or left intact. Pre-pasteurization measurements ("Pre-pasteurization} – black circle) were completed in triplicate on a single batch of powder. Pasteurization experiments were completed in biological triplicate for all conditions. Error bars represent standard deviation of measurements. Concentration of P100 in pre-dissolved ("Pasteurized pre-dissolved powder" – red triangles) and intact trehalose powder ("Pasteurized powder" – blue square) fell BLOQ (100 PFU/ml) upon pasteurization. Statistical analysis was performed on the log-transformed values, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, $0.001 \leq p < 0.01$ - **, $0.0001 \leq p < 0.001$ - ***, and $p \leq 0.0001$ - ****. For measurements BLOQ, values were analyzed at the limit of quantification as a conservative estimate. Abbreviations: BLOQ – below limit of quantification.



Supplementary Figure 5. Anti-*listeria* phage P100 can be encapsulated within pullulan-trehalose films; films protect P100 from thermal degradation by HTST pasteurization as compared to pre-dissolved P100. a. Schematic of the preparation of pullulan-trehalose multi-layered films. First, a 10% w/v pullulan+0.5M trehalose+P100 bacteriophage suspension is added to a microcentrifuge tube. 10% w/v pullulan only solution is then added on top, due to the high viscosity of the mixtures there is a delayed mixing effect. The multilayered system is then dried via vacuum desiccation. **b.** Picture of the clear pullulan-trehalose film. **c.** Encapsulation of P100 bacteriophage within films undergoes approximately 1.8 log loss on drying. Initial phage concentration prior to desiccation was measured out to be 1×10^9 PFU/ml ("Feedstock" - orange circle). Three distinct replicate films are plotted, standard deviation is represented by error bars ("Film" – red triangle). **d-e.** Concentration of phage P100 encapsulated films when subjected to pasteurization within 2% milk. **(d)** Concentration of pre-dissolved phage film ("PDF" - red triangle) is below the limit of quantification (BLOQ) (50 PFU/ml), significantly lower than the concentration before pasteurization ("BP" - orange circle). All measurements were completed on separate triplicate samples and plotted. (**e)** P100 concentration of phage film that underwent pasteurization ("PF"- burgundy square) is 1.1 log lower than the initial concentration ("BP" – orange circle). All measurements were completed on separate samples in triplicate and plotted. Standard deviation is represented by error bars. Statistical analysis was performed on the log-transformed values, where $0.001\leq p<0.01$, $0.0001\leq p<0.001$ - ***, and $p\leq0.0001$ - ****.

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Chapter 4: Spray dried leucine-lactose and leucine-lactose-maltodextrin Felix O1 bacteriophage platform is suitable for intermixing and storage with commercial powdered infant formula

Preface

In this chapter we consider bacteriophages as a method of intervention against bacterial contamination in powdered infant formula. However, bacteriophages are typically formulated as a liquid, so they must be stabilized within a powder that can be intermixed with commercial infant formula. We designed the commercial friendly powder system to be commercial friendly using the anti-*Salmonella* phage Felix O1 as a model phage. Powders were produced via spray drying, a common method for producing powdered infant formula. Furthermore, we designed a leucine-lactose and a leucine-maltodextrin-lactose stabilizing system, as these ingredients are already present in commercial infant formulas. This stabilizing system was assessed for phage encapsulation efficiency during production and the compatibility with two commercial infant formulas. Lastly, we subjected the powder to storage under realistic packaging conditions for 6 months, as well as conditions mimicking formula storage after the package has been opened. Both the leucine-lactose and the leucine-maltodextrin-lactose powders are suitable for use as a method to administer bacteriophages with powdered infant formula. Use of this presentation has the potential to reduce bacterial outbreaks in infants.

The concept of this work is based off my comprehensive exam, wherein my supervisory committee was composed of Zeinab Hosseinidoust, Carlos Filipe, Michael Thompson, and Myrna Dolovich. I was responsible for concept development, all experiment design, all data collection, all data analysis, all visualization, and manuscript composition. Michael Thompson was a supervisory author who provided equipment resources. Carlos Filipe and Zeinab Hosseinidoust were supervisory authors who provided resources and were involved in discussion of results and discussion of experimental design.

Citation

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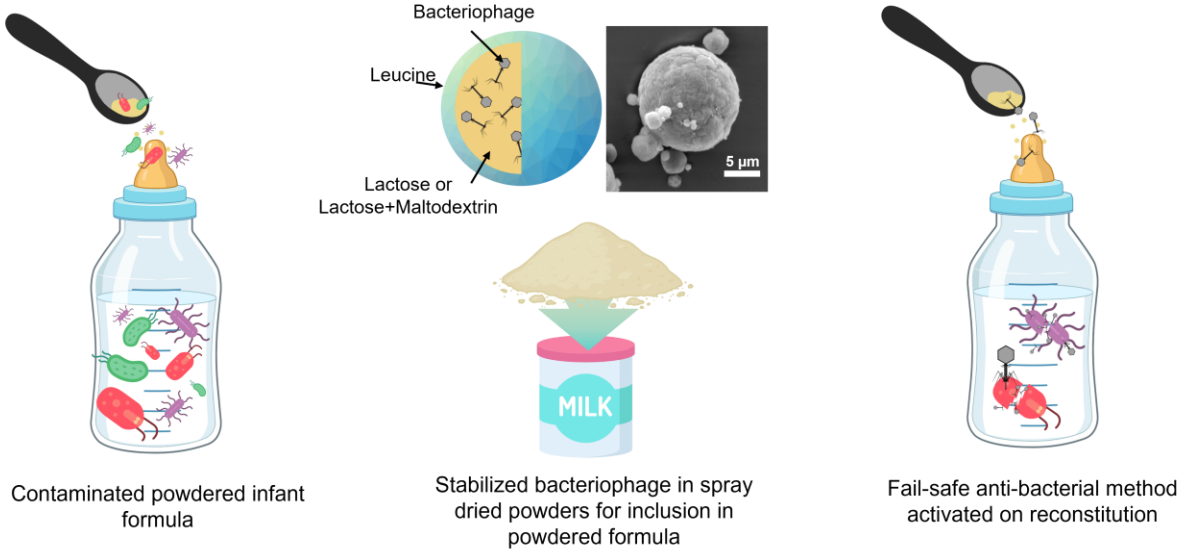
Development of spray dried bacteriophage platform for inclusion in powdered infant formula. **Mellissa Gomez**, Michael Thompson, Zeinab Hosseinidoust, and Carlos Filipe.

Abstract

Post-processing bacterial contamination of food is a significant global health risk. Inclusion of bacteriophages, viruses that infect bacteria, into food matrices may provide a built-in fail-safe method of protection. For instance, powdered infant formula is not sterile. However, addition of the liquid bacteriophage as a protectant is not possible. A spray dried bacteriophage powder was designed to be directly mixed with powdered infant formula. Upon reconstitution the bacteriophage would be released in the liquid formula and eliminate present harmful bacteria. Two stabilizing excipient systems were designed using ingredients already present in infant formula. Both consist of the disaccharide lactose as a stabilizer and the essential amino acid leucine as a shell former, with one formulation also including maltodextrin. Anti-*salmonella* bacteriophage Felix O1 was used as a model bacteriophage. Felix O1 underwent <0.35 log processing loss over spray drying. Inclusion of the powders in powdered infant formula did not significantly change the composition, pH, or dissolution pattern. A six-month stability study to assess shelf, and a six-week stability study to evaluate shelf life after package opening, were conducted. Felix O1 powder stabilized after one or two months of room temperature storage, with no significant change in concentration for the remainder of the study. The powders appeared to be biologically stable (insignificant loss) over a secondary shelf life of six weeks, rendering it compatible with infant formula's recommended secondary shelf life of one month. Investigated excipient systems would allow for the inclusion of stabilized bacteriophages with powdered infant formula as a fail-safe protection.

Keywords: powdered milk, *salmonella*, Felix O1, stabilization, bacterial contamination, food safety



4.1. Introduction

Powdered infant formula is a necessary substitute/supplement for human milk for many infants. The ease of storage and global availability of powdered infant formula are great advantages over human donor milk and liquid formula. Unopened powdered infant formula has a shelf-life of 18 months and a secondary shelf-life of one month after opening[1]. However, powdered infant formula is not sterile, with the World Health Organization (WHO) listing *Salmonella enterica* and *Cronobacter sakazakii* as the two pathogens of greatest concern[2]. There have been many cases of *S. enterica* and *C. sakazakii* foodborne illnesses linked to infant milk products. In 2022, four cases of *C. sakazakii* infection were linked to powdered infant formula in the USA, which is suspected to have contributed to two infant deaths[3]. Further inspection of the facility lead to a temporary plant shutdown upon discovery of unsanitary conditions[3], contributing to a powdered infant formula shortage. Exportation of contaminated infant formula can also lead to multi-country outbreaks, such as the 39 cases of *Salmonella* Agona in 2017[4], and the 32 cases of *Salmonella* Poona in 2018[5]. Outbreak severity may be exacerbated as government agencies issue recalls inefficiently and ineffectively. The addition of a "fail-safe" for powdered infant formula may reduce the risk that contamination poses to infant health.

Ideally, powdered formula would be heat-treated prior to packaging. However, powdered formula cannot be heat-treated post-production without significant reduction in stability and quality[6–8]. Infection can also occur to due domestic cross-contamination[9], and many sterilization methods do not prevent further downstream contamination. Given these constraints, an ideal method of eliminating bacterial contamination would be the inclusion of a thermostable, ingestion-safe bactericide that will activate upon reconstitution. A strain- or species-specific method of eradication would also prevent the destruction of the beneficial bacteria that may be added to formula to mimic breast milk. One solution is the use of bacteriophages, also known as phages, highly specific viruses that infect bacteria.

There are many phages that have been isolated and show promise to reduce *C. sakazakii* or *S. enterica* contamination. *S. enterica* phages have been assessed for control in dairy milk[10–13], whereas *C. sakazakii* phages have been assessed for control in both dairy milk[14,15] and rehydrated infant formula[15–19]. These studies have investigated efficacy under both refrigeration and temperatures up to 37 °C. While there have been some promising results in the rehydrated powdered infant formula, these studies do not offer a method for integrating the phage product with the infant formula powder format. Only one recent paper by Imm and Chang[20] present a method of stabilizing phage within a dry product that can be added to powdered infant formula.

Imm and Chang[20] have presented lyophilization of an anti-*C. sakazakii* phage using collagen peptides and trehalose as a stabilizing system. Their results are very promising, with demonstrated stability of 4 weeks of storage at room temperature. Furthermore, the rehydrated product was able to significantly reduce *C. sakazakii* contamination in commercial powdered infant formula at room temperature. The results are encouraging, with opportunity for improvements in this emerging field. For instance, spray drying could be used instead as a more production friendly method of desiccation, wherein atomized droplets are evaporated to form a powder composed of microparticles.

Spray drying is a critical step in the manufacturing process of powdered infant formula[21,22]. The same manufacturing method may be used to prepare powdered phage products. The spray dried combination of leucine and a disaccharide, such as lactose, has been investigated extensively as an inhalable stabilizing excipient system[23,24]. This system has been used to spray dry different phages to create an inhalable dry powder treatments for respiratory infections[25]. Leucine has a hydrophobic side chain that allows it to act as a weak surfactant in water[26,27]. As such, leucine accumulates on the surface of drying droplets during spray drying, generally forming an outer layer of crystalline leucine on the final particle. The presence of hydrophobic leucine as a shell also improves stability of the powder as it mitigates water-based degradation reactions and prevent lowering of the T_g due to moisture sorption. Thus, this combination not only creates highly inhalable biological powders, but can also improve the room temperature storage stability in a dry format[28].

To this end, a spray dried phage formulation was developed for inclusion in infant formula. Anti-*salmonella* Felix O1 was chosen as a representative phage for this study. The spray dried phage product must be composed of infant safe ingredients, undergo minimal processing loss and be compatible with the powdered infant formula and its storage. Two systems using established infant-safe ingredients were investigated: a lactose-leucine and a lactose-leucine-maltodextrin system. Both formulations were designed such that particles would be stable for conditions relevant to powdered infant formula storage. The spray dried formulations were assessed for processing loss, infant formula compatibility in terms of composition and properties, and storage stability. The aim of the project was to prepare a spray dried platform that could be used to encapsulate bacteriophages for simple intermixing with powdered infant formula. Upon reconstitution, the phage would be released and free to deactivate harmful bacteria within the formula.

4.2. Theory

Briefly, spray drying is a technique wherein a feed solution is atomized into a drying chamber with a flowing gas. The droplets are then dried into particles through evaporation and subsequently separated from the airflow. The final product is a powder wherein resultant properties are dependent on material properties and processing parameters. Understanding of how the spray drying parameters influence the particle formation is crucial to engineering spray dried particles. The rate of droplet evaporation is dependent on the properties of the solvent and the inlet temperature. Complex computation models have been developed to estimate evaporation rates of different systems[29]. The lifetime of a given droplet can be calculated based on the initial diameter and the evaporation rate[30].

The distribution within a multi-composite particle is unlikely to be even. The surface of droplet will recede as it dries. Concurrently, the components within the droplet will diffuse away from the surface towards the center of the droplet. The distribution of a given component within a particle dependent on the material properties affecting diffusion within the drying droplets as well the spray drying processing parameters. Both elements will influence the mass transfer within the droplet during drying. The diffusion of a given component is primarily dependent on the size and shape of the molecule, the solvent itself and its viscosity. The diffusion of a component through a liquid

can be approximated by the Stokes-Einstein equation (Eq. 1), where D is the diffusion coefficient, k_B is Boltzmann's constant, T is the absolute temperature, η is the dynamic viscosity, and r is the radius of the particle. This formula does not account for charged particles, assumes sphericity of the component, and assumes non-turbulent flow.

$$D = \frac{k_B T}{6\pi\eta r} \quad \text{Eq. 1}$$

Molecules that diffuse significantly slower than the rate of evaporation are expected to accumulate on or near the surface of the droplet whereas molecules that diffuse faster are expected to form evenly distributed spherical particles[30]. This distribution of components can be predicted using the dimensionless Peclet number, Pe (Eq. 2), where κ is the evaporation rate. For $Pe \approx 1$ suggests even distribution whereas $Pe >> 1$ suggests surface accumulation.

$$Pe = \frac{\kappa}{8D} \quad \text{Eq. 2}$$

Encapsulation of large components expected to accumulate near the droplet surface, such as bacteriophages or emulsions, can be achieved by increasing the overall solids content and increasing the temperature of the drying gas [31]. Increasing the overall solids content of the solution increases the viscosity of the droplet. This increase in viscosity will reduce the diffusion coefficient thereby reducing the speed of redistribution of the components during drying. Increasing the drying gas temperature leads to a faster formation of the initial semi-permeable "crust" at the droplet surface. A fast crust formation of this crust prevents large components from accumulating at the droplet interface, wherein biological components may be inactivated at the solid interface.

In addition to determining the distribution of components with a particle, spray drying parameters can affect the phase of the solids. This is especially critical for the stabilizers within a spray dried product. Phages are very susceptible to degradation when held at elevated temperatures. Prevention of degradation is primarily accomplished using amorphous glass stabilizers, such as amorphous lactose[32]. Amorphous glass sugars stabilize through two mechanisms: vitrification and water replacement[33].

Vitrification is the immobilization of components within a bulk system, minimizing the mobility available for degradation. Water replacement theory assumes that the stability of a component within water is due to hydrogen bonding. Therefore, water replacement theory refers to stability conferred when the hydrogen bonds formed with water are replaced with hydrogen bonds with the stabilizing sugar. This mechanism is most effective for sugars that have many spaces available for hydrogen bonding and are small enough to closely match against complex structures[34]. These mechanisms of stabilization are only relevant for stabilizers within an amorphous glass form. Therefore, a successful powder utilizing an amorphous glass stabilizing system must produce an amorphous glass form upon spray drying and must maintain a glassy amorphous form during storage.

Crystal nucleation occurs when a component reaches supersaturation. This typically occurs during spray drying at the droplet interface where the receding surface and evaporation lead to increased concentration. The time for a component to reach saturation at the droplet surface, t_{sat}, can be calculated relative to the droplet lifetime, t_{d}, using Eq. 3 for components with a *Pe* less than 20, where c_0 is the initial concentration of the component, and c_{sol} is the solubility[30]. Crystallization occurs after the surface becomes supersaturated. Based on the equation for t_{sat}, we find that crystallization is more likely to occur for components with a lower solubility, and with higher feedstock concentration. A long crystallization window will allow more time for crystal growth[35].

$$t_{\mathrm{sat}} = t_{\mathrm{d}}\left(1 - \left(\frac{c_0\left(1 + \frac{Pe}{5} + \frac{Pe^2}{100} - \frac{Pe^3}{4000}\right)}{c_{\mathrm{sol}}}\right)^{2/3}\right) \qquad \text{Eq. 3}$$

For components that are not expected to crystallize over the short drying times associated with spray drying, the time at which amorphous shell formation is to commence, t_{t}, can be calculated using Eq. 4, where ρ_{t} is the true density.

$$t_{\mathrm{t}} = t_{\mathrm{d}}\left(1 - \left(\frac{c_0\left(1 + \frac{Pe}{5} + \frac{Pe^2}{100} - \frac{Pe^3}{4000}\right)}{\rho_{\mathrm{t}}}\right)^{2/3}\right) \qquad \text{Eq. 4}$$

Yan et al.[36] have demonstrated that spray drying at a higher solids content produced more stable bacteriophage powder. This phenomenon may be due to the larger particles produced at the higher feed concentration, where there is proportionally more bulk composition available for stabilization. The final particle size can be engineered based on process parameters and formulation parameters. Assuming a spherical solid particle, final particle diameter, d_{g}, can be calculated based on a mass balance before and after spray drying, as given Eq. 5, where the c_{F} is the solids concentration of the feedstock, p_{ρ} is the particle density, and d_0 is the initial droplet diameter [37]. From this equation we find that increased solids content and larger initial droplet diameters will lead to larger particles.

$$d_g = \sqrt[3]{\frac{c_F}{\rho_P}}\, d_0 \qquad \text{Eq. 5}$$

The excipient system must be composed of safe ingredients and should not significantly alter the composition of infant formula such that the phage powder can be directly mixed in with powdered formula before packaging. Preliminary work to establish a set of spray drying parameters and two lead excipient candidates is detailed in Supplementary Note 1.

4.3. Methods

4.3.1. Chemicals

Investigative excipients for spray drying were D-mannitol (CAS 69-65-8; Fisher Scientific; Ottawa, ON, Canada) D-lactose monohydrate (CAS 64044-51-5; Sigma Aldrich; Oakville, ON, Canada), L-leucine (CAS 61-90-5, Fisher Scientific; Ottawa, ON, Canada) and maltodextrin DE 13-17 (CAS 9050-36-6; Sigma Aldrich; Oakville, ON, Canada).

Tryptic soy broth (TSB) (DF0370-17-3), salmonella shigella (SS) agar (B11597), agar (CAS 9002-18-0), sodium chloride (CAS 7647-14-5), and tris base (CAS 77-86-1), were purchased from Fisher Scientific (Ottawa, ON, Canada). Agarose (CAS 9012-36-6) and gelatin (CAS 9000-70-8) was purchased from Sigma Aldrich (Oakville, ON, Canada). Magnesium sulfate (CAS 7487-88-9),) and hydrochloric acid (CAS 7647-01-0) purchased from VWR (Mississauga, ON, Canada).

TSB (30 g/L) was used as the growth media for bacteria, and agar plates were poured from TSB (30 g/L) and agar (15 g/L) solution. Soft agar, as used for bacteriophage enumeration, was prepared from TSB (30 g/L) and agarose (5 g/L) solution. Sodium-magnesium (SM) buffer [0.1 M NaCl, 8mM $MgSO_4$, 50 mM of 1M Tris-HCl (pH 7.5), 0.1% gelatin][38] was used to dilute bacteria and bacteriophage. All aforementioned media and buffers were prepared in deionized water and autoclaved after preparation for sterility. SS agar was prepared according to manufacturer's instructions.

4.3.2. Bacteria and bacteriophage

The anti-*Salmonella myovirus* Felix O1 (DSM 18524) was used as the model phage in the study. *Salmonella enterica* serovar Typhimurium (ATCC 700720) was used as the bacterial host. A high concentration stock of Felix O1 (10^{10} plaque forming units per ml [PFU/ml]) was propagated in *S. enterica* for this study.

Felix O1 was rehydrated from lyophilized format as per vendor instructions. *S. enterica* overnight culture was prepared in TSB media from a frozen stock. This culture was placed in a shaking incubator set to 180 rpm and 37 °C for 24 h. 30 µL of the subsequent overnight culture was added to 30 ml of TSB. The resulting *S. enterica* subculture was then placed into the shaking incubator for 4 h to reach exponential phase. At this point, 30 µL of Felix O1 was added to the subculture and the resulting suspension was placed back into the incubator to allow for propagation overnight. 10 µL of chloroform was added to the suspension and allowed to rest for 15 min to release any phages contained within bacteria. The suspension was then centrifuged at 7000 rcf for 15 min to pellet the bacteria. The supernatant was removed and filter sterilized to obtain the high concentration stock of Felix O1. The Felix O1 stock was placed in the refrigerator for storage until use.

4.3.3. Biological enumeration

The concentration of liquid Felix O1 samples were quantified using a standard plaque overlay[39] assay. 100 uL of an overnight *S. enterica* culture was added to 3.5 mL of soft agar then mixed. The mixed culture was then poured onto a TSB agar plate. The phage suspensions were diluted in SM buffer. Once the agar plate had dried, 10 µL of the diluted phage were spot tested[40] on top of the bacterial layer of the agar plate. Plates were incubated over approximately 24 h at 37 °C to

allow for plaque formation. Plaques were counted to obtain phage concentration of the original sample as PFU/ml.

4.3.4. Spray drying

Excipients (mannitol, leucine, lactose, maltodextrin) were dissolved in deionized water on a stir plate overnight. The dissolved solution was then volume adjusted to the correct concentration. The resulting solution was filter-sterilized using a 0.2 µm filter and transferred to a sterile tube. An aliquot of the high concentration Felix O1 stock was added to the filtered solutions to achieve a phage concentration of ~10^9 PFU/ml. 1 ml of the suspension removed prior to spray drying as a feedstock sample. Powder production was accomplished using a laboratory scale spray dryer (B-290; BUCHI Corporation; New Castle, DE, USA). Lead candidates and processing parameters were determined based on literature and preliminary work (Supplementary Note 1).

Two formulations were investigated as lead stabilizing excipient systems: a leucine-lactose formulation (LL) and a leucine-maltodextrin-lactose formulation (LML). Both formulations consisted of a total solids content of 72 mg/ml. The LL formulation contained 14.4 mg/ml of leucine and 57.6 mg/ml of lactose for a mass ratio of 20:80 leucine to lactose in the dry powder. The LML formulation contained 12 mg/ml of leucine, 12 mg/ml of maltodextrin, and 48 mg/ml of lactose for a mass ratio of 16.7:16.7:66.7 leucine to maltodextrin to lactose.

Clean dry air was used as the drying gas where the inlet temperature was set to 100 °C. Aspirator flow rate was set to 100% (35 m^3/h)[41], atomizing gas height set to 40 mm (667 L/h)[41], and the pump speed set to 10% (feed flow rate of 3 ml/min)[41]. Outlet temperature varied from 61-64 °C, and outlet relative humidity was estimated to be 7.5% [42].

4.3.5. Powder yield

Powder yield was determined by dividing the powder mass collected by the total solids content spray dried. Calculated powder yield included loss of powder on transfer from the spray dryer collection port to the vial.

4.3.6. Bacteriophage enumeration within powder and processing loss

The spray dried powder was weighed out to 72 ± 3 mg and reconstituted with 1 ml of sterile deionized water to achieve the same solids concentration as the feedstock. Bacteriophage enumeration was then completed on the reconstituted powder as described above. Enumeration was conducted on three powder aliquots for a given sample, and each reconstituted aliquot was plated in duplicate.

Prior to spray drying, 1 mL of the feedstock was removed and set aside to determine the initial concentration. This sample was plated in triplicate. Bacteriophage processing loss was calculated by comparison of the feedstock concentration to that of the reconstituted powder.

4.3.7. Salmonella enterica challenge in powdered infant formula

The ability of stock Felix O1 bacteriophage to eliminate *S. enterica* in commercial infant formula (Similac; Saint-Laurent, QC, Canada) was assessed under several relevant conditions as part of the preliminary work. Commercial powdered infant formula was prepared according to manufacturer's instructions, then infected with overnight *S. enterica* to obtain a final concentration

of $6.5x10^3$, or $6.5x10^4$ colony forming units (CFU) per ml. Samples were then treated with 10^7 PFU/ml of Felix O1 stock and held for 24 h under refrigeration (4 °C), room temperature (~21 °C) and at 37 °C. Control samples were infected with *S. enterica* but left untreated with Felix O1. Initial conditions and endpoints were plated on selective SS agar and incubated for 24 h at 37 °C. Colonies were then counted to determine *S. enterica* growth in terms of CFU/ml at the endpoint. Experiments were completed once per condition.

4.3.8. Salmonella enterica kinetic curves

The lytic ability of spray dried Felix O1 encapsulated within LL or LML formulations was compared to that of Felix O1 stock. *S. enterica* cultures were infected with the phage preparations and a growth kinetic profile was conducted using a plate reader (Synergy Neo2; Biotek; Winooski, VT, USA). The bacterial concentration over time was measured in terms of culture optical density (OD). Separate tubes containing 5 mg/ml of phage free LL, phage free LML, Felix O1 encapsulated within LL, or Felix O1 encapsulated within LML was dissolved within TSB media. The resulting suspension was then sterile filtered with a 0.2 µm filter. Felix O1 concentration within the dissolved LL and LML formulations was measured to be $1.4x10^7$ PFU/ml and $1.8x10^7$ PFU/ml, respectively. Felix O1 stock was added to the TSB tubes that contained 5 mg/ml of phage-free LL or LML, to a final concentration of $1.7x10^7$ PFU/ml. *S. enterica* overnight culture diluted in TSB and subsequently added to all formulations for a final concentration of $7.0x10^2$ CFU/ml to simulate a low contamination. Uninfected samples, containing *S. enterica* and phage-free LL or LML, were also prepared as a control. The six subcultures (Figure 1) were added to a 96 well plate (10861-562; VWR; Mississauga, ON, Canada). The optical density at a wavelength of 600 nm was recorded over 24 h every 10 min. The incubation temperature was set to 37 °C and the well plate was subjected to constant agitation through continuous shaking at a frequency of 425 cycles per minute (cpm). Experiment was completed in triplicate on distinct samples. All OD readings were normalized by subtracting the OD of TSB media, 0.095. Host bacteria, medium, and temperature were chosen based on the recommendations of the manufacturer for Felix O1 propagation.

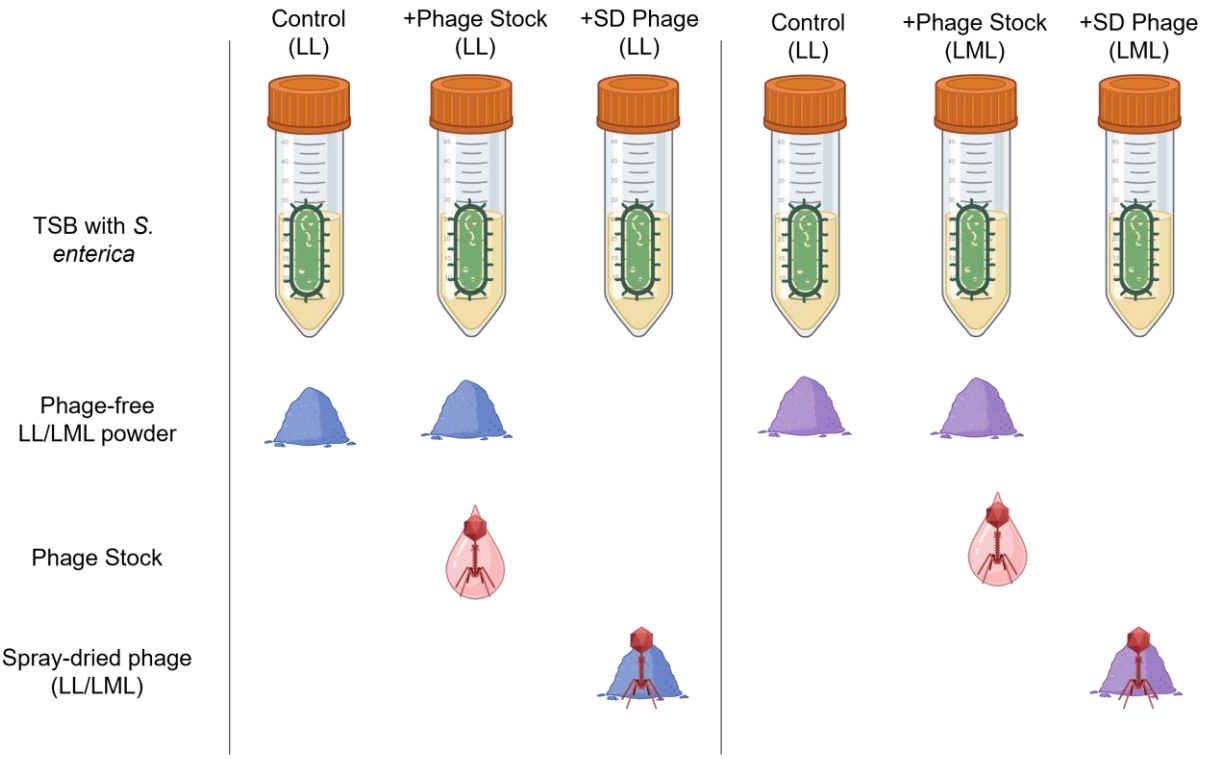


Figure 1 Samples prepared to compare performance of spray dried Felix O1 to stock Felix O1. 6 total samples were prepared. All samples contained TSB media with *S. enterica* as the host bacterium. Control and +Phage Stock vials included phage-free spray dried LL or LML powder. Felix O1 stock was then added to the +Phage Stock vials. Spray dried Felix O1 encapsulated in LL or LML powders were added to the +SD Phage vials. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose, SD – spray dried.

Kinetic curves were analyzed with the three phase linear model[43] to solve for lag phase duration, λ (h), and bacterial growth rate, μ (h^{-1}). The curves were plotted as ln(OD) with respect to time to determine the exponential growth phase by inspection. The growth phase was fit to the Eq. 6[44] in GraphPad software (Graphpad Prism; San Diego, CA, USA) using a least squares fit, where OD_λ was constrained to initial OD for all phage-free experiments and constrained to the OD post-suppressed growth for phage-containing experiments.

$$OD(t) = OD_\lambda * e^{\mu(t-\lambda)} \qquad \text{Eq. 6}$$

R^2, sum of squares, λ, μ, and the respective 95% confidence intervals were reported.

4.3.9. Comparison to commercial powdered infant formula

Two types of commercial powdered infant formula were purchased for comparison with the developed phage product. A dairy milk based iron-fortified formula (Similac; Saint-Laurent, QC, Canada) and a maltodextrin-inclusive milk based formula (Good Start Plus 1; Nestle; North York, ON, Canada) were purchased. The former will be referred to as PIF (powdered infant formula) A and the latter as PIF B. Both powdered infant formulas are for infants aged 0-6 months of age, the most vulnerable age. As per manufacturer instructions, PIF A and PIF B were reconstituted to 132 mg/ml and 145 mg/ml, respectively. Room temperature deionized water was used for rehydration.

The influence of the spray dried powder on the pH of the infant formula was tested using a pH meter (SevenExcellence S400; Mettler Toledo; Mississauga, ON, Canada). 20 mL vials of PIF A and of PIF B were prepared according to package instructions using deionized water at room temperature. 5 mg/ml of phage-free formulations of LL and the LML spray dried powder was added to PIF A and PIF B, respectively. The pH of the solutions was then measured in technical duplicate. The pH of prepared PIF A and PIF B, without spray dried powder, was also measured as a control. Experiments were completed in triplicate on distinct samples.

Effect of the spray dried powder on the dissolution of powdered infant formula was assessed using the microplate reader. An analytical balance (XS105; Mettler Toledo; Mississauga ON Canada) was used to measure out 25 mg of either phage-free LL or phage-free LML spray dried powder. The LL and LML powder were then added to 660 mg of PIF A and 725 mg of PIF B, respectively. The microplate reader was set to run a kinetic study over 10 min with continuous mixing at 548 cpm (2mm), where an optical density reading occurred every 10 s under ambient conditions. 5 ml of deionized water at 21 °C was transferred to a single well of a 6 well plate (10861-554; VWR; Mississauga, ON, Canada). The powder sample was added to the water immediately preceding the beginning of the kinetic cycle. Control dissolution readings were performed on PIF A and PIF B only powder samples. Readings were set to 500 nm. Spectral scanning of PIF A and PIF B determined that scanning at wavelengths smaller than 500 nm would lead to values above the upper limit of quantification. Larger wavelengths would not provide significant data on dissolution as the refractory index increases with decreasing wavelengths. All experiments were completed in triplicate on separate samples.

4.3.10. Scanning electron microscopy

Particle morphology was assessed visually through scanning electron microscopy (TESCAN VEGA-II LSU SEM; TESCAN; Brno, Czech Republic). Powders were mounted onto either carbon tape- or copper tape-covered aluminum stubs and coated with 15 nm of gold (Q300T D Plus; Quorum; Laughton, East Sussex, UK) prior to imaging.

4.3.11. Stability study

LL and LML powders were spray dried and packaged the same day for the stability studies. As a timepoint 0 measurement, powder was also enumerated and imaged via SEM. For the remaining timepoints, the LL and LML powders were aliquoted into separate glass vials and capped. The vials were then sealed with parafilm and placed into a moisture-resistant resealable aluminum bag (2178K19; McMaster-Carr; Elmhurst, IL, USA). Four food grade oxygen absorbers (ASIN: B083SGY3H5; Amazon; Seattle, WA, USA) and two humidity sponges (EW-07193-13; Cole-Parmer; Quebec City, QC, Canada) were also added to the package. 50 g of bulk colour indicating desiccant (2181K93; McMaster-Carr; Elmhurst, IL, USA) was also included for visual confirmation of package integrity. The package was then sealed via the zip lock closure then further reinforced with tape. This procedure was repeated to prepare packaged powder samples for each timepoint of storage: 2 weeks, 1 month, 2 months, 3 months, and 6 month. At each timepoint the powder was analyzed for phage concentration and particle morphology. At the 6 month timepoint the colour of the powder was measured.

Another protective package was prepared to assess the secondary stability of the powders. The powder samples were packaged as outlined above, with a sensor (HT.w; SensorPush; New York, NY, USA) added to measure humidity and temperature throughout storage. After 5 weeks of storage, this package was opened and all protection (i.e. desiccant, humidity sponges, de-oxidizers, parafilm) was removed. Powder samples were then taken as timepoint 0. The vials and sensor were then placed back within the empty resealable aluminum bag and the bag was stored at room temperature for an additional month. The bulk powder was sampled at 1 week, 2 weeks, 4 weeks, and 6 weeks of unprotected storage. At each timepoint the powder was measured for phage concentration and particle morphology using the procedures as outlined above.

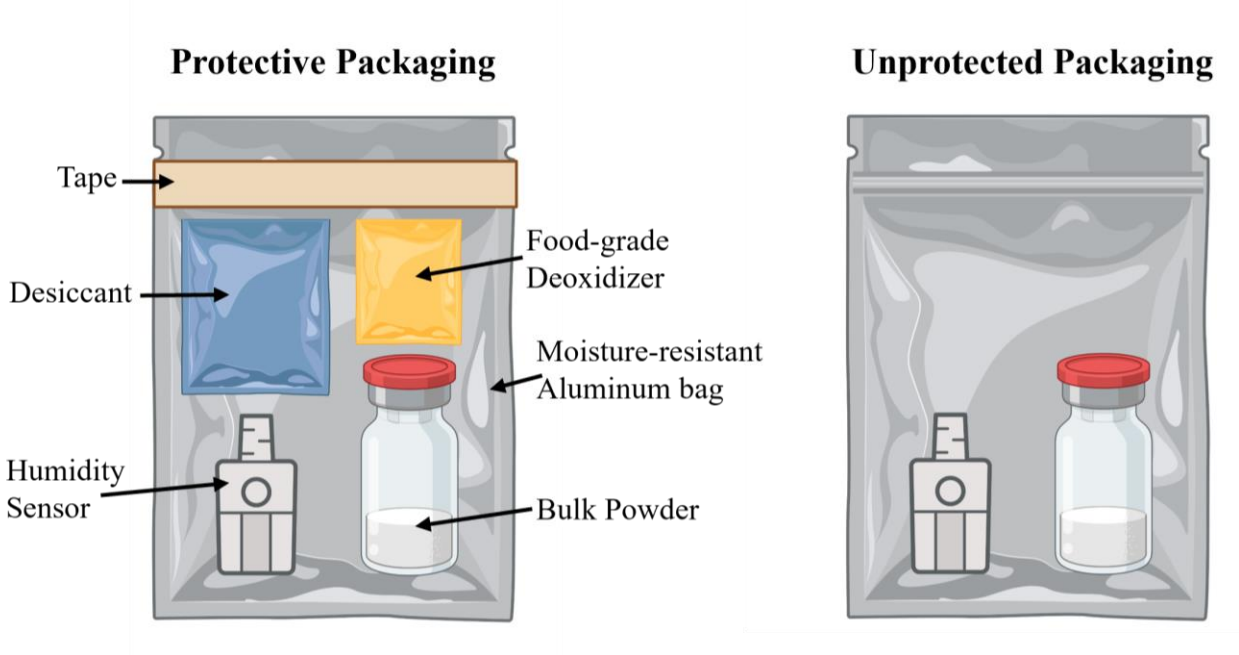


Figure 2 Schematic of packaging to simulate storage of powdered infant formula prior to opening (left) and after opening (right). Figure prepared using icons from Biorender.com.

4.3.12. Powder colour analysis

Determination of colour change over time was completed using the image processing package Fiji[45]. Powder colour was not calibrated to a white standard as the experiment was focused on the relative change in powder colour. Spray dried Felix 01, LL or LML powder, was placed into a clear 60 mm petri dish and scanned with a black background (Epson Perfection V850 Pro; Epson; Markham, ON, Canada) under default scanning parameters. The resulting images were then analyzed via Fiji's Colour Inspector 3D plugin. The *L*a*b* values were then analyzed over a region of interest of each powder image, where a and b refer to chromaticity (*a* – magenta to green axis, *b* – blue to yellow axis) and *L* refers to lightness, as per standard CIELAB colour space[46]. *L* ranges from 0 to 100, and *a* and *b* range from −128 to 127. Over one million pixels were analyzed in a given region of interest. A histogram of the corresponding *L*a*b* values was examined to determine the weighted average, based on colour frequency, for each sample. The colour change over 6 months of storage was determined by measuring the Euclidian distance (Eq. 7), where the

subscripts 0 and 6M refer to samples that were just spray dried and samples that were stored for 6 months, respectively.

$$\Delta E^*_{Lab} = \sqrt{(L^*_{6\mathrm{M}} - L^*_0)^2 + (a^*_{6\mathrm{M}} - a^*_0)^2 + (b^*_{6\mathrm{M}} - b^*_0)^2} \qquad \text{Eq. 7}$$

4.3.13. Statistical analysis

Statistical significance was generally evaluated using an unpaired t-test via Graphpad software. One way ANOVA was conducted on the phage concentration results over the course of the stability study and secondary stability study. Multiple comparisons were corrected using the Tukey test to obtain multiplicity adjusted *p* values[47]. For the evaluation of phage concentrations, all values were log-transformed prior to analysis.

4.4. Results

4.4.1. Encapsulation of anti-Salmonella bacteriophage Felix O1 within infant-formula compatible powders

Average powder yield of spray dried LL formulation was 75.1% and the LML formulation is 71.9%, across three batches. High yield is likely due to leucine accumulation at powder interface, reducing loss due to sticking to spray dryer inner surfaces. Processing loss of Felix O1 when spray dried with LL and LML formulations is shown in Figure 3a and Figure 3b, respectively. LL Batch 1 and all of LML batches showed statistically insignificant processing loss. For LL Batch 2 and Batch 3, the processing loss was 0.19 and 0.33 log, respectively. All batches met the criteria of <1.0 log loss over spray drying, indicating that spray drying of LL and LML powder has a high batch producibility in the encapsulation of Felix O1.

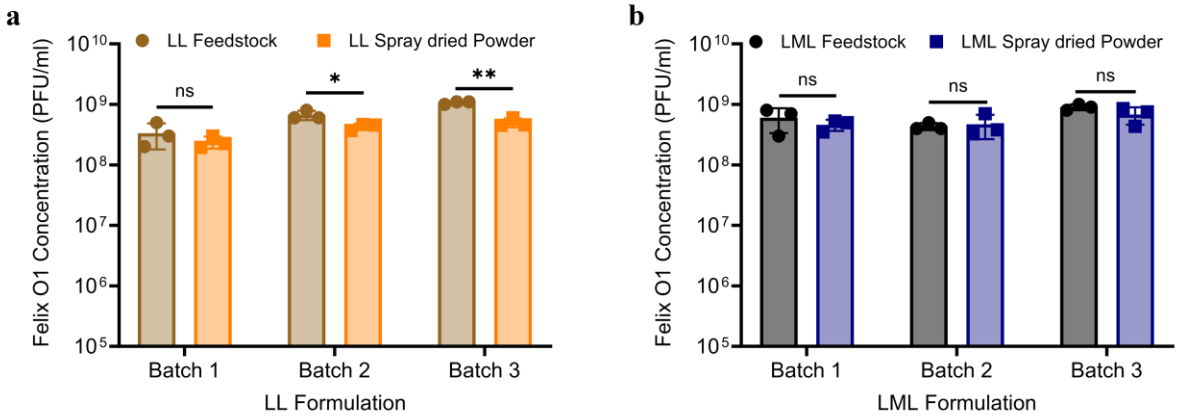


Figure 3 Felix O1 undergoes <0.35 log processing loss over three different batches when spray dried with either the LL or LML formulation. a-b. Difference between the concentration of Felix O1 in the feedstock prior to spray drying compared to the spray dried powder for both the (a) LL and (b) LML formulations. LL Feedstock – brown circles, LL Spray dried Powder – orange square, LML Feedstock – black circles, LML Spray dried Powder – blue squares. Feedstock results shown are triplicate measurements of the feedstock. Spray dried powder results are measurements of three distinct, reconsituted powder samples from the same batch. Error bars represent standard deviation. Statistical analysis was performed, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, and $0.001 \leq p < 0.01$ - **. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose.

Powder appearance and particle morphology of the LL and LML formulations is shown in Figure 4. Both the LL and LML formulations appear as uniform, fine, white powders (Figure 4a, and

Figure 4c, respectively). The white colour of both powders meets the requirements of Grade 1 spray dried dairy products for Canada[48]. Both the LL and LML powders are composed of polydisperse particles (Figure 4b, and Figure 4d, respectively). In both cases, particles are generally spherical, likely due to the high relative concentration of lactose[49]. Particles exhibit a rough exterior, due to preferential leucine accumulation at the droplet interface. The presence of maltodextrin in the LML formulation did not appear to influence the powder appearance at the macroscopic or microscopy level. Single excipient lactose, maltodextrin, and leucine particle morphology is shown in Figure S 1 as part of Supplementary Note 2. Particle morphology is similar to that of other spray dried lactose-leucine encapsulated phage[25].

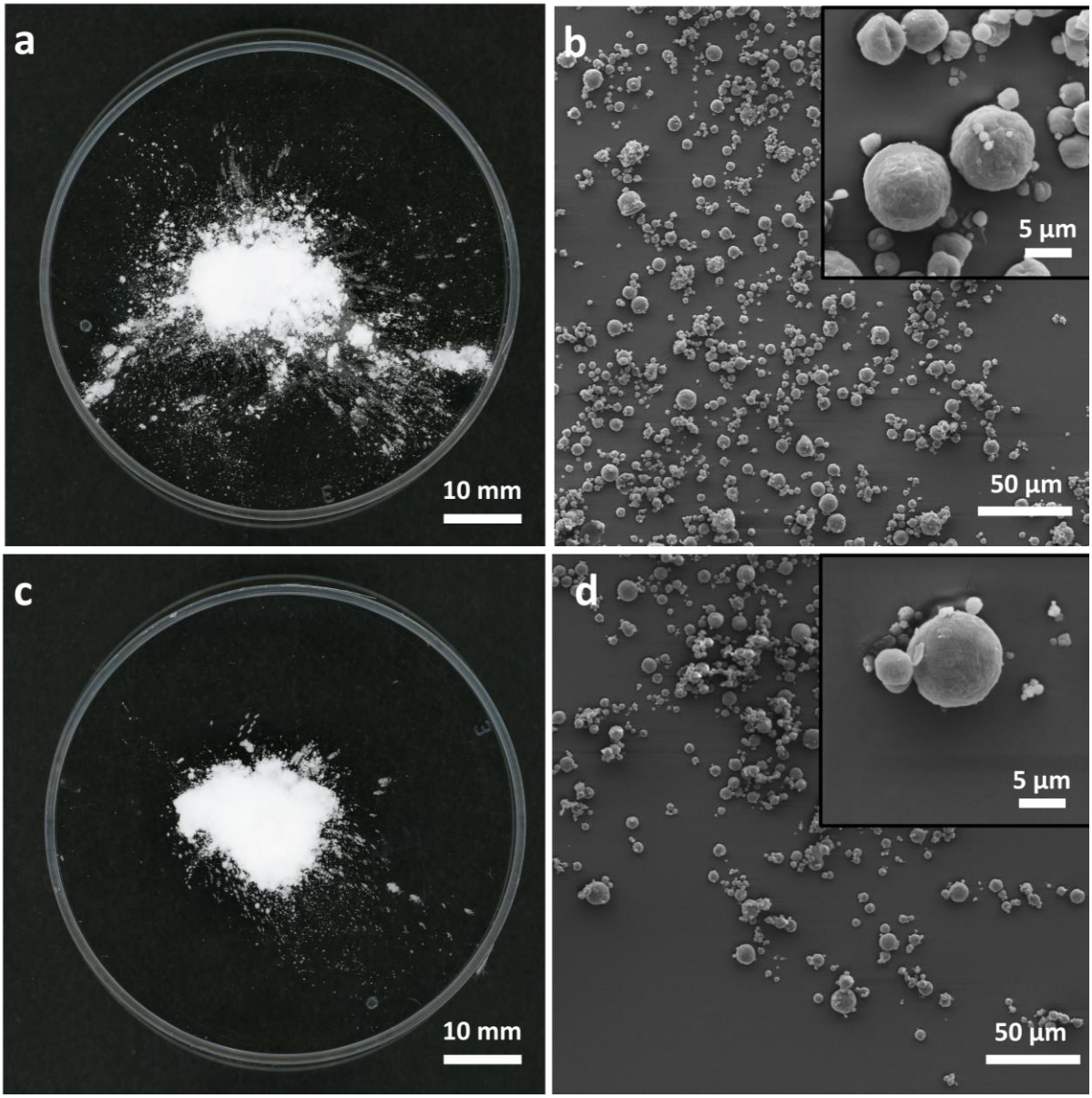


Figure 4 Powder appearance and particle morphology of spray dried Felix O1 encapsulated within LL powders (top) and LML powders (bottom). a. Macroscopic image of spray dried LL powder. **b.** Scanning electron microscopy of LL formulation. **c.** Macroscopic image of spray dried LML powder. **d.** Scanning electron microscopy of LML formulation. Spray drying of both the LL and LML powders produce fine, white powders. These powders are comprised of polydisperse spherical

microparticle with a rough surface morphology due to leucine accumulation. Visually, there is no significant difference between the LL and LML formulation. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose

Addition of approximately 5 mg/ml of spray dried LL or LML powder to rehydrated PIF was targeted. A 5 mg/ml addition would account for approximately 3.6% and 3.4% of the powder mass for LL added to PIF A and LML added to PIF A, respectively. A relatively low powder mass and relatively low concentration was targeted to minimize influence of the LL or LML powders on PIF composition and properties. LL and LML platform are composed of sugars/starch and an amino acid only. The influence of LL and LML on the carbohydrate and protein content was assessed and compared with WHO standard[50] on PIF composition (Table 1). PIF A with the addition of LL, and PIF B with the addition of LML, is well within the WHO standards. No other nutrients were considered (e.g. fats).

Table 1 Added LL or LML powder to PIF A or B, respectively, maintains WHO standard composition of powdered infant formula. Total carbohydrates and proteins of powdered infant formula was compared to the WHO standard. Carbohydrates and proteins within commercial infant formula (PIF A and PIF B) are well within WHO standard even with addition of LL and LML powders. WHO standards are reported in terms of /100 kcal. A conversion factor of 67.5 kcal/100 ml was used based on average reported energy for PIF A[51] and PIF B[52]. Abbreviations: WHO – World Health Organization, PIF – powdered infant formula, LL – leucine lactose, LML – leucine maltodextrin lactose. *Maximum total protein content allowed by the Canadian government is 2.7 g/100 ml [4.0 g/100 kcal][53]. Other relevant standards are in line with the WHO. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose, PIF – powdered infant formula.

Powder	Total Carbohydrates (g/100 ml)		Total Proteins (g/100 ml)	
WHO Standard[50]	6.1 (MIN)	9.5 (MAX)	1.2 (MIN)	2.0 (MAX)*
PIF A[51]	7.6		1.3	
PIF B[52]	7.5		1.5	
LL (5 mg/ml)	0.4		0.1	
LML (5 mg/ml)	0.42		0.08	
PIF A + LL	8		1.4	
PIF B + LML	7.9		1.6	

Data on the essential amino acid composition of PIF A and PIF B is unavailable. WHO standards provide the average reported content of essential amino acids in breast milk. Average leucine level in breast milk is reported to be 169 mg/100 kcal (assuming minimum protein content of 1.8g/100 kcal)[50]. Assuming a conversion factor of 67.5 kcal/100 ml[51,52], average leucine level in breast milk can be approximated to 114 mg/100 ml. Leucine concentration of LL and LML formulations when rehydrated to 5 mg/ml is 100 mg/100 ml and 83 mg/100 ml, respectively. As leucine content with PIF A and PIF B was unknown, a leucine concentration under the average leucine level of breast milk was targeted to account for the presence of leucine in PIF A and PIF B. The WHO does not indicate that there are maximum levels for leucine.

The pH of rehydrated PIF A was measured to be 6.82 ± 0.03, and the pH of PIF A + 5 mg/ml of LL (phage-free) powder was 6.79 ± 0.02. The pH of PIF B was 6.93 ± 0.02 and was measured to be 6.94 ± 0.02 when 5 mg/ml of LML (phage-free) was added. In both cases the difference in pH of the powdered infant formula was statistically insignificant as compared to when phage powder

was added. This result indicates that the addition of LL or LML at 5 mg/ml does not alter the pH of powdered infant formula. PIF A+LL and PIF B+LML dissolution pattern is similar to their controls (Figure S 3). Lack of significant peaks or troughs suggests that the PIF and the phage-free powder had fully dissolved by the end of the investigated timeframe, with no large clumps to cause substantial refraction.

4.4.2. Felix O1 infectivity maintained post-spray drying

Felix O1 is unable to control *S. enterica* growth in rehydrated powdered infant formula over 24 h at 4, 21 or 37 °C (Figure S 4). Comparison of spray dried or stock Felix O1 infectivity was instead conducted in TSB media at 37 °C for optimal bacterial growth. *S. enterica* kinetic curves of samples grown in the presence of spray dried lactose leucine (LL) at 5 mg/ml and spray dried lactose maltodextrin leucine (LML) at 5 mg/ml are shown in Figure 5. Addition of Felix O1 phage, either from the stock or as a spray dried sample, temporarily depressed *S. enterica* growth (Figure 5a, b). Both the stock Felix O1 and the spray dried Felix O1 phage significantly extended lag phase duration by 7.3-8.3 h in LL and LML formulations (Figure 5c, d). Similarly, both the Felix O1 and spray dried Felix O1 phage significantly reduced the growth rate (Figure 5e,f). Comparison of the Felix O1 phage stock to the spray dried phage within their respective formulations found no significant difference in performance in terms of lag phase duration and growth rate.

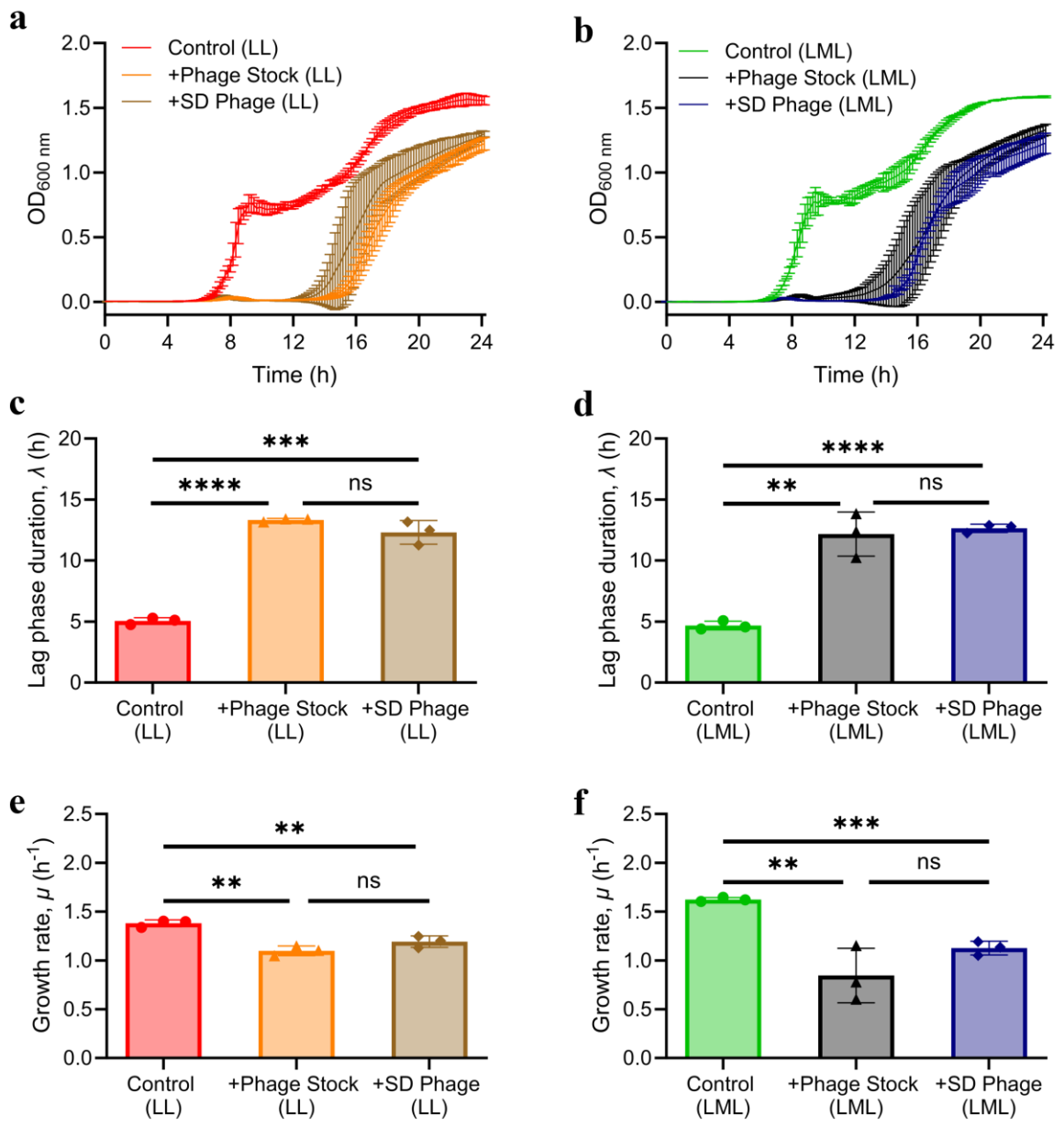


Figure 5 Suppression of *S. enterica* at 37 °C in TSB+LL or TSB+LML is the same when treated with Felix O1 phage stock or spray dried Felix O1. a. Kinetic curves of untreated *S. enterica* [Control (LL)], *S. enterica* treated with Felix O1 stock [+Phage Stock (LL)], and *S. enterica* treated with spray dried Felix O1 [+SD Phage (LL)], grown in TSB+LL. **b.** Kinetic curves of untreated *S. enterica* [Control (LML)], *S. enterica* treated with Felix O1 stock [+Phage Stock (LML)], and *S. enterica* treated with spray dried Felix O1 [+SD Phage (LML)], grown in TSB+LML. **c-d.** Calculated lag phase duration of untreated *S. enterica*, *S. enterica* treated with Felix O1 stock, and *S. enterica* treated with spray dried Felix O1 when grown in TSB+LL (c) and TSB+LML (d). **e-f.** Calculated growth rate of untreated *S. enterica*, *S. enterica* treated with Felix O1 stock, and *S. enterica* treated with spray dried Felix O1 when grown in TSB+LL (e) and TSB+LML (f). Addition of phage significantly increased lag phase duration and significantly decreased the growth rate as compared to the untreated control. Difference in lag phase and growth rate between the Felix O1 stock and spray dried Felix O1 was statistically insignificant. Error bars represent the standard deviation of triplicate experiments. Statistical analysis was performed, where $p \geq 0.05$ – not significant, $0.001 \leq p < 0.01$ - **, $0.0001 \leq p < 0.001$ - ***, and $p \leq 0.0001$ - ****. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose, SD – spray dried.

4.4.3. Stability of Felix O1 powders over storage

LL formulation undergoes significant loss in Felix O1 concentration over the first month of storage (Table S 8), from $2.4x10^8$ PFU/ml to $6.9x10^7$ PFU/ml but then stabilizes for the remaining 5 months of the stability study (Figure 6). The LL formulation stabilizes to approximately same concentration as LML formulation over 6 months of storage ($3.8x10^7$ and $4.0x10^7$ PFU/ml, respectively). However, the LML formulation undergoes a longer period of instability over the initial 2 months of storage (Table S 9), with phage concentration decreasing from $4.6x10^8$ PFU/ml to $8.0x10^7$ PFU/ml (Figure 6).

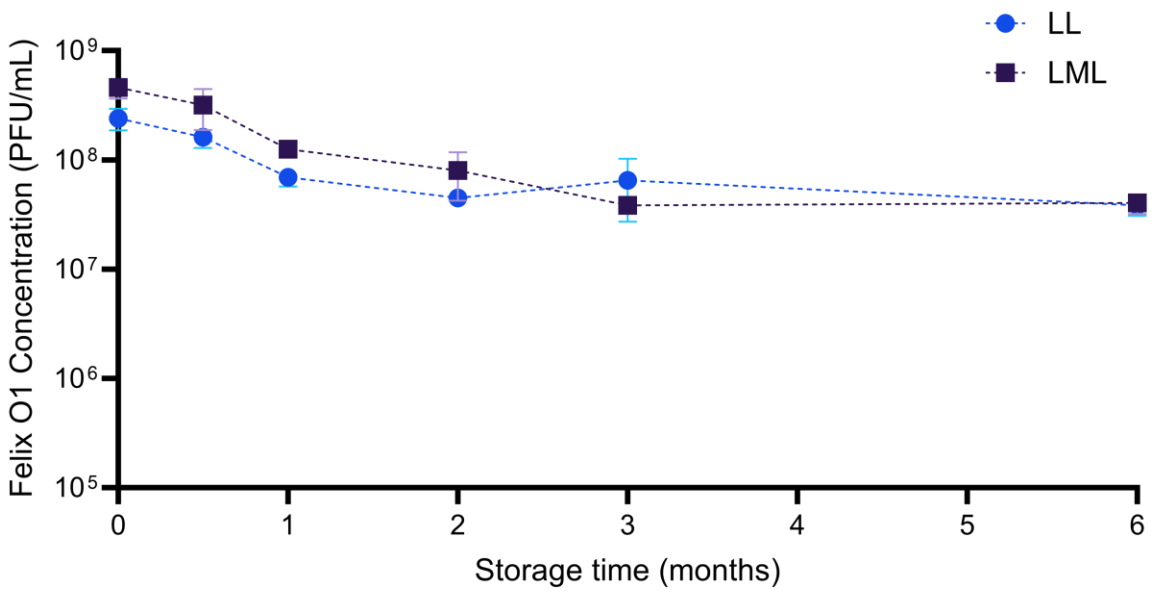


Figure 6 Stability of the spray dried Felix O1 powder over six months of storage under ambient conditions. Bacteriophage Felix O1 concentration of the LL (blue circle) and LML (purple square) formulations over six months when stored within protective packaging. Both LL and LML formulations are highly stable over this timeframe. Felix O1 concentration is plotted as the average of three reconstituted powder aliquots, where duplicate technical replicates for each aliquot was averaged. Error bars represent the standard deviation of the triplicate aliquots. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose.

Particle morphology of the LL and LML formulations over 6 months of storage within protective packaging is given in Figure 7 and Figure 8, respectively. Particle morphology for both formulations is stable across all timepoints, with maintenance of the surface morphology and no indication of particle fusing. Results indicate that the protective packaging, designed to simulate real world packaging, is very effective at maintaining physical stability of phage powder.

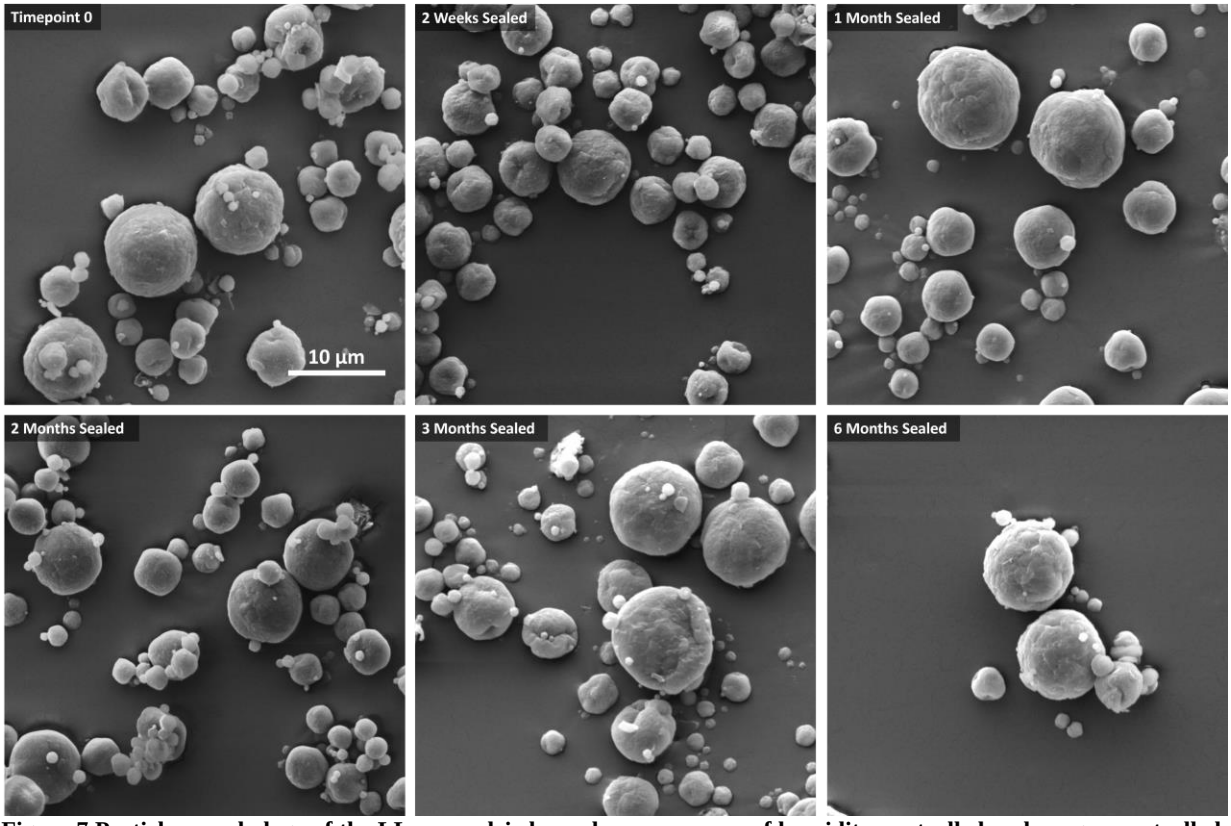


Figure 7 Particle morphology of the LL spray dried powder over course of humidity-controlled and oxygen-controlled storage. Particle morphology of the LL powder is maintained over 6 months of protected storage, with no significant sign of particle fusing. Displayed scale bar applies to all images.

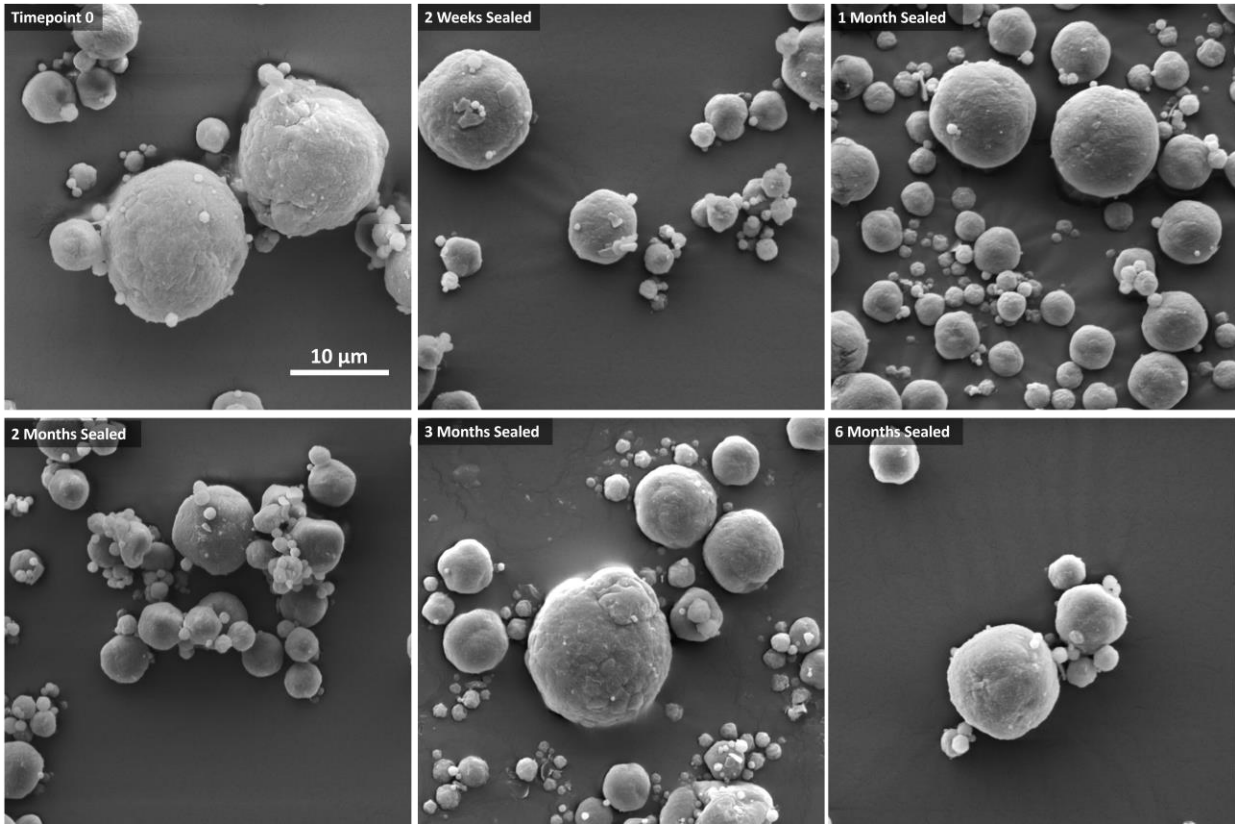


Figure 8 Particle morphology of the LML spray dried powder over course of humidity-controlled and oxygen-controlled storage. Particle morphology of the LML powder is maintained over 6 months of protected storage, with no significant sign of particle fusing. Displayed scale bar applies to all images.

Visually, the LL and LML powders appear to maintain their macroscopic appearance after 6 months of storage (Figure 9). There is no visual difference between the LL and LML powders, where both appear as uniform, white, fine powders. Results of CIELAB analysis on just spray-dried LL and LML (Figure 4) as compared to when stored for 6 months (Figure 9) is given in Table 2. *L*a*b** values were calculated based on a weighted average of frequency of pixel colours in a given image (Table S 10).

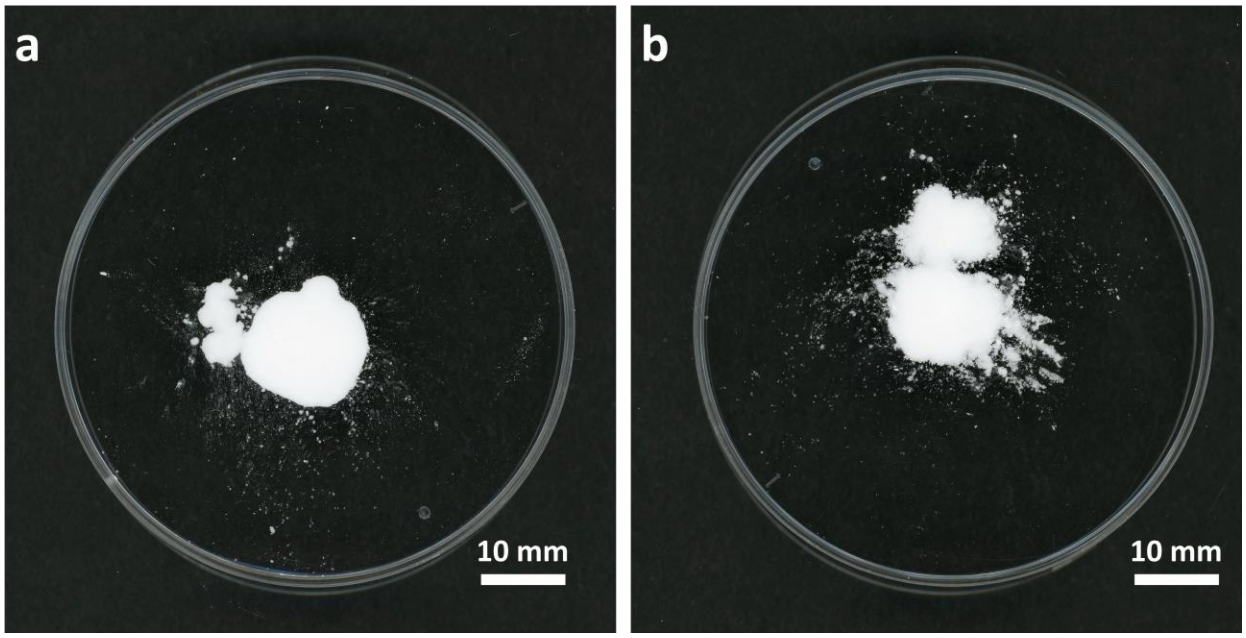


Figure 9 Spray dried Felix O1 encapsulated within (a) LL and (b) LML visually maintains white colour after 6 months of protected storage. Scale bar is shown on respective images.

The LL and LML powders show a maintenance in *a** and slight decrease in *b** over time, confirming that the powders are not yellowing or browning. The overall change in powder colour for LL and LML powders after 6 months of storage is 0.6 and 0.7, respectively. Higher ΔE^*_{ab} would indicate a larger colour difference between the initial sample and the stored sample. This colour change is far below the human perception of colour change (ΔE^*_{ab} = 2.3[54]).

Table 2 Powder colour change, ΔE^*_{Lab}, as determined by the CIELAB colour values, of the LL and LML powders after 6 months of storage is imperceptible to the human eye (ΔE^*_{ab}<2.3[54]). *L**, *a**, *b** values are reported as a weighted average across the region of interest within the powder image.

Sample	*L**	*a**	*b**	ΔE^*_{Lab}
LL, timepoint 0	96.1	0.0	0.1	0.6
LL, 6 month	96.7	0.0	0.0	
LML, timepoint 0	96.9	0.0	0.6	0.7
LML, 6 month	96.5	0.0	0.1	

4.4.4. Stability of Felix O1 powders post-product opening

Felix O1 concentration over 6 weeks of unprotected bulk storage is shown in Figure 10. Average temperature over the course of the study was 21 °C and average relative humidity prior to opening was 22% (Figure S 5). Timepoint 0 occurs 5 weeks post-production, where the powder was stored in protective packaging prior to opening. At timepoint 0 the Felix O1 concentration for the LL and LML formulation was 9.2×10^7 PFU/ml and 9.7×10^7 PFU/ml, respectively. Both the LL formulation and LML formulation are highly stable, with no significant changes in concentration for either formulation (Table S 11, Table S 12). The concentration of Felix O1 after 6 weeks of storage was 7.8×10^7 PFU/ml for both formulations. Powder appears to undergo significant loss of

Felix O1 in the 5 weeks of protected storage prior to the secondary stability study. These results are consistent with the results of the stability study where both the LL and LML powders undergo significant loss in the month post-spray drying, followed by stabilization.

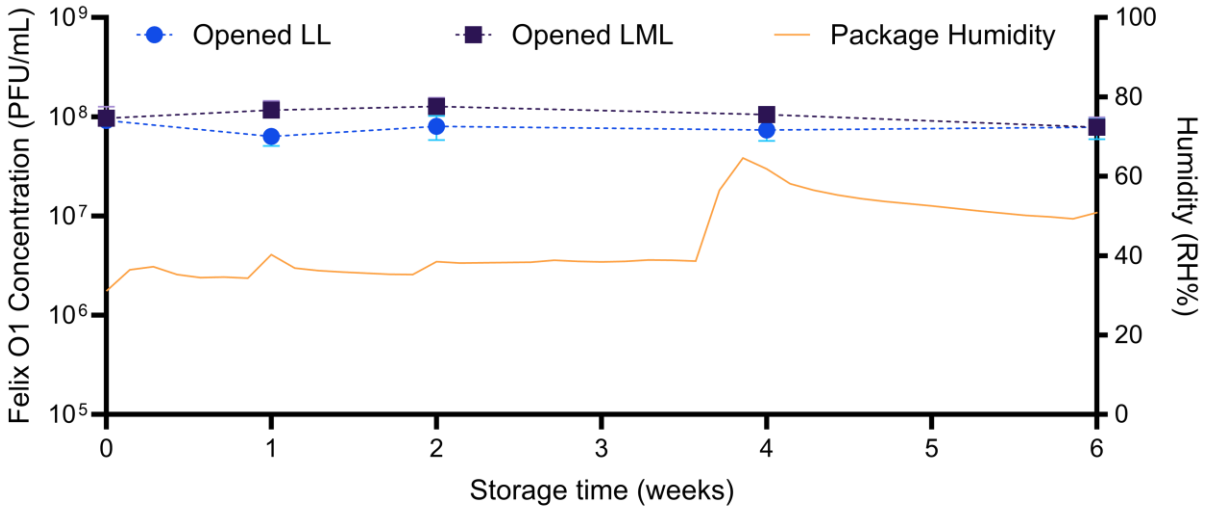


Figure 10 Stability of the spray dried Felix O1 powder after package opening when stored under ambient conditions. Bacteriophage Felix O1 concentration of the LL (blue circle) and LML (purple square) formulations over six weeks when stored within resealable containers without protective packaging. Humidity of the package (orange line) was also recorded over the course of the secondary stability study. Both powders underwent <0.1 log loss over this timeframe. Felix O1 concentration is plotted as the average of three reconstituted powder aliquots, where duplicate technical replicates for each aliquot was averaged. Error bars represent the standard deviation of the triplicate aliquots. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose.

Particle morphology of the LL and LML formulations over 6 weeks of ambient storage within aluminum packaging is given in Figure 11 and Figure 12, respectively. Particle morphology for both formulations is stable across all timepoints, with no significant signs of fusing and maintenance of the surface morphology. Results indicate that powder is highly physically stable over the course of powdered infant formula's secondary shelf life. Furthermore, humidity varied from 31-65% RH, with an average of 43% RH, and powder maintained both physical and biological stability throughout exposure.

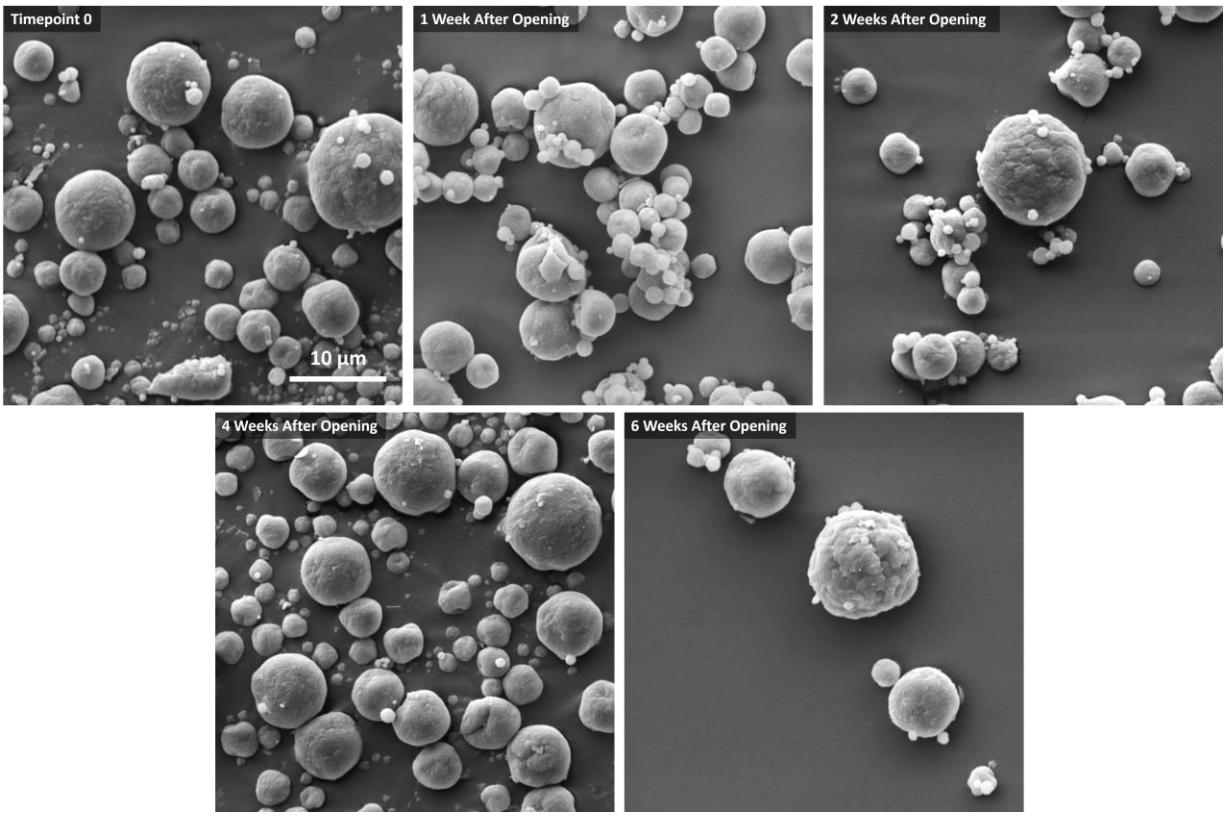


Figure 11 Particle morphology of the LL spray dried powder over course of unprotected storage. Particle morphology of the LL powder is maintained over 6 weeks of storage under ambient conditions, with no significant sign of particle fusing.

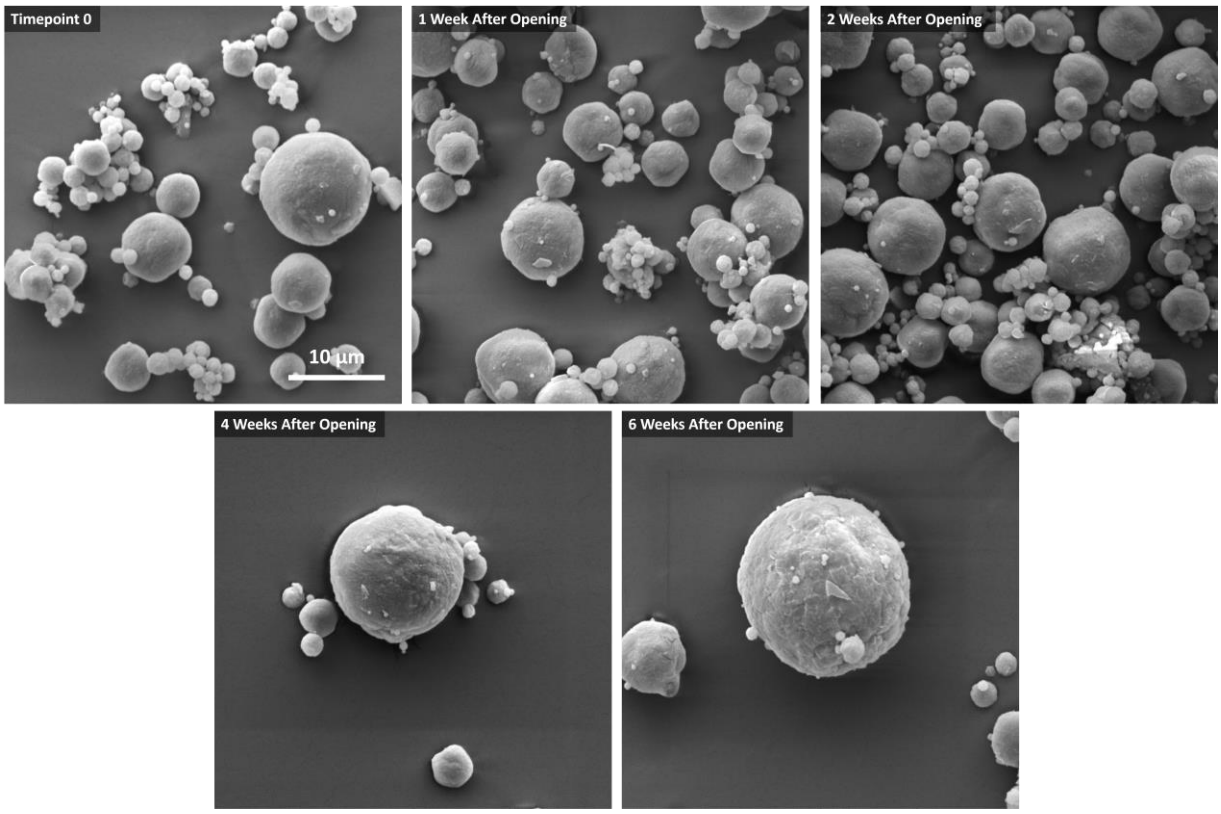


Figure 12 Particle morphology of the LML spray dried powder over course of unprotected storage. Particle morphology of the LML powder is maintained over 6 weeks of storage under ambient conditions, with no significant sign of particle fusing.

4.5. Discussion

Health Canada allows for the inclusion of live microorganisms in infant formula, provided that the microorganism has been deemed safe for infant ingestion[55].This research sought to evaluate the use of the leucine-lactose excipient system to stabilize bacteriophages for inclusion in powdered infant formula, as well as a powder composed of leucine-lactose-maltodextrin. Lactose is the primary component (35-50%) of most powdered infant formulas[21]. Lactose is also a known stabilizer in its glass amorphous form[56], with a relatively high glass transition temperature (~100 °C)[57]. Leucine is an essential amino acid that is present in infant formula[22,50]. Maltodextrin, a glucose polymer, may also be present in powdered infant formula, comprising up to 30% of the carbohydrates[50]. In the investigated systems, LL and LML, were designed to have lactose as the highest relative concentration within the system, 80% and 67%, respectively. A significantly higher relative concentration of lactose (≥80%) compared to leucine has been led to long term stability of the phage within powder[28]. Additionally, with a high concentration of lactose more phage powder can be added to powdered infant formula without significantly changing the composition. This will increase the relative amount of phage that can be present in the system.

The L form of the amino acid leucine was used in this study, as per WHO standards on the addition of free amino acids to infant formula[50]. Leucine concentration was designed to form a crystalline outer shell. The addition of this component was designed to reduce moisture degradation pathways by limiting the moisture absorption during storage due to the hydrophobic crystalline shell.

Maillard reactions are one such pathway that are exacerbated with the presence of moisture. Due to lactose being a reducing sugar, this degradation pathway is a concern. To mitigate the weaknesses of lactose, maltodextrin was also included as a secondary stabilizing excipient. Masum et. al's [58] results suggested that the addition of maltodextrin (dextrose equivalent [DE] 20) to a lactose powder reduced the extent of Maillard browning. Specifically, maltodextrin DE 13-17 is chosen for this study. DE 13-17 will provide better protection against crystallization than lower DE[59] while balancing the higher T_g found in lower DE. Additionally, the addition of maltodextrin was added to improve the long term stability of the powder as maltodextrin[60] has a higher glass transition temperature than lactose[61].

Spray drying the LL and LML formulations produced powders with relatively high yield for laboratory scale spray dryers. The high powder yield is likely due to the inclusion of leucine wherein the crystalline outer layer would reduce the powder stickiness and thereby loss within the system. This result is consistent with the increased protein to lactose ratios increasing powder yield of spray dried formula[21]. Both powders produced spherical particles with rugose crystalline shells. Particle morphology is in accordance with the theory. Material properties (Table S 4) of each component was used to calculate the time to saturation and time to true density (Table S 5). Calculations estimate that leucine will reach saturation at the droplet interface 22% into the droplet lifetime, whereas lactose and maltodextrin would not form a shell until >87% of the droplet lifetime. The early time to saturation would also allow for more time for crystal growth. Furthermore, the calculations do not consider the hydrophobicity of leucine. Leucine's hydrophobic tail would promote accumulation at the air-liquid interface[26], likely leading to even earlier shell formation and crystal growth than predicted by *Pe*. The prevalence of lactose is likely responsible for the spherical shape of the particles. Single excipient lactose particles are spherical with smooth surfaces, whereas spray dried maltodextrin produces highly corrugated particles[62].

The designed fast encapsulation time and particle morphology led to high encapsulation efficiency. Spray drying of the model phage Felix O1 led to low processing loss of ≤0.35 log loss in the LL and LML formulations. This result was determined over three separate batches for each formulation, indicating high repeatability. Both formulations were able to achieve a very high loading of phage within the powder, with an average of 5.4×10^9 PFU and 7.4×10^9 PFU of Felix O1 per gram of powder across three batches for the LL and LML powders, respectively. The LML formulation showed no significant processing loss, suggesting an advantage in terms of phage yield over the LL formulation. The LL and LML excipient systems also preserved phage fecundity. Comparison of lytic curves against *S. enterica* found that the spray dried phage performed as well as the stock phage when administered at approximately the same concentration in the same environment. The excellent ability of lactose-leucine excipient formulations to spray dry phages with low processing loss has been previously shown by Chang et al.[28]. The authors spray dried three different anti-*pseudomonas* phages in varying relative concentrations of leucine-lactose, where all phages underwent ≤0.6 log processing loss. The addition of leucine may have also improved encapsulation efficiency. Phages, due to their larger size, are expected to accumulate at the surface and may be lost to drying. The preferential accumulation of leucine at the interface may have kept phage away from the surface and subsequent degradation due to interactions at the air-liquid interface. Felix O1 appears to be a robust bacteriophage when spray dried with an

appropriate system. Spray drying of Felix O1 with formulations composed of Eudragit and trehalose also found no statistically significant loss in titer, whereas without trehalose it underwent several log reduction in titer[63] .

Spray dried Felix O1 in LL or LML powders were stored under conditions mimicking powdered infant formula packaging. Packaging simulated the aluminum packaging of PIF. The addition of oxygen adsorbers and desiccant was to mimic PIF headspace filled or flushed with nitrogen[64]. Initial Felix O1 concentration was $3.3x10^9$ PFU/g powder ($2.4x10^8$ PFU/ml when rehydrated to 72 mg/ml) for the LL powder and $6.4x10^9$ PFU/g for the LML powder ($4.6x10^8$ PFU/ml). LL formulation underwent 0.80 log loss of Felix O1 over 6 months, LML underwent 1.1 log loss. The addition of maltodextrin and the lower relative leucine concentration appeared to provide the LML formulation reduced processing loss over the LL formulation, it appears that the LL formulation appeared to undergo less absolute loss over the course of the stability study.

The secondary stability study, which occurred 5 weeks post-spray drying, showed that Felix O1 encapsulated within LL and LML powders were highly stable when stored at ambient conditions for 6 weeks, experiencing 0.07 and 0.09 log loss, respectively. The powder appeared to have stabilized after 5 weeks of storage under protective conditions. Once the package was opened, the presence of leucine likely protected against moisture sorption even when subjected to environmental humidity as high as 65% RH. Particle morphology did not change during this timeframe, further indicating that the leucine shell prevented plasticization and fusing of the particles. This result is consistent when Zheng et al's[65] work, where they showed that even small amounts of leucine protected spray dried trehalose from humidity induced reduction in physical stability.

Comparison of stability results to literature is difficult as no other study has investigated the same phage and excipient system combination, initial phage concentration, and storage conditions. Stability is dependent on all these factors[66,67], and dried phage can completely degrade in a few weeks of ambient storage[67,68]. Chang et. al's[69] work on spray drying phage PEV20 in a 80%lactose and 20%leucine powder (~$1x10^7$ PFU/mg) exhibited a similar degradation pattern as the LL and LML formulation. This degradation pattern can be characterized as an initial period of titer loss, followed by no significant loss for the remainder of the stability study. In their work, the spray dried phage underwent ~1.1 log loss over the first 7 d of storage at 20 °C and 15% RH, followed by no significant loss for the remainder of the 250 d study. Similarly, the results in this study found that the majority of loss occurred over the first two months of storage, with the LL and LML formulations undergoing 0.70 and 0.64 log loss, respectively, but no significant loss thereafter. A different degradation pattern of dried phage was found in Yazdi et al's[63] powders, where Felix O1 spray dried with Eudragit-trehalose and stored for 12 months at 20 °C in sealed vials, with initial concentration of >10^9 PFU/g[63]. Their two lead candidates underwent approximately 0.7 log and 1.2 log loss after 6 months of storage. This loss is comparable to the absolute degree of titer loss at the 6 month timepoint of this study, however, the Yazdi et al.'s[63] powders underwent no significant loss for the first few months of storage. Degradation continued with time, with 2-3 log loss by the 12 month timepoint. It is possible to achieve highly stable powders with either lactose-leucine formulations or with spray dried Felix O1. Chang et al.[28] spray dried three anti-

pseudomonas phages in different lactose-leucine formulations and stored the powders at 20 °C. Powders were packaged under low RH and heat sealed in aluminum bags. The best performing powders underwent approximately 0.5 log (PFU/mg) loss over 12 months of storage where starting titer was between 10^5-10^7 PFU/mg. Ergin[70] spray dried Felix O1 using a combination of whey protein isolate-inulin-gum arabic-tween 80 to an initial concentration of 10^7 PFU/g. The samples were stored in a falcon tube for 180 d under ambient conditions with no other protective packaging, and only reduced by 0.49 log (PFU/g). Further work is needed to determine the mechanism of initial degradation in this study. Formulation optimization may reduce degradation; however, components of the excipient system for this work are significantly restricted by infant formula requirements.

The recent study by Imm and Chang[20] is the only other work to propose the stabilization of phage in a dry format for inclusion with PIF as an intervention against bacterial contamination. They isolated and then stabilized the anti-*C. sakazakii* phage SG01 within a collagen peptide-trehalose system via freeze-drying at approximately 10^8 PFU/ml. The powder has shown excellent ability to reduce *C. sakazakii* when added to commercial PIF, with 1 log loss at after only 30 min at 25 °C. The results are very promising, especially the isolated phage SG01. Comparatively, Felix O1 acts as a poor *S. enterica* treatment in infant formula. Felix O1 specifically may be ill-suited for use in milk. Certain phages may have high binding affinity for the fat or proteins in milk[71–73], limiting their ability to infect bacteria in this matrix.

The freeze-dried collagen peptide system proposed by Imm and Chang[20] has not been optimized nor assessed for inclusion with powdered infant formula in terms of composition, production or powder properties. Collagen peptides may be included as a hydrolyzed protein, and trehalose, as a glucose polymer, is by definition allowed in infant formula[50]. Furthermore, trehalose is a non-reducing sugar, unlike lactose. Trehalose is also an excellent sweetener alternative for those with lactose intolerance. However, trehalose is explicitly excluded from approval for use in infant formula in the USA[74] and the EU[75]. Trehalose was also not considered for safety for children under 2 by Australia and New Zealand[76]. Furthermore, lactose is the preferred carbohydrate for infant formula based on WHO standards, where it should account for majority of the carbohydrate[50].

The collagen peptide-trehalose system is produced via freeze drying[20]. However, powdered infant formula is typically produced by spray drying. Freeze drying is a batch process, so it is less high throughput than spray drying. Freeze drying also results in the formation of a dry "cake", requiring an additional processing step to prepare a powder formant. The collagen peptide-trehalose system was able to stabilize the SG01 phage for room temperature storage, with approximately 0.4 log loss after 4 weeks of storage[20]. For comparison, the LL and LML formulations underwent insignificant loss (<0.1 log loss) after 6 weeks of storage under the secondary stability study. A longer stability study is necessary to assess compatibility with long term powdered infant formula storage.

The spray dried LL and LML powders also meet several requirements for classification Grade 1 milk powder by Health Canada[48]. Both the LL and LML powders are free flowing and uniformly white in appearance, with no visible black or brown particles, meeting the visual requirements for a Grade 1 milk powder. Both powders also maintained their colour over long term storage. Milk

powders, especially wherein lactose is amorphous, will undergo Maillard degradation reaction when subjected to representative spray dryer outlet temperatures[77]. Maillard degradation was a concern given that Felix O1 expresses lysine[78], and reduction caused by the presence of lactose has been thought to damage phage integrity in spray dried powder[67].

The LL and LML formulations were designed to be as commercial friendly as possible. Production of LL and LML powders are compatible with the manufacture of powdered infant formula. LL and LML powders were composed of inexpensive ingredients and produced via the industry process used for infant formula. The spray dried LL and LML powders are fine, non-stick dairy powders with no visible lumps. These powders may be further optimized for uniform blending with powdered infant formula prior to packaging. Both the LL and LML formulations appear to be effective vehicles for bacteriophage addition to powdered infant formula. LL and LML powders should be spray dried with other experimental bacteriophages to evaluate their ability to reduce contamination in infant formula.

4.6. Conclusion

Powdered infant formula is consumed globally, however, bacterial post-processing contamination has led to infant death. The WHO recommended method of eliminating post-processing contamination, reconstituting powder with sterile water heated to >70 °C, is not widely practiced either due to messaging inconsistency or lack of access to heated water. This method does not consider contact of the reconstituted formula with contaminated equipment (e.g., bottles), nor the potential killing of included beneficial bacteria. A thermostable phage powder within formula provides a built-in method to eliminate contamination that is effective even after long term storage.

Two stabilizing excipient systems were developed for this work: one that was comprised of only lactose and leucine (LL), the other comprised of lactose, leucine, and maltodextrin (LML). The spray dried LL and LML powders were specifically designed for inclusion of bacteriophages in powdered infant formula. Both powders were able to encapsulate the model phage Felix O1 at a high concentration of phage to powder (>10^9 PFU/g). Both the LL and LML powders used ingredients that were explicitly in accordance with the WHO standards for powdered infant formula composition. Furthermore, when added to commercial powdered infant formula at a concentration of 5 mg/ml, the LL and LML powders were found to not significantly affect the protein and carbohydrate requirements of two formulas. Analysis of phage-free LL and LML powders when added to the two commercial infant formulas found that there was no change in pH or dissolution pattern. The recommended secondary shelf life of powdered infant formula is 1 month. The high stability of Felix O1 in the LL and LML samples during the six-week secondary stability study suggests that the powder is suitable for the increased humidity that it would experience after package opening until end of recommended use. High stability was likely conferred by the designed protective leucine shell and sugar/starch core. While improved storage stability may be achieved with further formulation development, the allowed excipients are heavily constrained due to concern for infant health.

Further work is needed to confirm long-term stability and development of lactose-free formulations. Powdered infant formula has a shelf life of more than one year when unopened. Further timepoints are needed to evaluate the spray dried excipient system when stored for longer

than six months. The vast majority of infant formulas are dairy-based, however, neither formulation is compatible with a lactose-free powdered infant formula. Other sugars, including sucrose and fructose, are prohibited from use in infant formula. Additional work would need to be completed to develop a suitable stabilizing system for lactose-free formulas.

The investigated excipient systems are compatible with dairy milk based powdered infant formulas and provide a built-in failsafe method for reducing bacterial contamination. Addition and manufacture of this powder is compatible with the existing processes for manufacturing powdered infant formula. The addition of spray dried phage powder to infant formula has the potential to significantly reduce the risks bacterial outbreaks. This research was focused on providing a vehicle for bacteriophage interventions, this technique may be used to apply promising phage treatments to powdered infant formula.

4.7. Acknowledgements

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4.8. Supplementary Information

Supplementary Note 1: Preliminary formulation and processing parameter development

The two spray drying parameters were developed for testing using an existing model[42] for the Buchi B-290 lab-scale spray dryer. In both cases an outlet relative humidity of <10% was chosen to minimize plasticization of the powder during collection and reduce moisture content of powder. Additionally, a maximum outlet temperature of 65 °C was chosen to minimize thermal stress on the powder. Aspirator rate of 100% was chosen to achieve the highest possible yield based on the included cyclone.

Two target parameter sets were chosen to mitigate processing losses. Parameter set 1 was chosen to induce low temperature drying of small droplets. This parameter set is designed to minimize thermal stresses placed on the powder. Parameter set 2 was chosen to induce high temperature drying of large droplets. This formulation targeted the formation of large particles with a fast encapsulation rate to quickly stabilize phage within dry powders.

Table S 1 Proposed spray drying operating parameters for encapsulation of Felix O1 bacteriophage. Chosen parameters simulate either low temperature drying of small droplets (parameter set 1) or high temperature drying of large droplets (parameter set 2).

Spray drying parameter	Parameter set 1	Parameter set 2
T_{in}	65 °C	100 °C
Predicted T_{out}[42]	47 °C	62 °C
Aspirator rate (%)	100	100
Atomizer height (mm)	40	40
Feed rate (%)	3.3	10
Predicted RH[42]	9%	7.5%

Formulations containing different mass fractions of leucine, lactose, mannitol, and maltodextrin at varying solids content was assessed for encapsulation efficiency of bacteriophage Felix O1. The spray drying conditions investigated were either Parameter set 1 or Parameter set 2 (Table S 2). Target was <1.0 log loss over the course of spray drying. Results are given in Table S 2.

Table S 2 Summary of tested spray dried formulations for encapsulation of Felix O1 bacteriophage. Formulation details and spray dried parameters are given, as well the corresponding measured processing loss of Felix O1.

Experiment	Formulation (F) (mass %)	Total solids content (mg/ml)	Spray drying condition (P)	Processing loss (log(PFU/ml))
F1P1	Leucine - 20% Lactose - 80%	60 mg/ml	Parameter 1	1.9
F1P2	Leucine - 20% Lactose - 80%	60 mg/ml	Parameter 2	1.1
F2P1	Leucine - 17% Lactose - 67% Mannitol - 17%	72 mg/ml	Parameter 1	1.0

F2P2	Leucine - 17% Lactose - 67% Mannitol - 17%	72 mg/ml	Parameter 2	0.3
F3P1	Leucine - 17% Lactose - 67% Maltodextrin- 17%	72 mg/ml	Parameter 1	0.7
F3P2	Leucine - 17% Lactose - 67% Maltodextrin - 17%	72 mg/ml	Parameter 2	0.8
F4P2	Leucine - 15% Lactose - 85%	80 mg/ml	Parameter 2	0.9
F5P2	Lactose - 100%	72 mg/ml	Parameter 2	1.2
F6P2	Maltodextrin - 20% Lactose - 80%	72 mg/ml	Parameter 2	0.9
F7P2	Leucine - 25% Lactose - 75%	72 mg/ml	Parameter 2	0.5
F8P2	Leucine - 20% Lactose - 80%	40 mg/ml	Parameter 2	0.5

Comparison of F1P1 to F1P2, and F2P1 to F2P2 suggest that using Parameter set 2 reduced processing loss of Felix O1. Additionally, the use of mannitol also reduced processing loss. However, mannitol is an artificial sweetener that is not explicitly approved for infant ingestion. Therefore, we investigated inclusion of maltodextrin as an additional stabilizer. Comparison of the F3P1 and the F3P2 suggests that the difference of spray drying parameter did not significantly influence the Felix O1 retention rate for the maltodextrin inclusive formulation. Later experiments investigated changes in leucine to lactose ratio, as well as changes to total solids content. Spray drying parameter set 2 was held constant for these experiments. No correlation was found between these input parameters and Felix O1 retention.

Supplementary Note 2: Predicted particle formation

Estimation of the properties and evaporation rate of the atomized droplet was completed in order to predict the particle formation (Table S 3). Air liquid mass ratio was calculated to estimate the initial droplet diameter[79]. Evaporation rate was estimated through interpolation of the drying of a water droplet[30]. The droplet lifetime was estimated to be <0.02 s, significantly less than the expected residence time of the droplet within the spray dryer. The low droplet lifetime confirms that there is enough time for the droplet to dry prior to reaching the spray dryer outlet.

Table S 3 Calculated parameters for the drying droplet when spray dried. Air-liquid mass ratio calculated assuming air as incompressible gas with density of 1.225 kg/m^3 and water density of 997 kg/m^3.

Droplet parameters	Value
Atomizer air-liquid mass ratio	4.6
Initial droplet diameter (μm)[79]	8.5
Evaporation rate at T_{in} (m^2/s)[30]	6.2x10^9
Evaporation rate at T_{out} (m^2/s)[30]	3.6x10^9
Droplet lifetime (s)	0.01-0.02
Residence time (s)	0.82

The Pe number for leucine, maltodextrin, and lactose when spray dried under the chosen parameters is given in Table S 4. The calculated Pe for each material was used to predict the time for each component to form a shell when spray dried as part of the LL or LML formulations (Table S 5). Comparison of the t_{sat} or t_t shows that leucine is expected to form a shell before the other components in both the LL and LML formulation. These results confirm the chosen excipient formulation and spray drying parameters to promote the formation of a crystalline leucine shell at the particle surface.

Table S 4 Calculated *Pe* for each considered component in the spray dried LL and LML formulations – leucine, lactose, and maltodextrin. The density, solubility, and diffusion coefficient for each component was found in literature. *Pe* was calculated assuming the evaporation rate at the spray dryer inlet (6.2x10^9 m^2/s).

Excipient	Density (kg/m^3)	Solubility (mg/ml)	Diffusion coefficient m^2/s	*Pe*
Leucine	1170[80]	22[81]	6.30E-10[82]	1.23
Maltodextrin	1260[83]	1200[84]	2.20E-10[85]	3.52
Lactose	1546[86]	200[81]	6.00E-10[87]	1.29

Table S 5 Composition of each formulation under spray drying parameters and calculation of time for each component to begin shell formation, relative to the droplet lifetime. The sugars lactose and maltodextrin are expected to form amorphous powders and leucine is expected to form a crystalline powder. *C* refers to the solute concentration in the feedstock of the relevant component, t_d is the droplet lifetime, t_t is time to true density, and t_{sat} is time to saturation. Both t_t and t_{sat} are measurements of when the component will form a shell during drying.

Formulation	C_{lactose} (mg/ml)	C_{leucine} (mg/ml)	$C_{\text{maltodextrin}}$ (mg/ml)	$\frac{t_{t,\text{lactose}}}{t_d}$	$\frac{t_{sat,\text{leucine}}}{t_d}$	$\frac{t_{t,\text{maltodextrin}}}{t_d}$
LL	57.6	14.4	-	87%	22%	-

LML	48	12	12	88%	22%	93%

Scanning electron microscopy was completed on the single excipient system maltodextrin; lactose; and leucine (Table S 2). All formulations were spray dried using parameter set 2 as the operating conditions. Spray dried lactose produced highly spherical, smooth particle whereas spray dried maltodextrin produces highly corrugated particles. Spray dried leucine produced thin-shelled crystalline particles. Majority of particles appeared broken, intact particles displayed a folded morphology with a rough surface. In all cases the exhibited morphology is consistent [27,49,62].

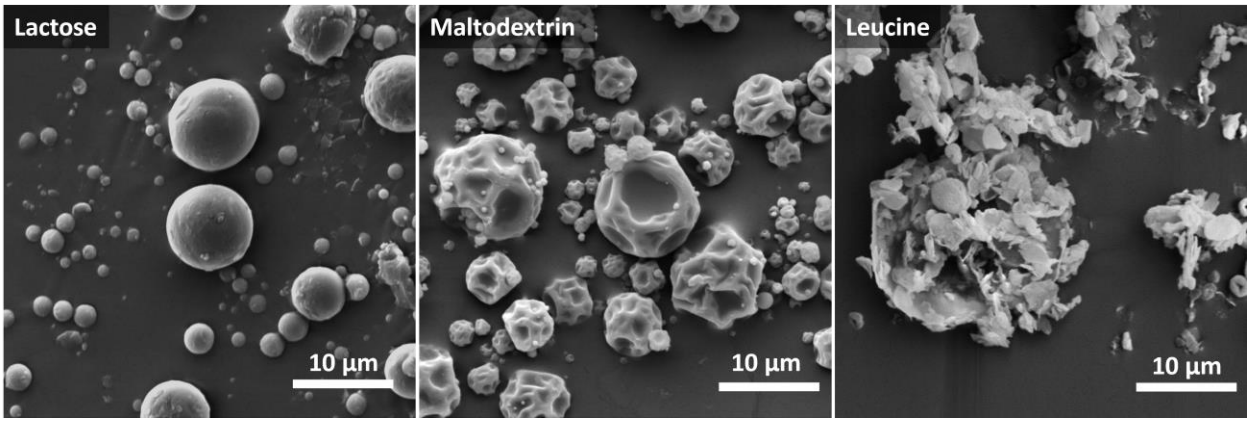

Figure S 1 Spray dried maltodextrin (72 mg/ml), lactose (72 mg/ml), and leucine (20 mg/ml) under parameter 2 processing conditions. Morphology of the resulting powder is dependent on the chosen excipient. Scale bars are shown on the respective image.

Commercial infant formula PIF A and PIF B are similar at the macroscopic and microscopic level (Figure S 2). PIF A (Figure S 2a) and PIF B (Figure S 2b) powders had similar flowability, however, PIF A was slightly more yellow than PIF B. Scanning electron microscopy shows that both formulations do not contain many distinct particles, rather, the particles have clumped and fused together to form various sized aggregates. The smooth, spherical features of these powders is consistent with fresh powdered infant formula with little to no lactose crystallization[21]. These aggregates overall have smooth surfaces, likely due to their high lactose content. The interior of the particles contain many small holes. These holes represent the encapsulated fat content of the powdered infant formula as these holes are consistent with morphology of spray dried emulsions[88].

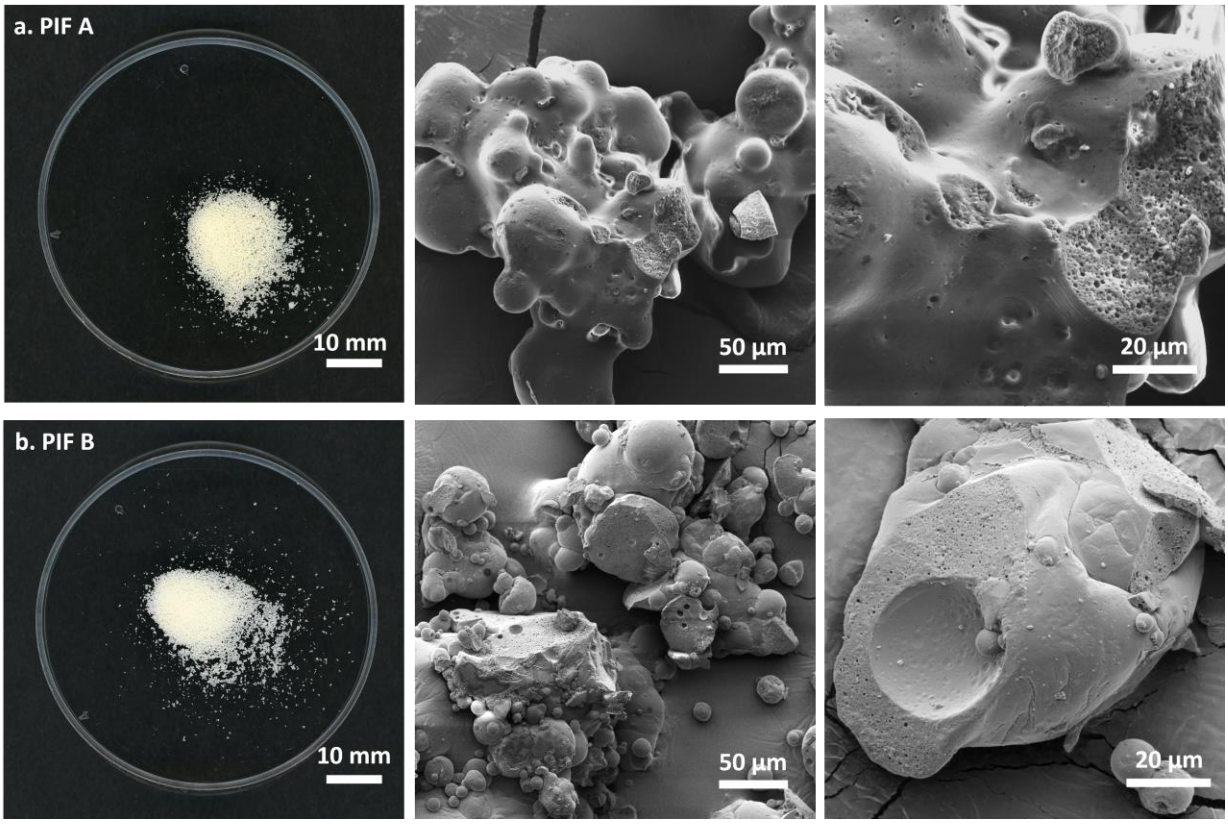

Figure S 2 Macroscopic and microscopic images of PIF A (maltodextrin-free Similac formula) (a) and PIF B (maltodextrin-containing Nestle formula) (b). Scale bars are provided on each respective image. Abbreviations: PIF – powdered infant formula.

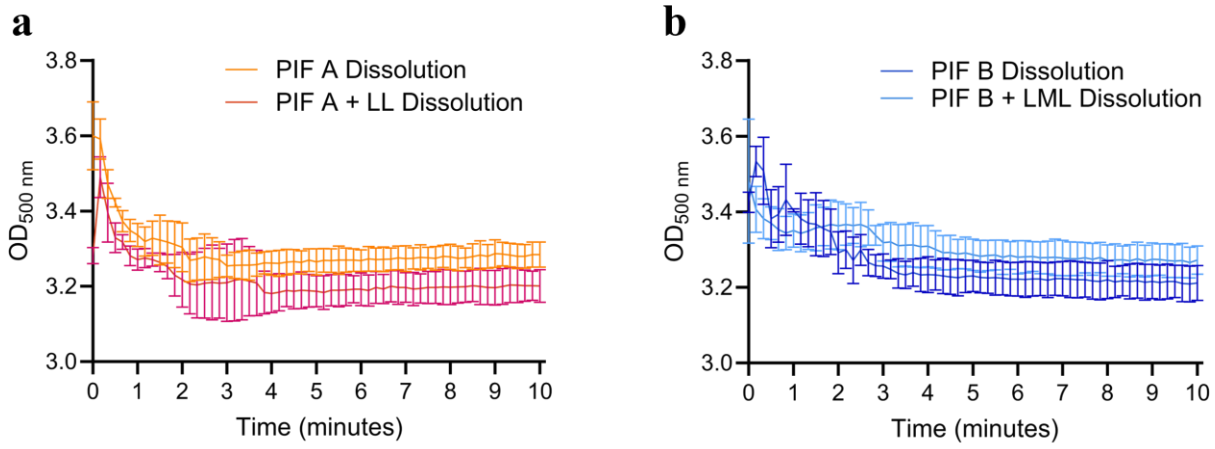

Figure S 3 Dissolution of phage-free PIF A, PIF A+LL, PIF B, and PIF B+LML powders in water under ambient temperature as a measure of optical density at 500 nm. Comparison of the dissolution of (a) PIF A+LL and (b) PIF B+LML to their control counterparts (PIF A and PIF B, respectively). Powders appear to follow the same dissolution pattern. Experiments were completed in triplicate on separate samples. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose, PIF – powdered infant formula.

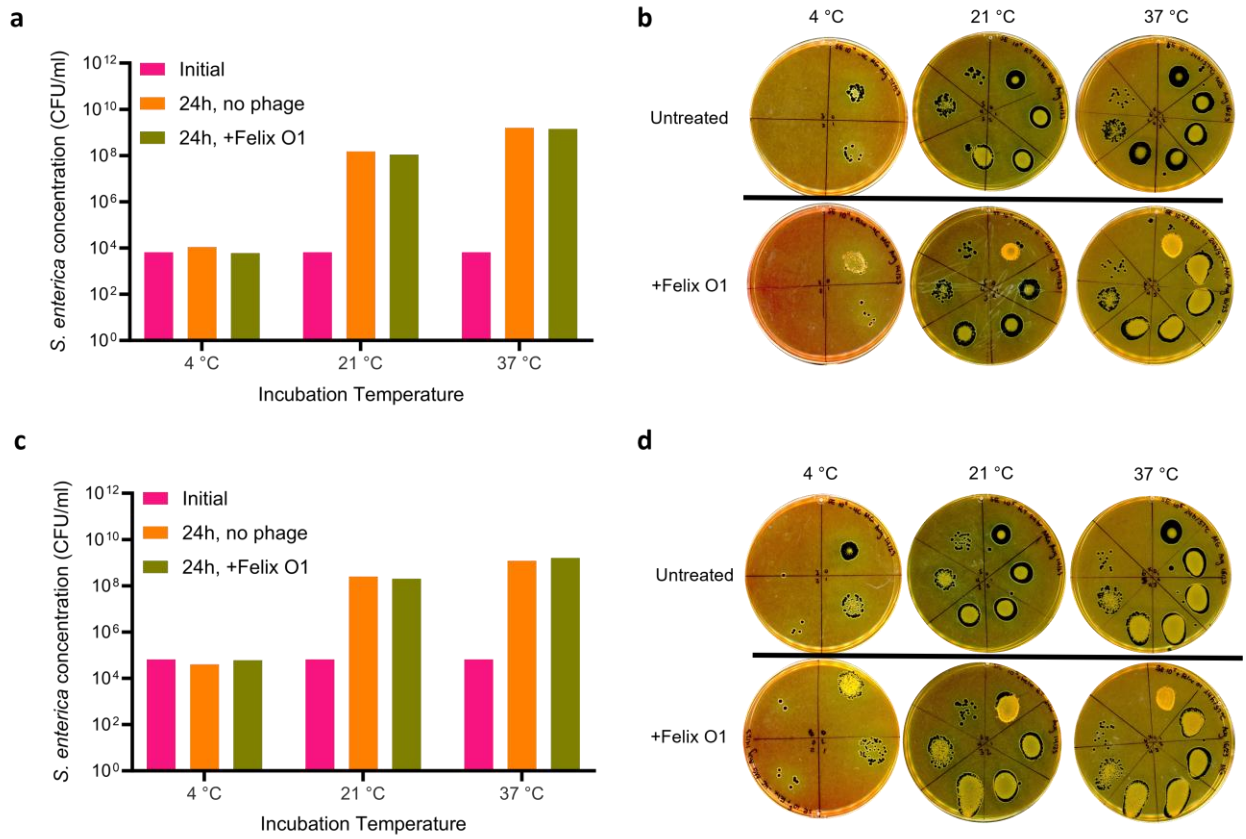

Figure S 4 Phage Felix O1 at 10^7 PFU/ml cannot suppress *S. enterica* growth in infant formula after 24 h of incubation at 4, 21, or 37 °C. Preliminary work investigating the infectivity of Felix O1 in rehydrated infant formula when challenged with **a-b.** $6.5x10^3$ CFU/ml and c-d. $6.5x10^4$ CFU/ml of *S. enterica* at different incubation temperatures relavent to infant formula storage. Concentration of *S. enterica* after 24 h of incubation is similar between the control and the Felix O1 treated samples. Experiments were performed once as a sanity check.

Table S 6 Results of fitting growth phase of experimental *S. enterica* kinetic curves of samples grown in the presence of spray dried lactose leucine (LL) at 5 mg/ml to an exponential function. The parameters growth rate, μ, and lag phase duration, λ, were solved for whereas OD_λ was constrained to experimental values. Exponential fit was completed in GraphPad using least squares fit. Analysis was completed for an untreated control experiment (Control), *S. enterica* treated with Felix O1 phage stock (Phage Stock), and *S. enterica* treated with spray dried Felix O1, where experiments were completed in triplicate.

Parameter	Control (LL)			Phage Stock (LL)			Spray-dried Phage (LL)		
Range analyzed (h)	5.6 to 8.9	5.2 to 8.2	5.6 to 8.2	14.1 to 16.4	14.2 to 16.9	13.8 to 16	13.3 to 16	11.7 to 14.8	14.0 to 16.4
R^2	0.9932	0.9932	0.988	0.9986	0.9988	0.9994	0.9981	0.9995	0.9989
Sum of Squares	0.005524	0.001525	0.00306	0.00012	0.000246	8.43E-05	0.000348	0.000244	0.000288
$OD_{\lambda,1}$	0.0050	0.0040	0.0060	-	-	-	-	-	-
$OD_{\lambda,2}$	-	-	-	0.0110	0.0110	0.0140	0.0070	0.0100	0.0090
μ (h^{-1}) (95% CI)	1.398 (1.313 to 1.489)	1.339 (1.258 to 1.426)	1.402 (1.280 to 1.533)	1.102 (1.069 to 1.135)	1.048 (1.021 to 1.076)	1.147 (1.125 to 1.170)	1.129 (1.093 to 1.165)	1.202 (1.182 to 1.222)	1.246 (1.214 to 1.280)
λ (h) (95% CI)	5.277 (5.064 to 5.479)	4.756 (4.553 to 4.949)	5.111 (4.848 to 5.353)	13.40 (13.32 to 13.47)	13.41 (13.33 to 13.49)	13.17 (13.12 to 13.22)	12.50 (12.39 to 12.60)	11.27 (11.22 to 11.32)	13.17 (13.09 to 13.24)

Table S 7 Results of fitting growth phase of experimental *S. enterica* kinetic curves of samples grown in the presence of spray dried lactose maltodextrin leucine (LML) formulation at 5 mg/ml to an exponential function. The parameters growth rate, μ, and lag phase duration, λ, were solved for whereas OD_λ was constrained to experimental values. Exponential fit was completed in GraphPad using least squares fit. Analysis was completed for an untreated control experiment (Control), *S. enterica* treated with Felix O1 phage stock (Phage Stock), and *S. enterica* treated with spray dried Felix O1, where experiments were completed in triplicate.

Parameter	Control (LML)			Phage Stock (LML)			Spray-dried Phage (LML)		
Range analyzed (h)	5.0 to 8.0	5.2 to 8.0	5.6 to 8.2	14.4 to 16.8	13.6 to 16.5	10.0 to 15.0	12.5-16.4	13.5-15.9	13.7-16.4
R^2	0.9957	0.9967	0.9967	0.9962	0.9964	0.9977	0.9553	0.9982	0.9958
Sum of Squares	0.0005099	0.0004958	0.000856	0.000276	0.001524	0.001987	0.01601	0.00031	0.001111
$OD_{\lambda,1}$	0.001	0.001	0.003	-	-	-	-	-	-
$OD_{\lambda,2}$	-	-	-	0.010	0.023	0.036	0.006	0.010	0.009
μ (h^{-1}) 95% CI	1.646 (1.562 to 1.734)	1.604 (1.530 to 1.682)	1.620 (1.541 to 1.704)	1.152 (1.096 to 1.210)	0.780 (0.750 to 0.811)	0.604 (0.589 to 0.618)	1.051 (0.909 to 1.215)	1.192 (1.153 to 1.233)	1.135 (1.081 to 1.192)
λ (h) 95% CI	4.574 (4.403 to 4.738)	4.389 (4.225 to 4.545)	5.082 (4.935 to 5.223)	13.85 (13.72 to 13.97)	12.40 (12.25 to 12.53)	10.26 (10.16 to 10.36)	12.25 (11.68 to 12.76)	12.79 (12.70 to 12.88)	12.89 (12.73 to 13.03)

Table S 8 Summary of multiple comparisons via one way ANOVA using Tukey's multiple comparisons test on spray dried Felix O1 encapsulated in LL when stored under protective packaging for 6 months under ambient conditions. Powders were packaged for stability study immediately after spray drying. One way ANOVA with multiple comparisons was performed, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, $0.001 \leq p < 0.01$ - **, $0.0001 \leq p < 0.001$ - ***, and $p \leq 0.0001$ - ****. Abbreviations: LL – leucine lactose.

Timepoint comparison (month)	Mean difference (log(PFU/mL))	95% Confidence interval	Adjusted *P* Value	Summary
0 vs. 0.5	0.1711	-0.2315 to 0.5737	0.7116	ns
0 vs. 1	0.5364	0.1338 to 0.9390	0.0076	**
0 vs. 2	0.7199	0.3173 to 1.122	0.0007	***
0 vs. 3	0.6254	0.2229 to 1.028	0.0023	**
0 vs. 6	0.7956	0.3931 to 1.198	0.0003	***
0.5 vs. 1	0.3653	-0.03725 to 0.7679	0.0837	ns
0.5 vs. 2	0.5488	0.1462 to 0.9514	0.0064	**
0.5 vs. 3	0.4544	0.05178 to 0.8569	0.0241	*
0.5 vs. 6	0.6246	0.2220 to 1.027	0.0023	**
1 vs. 2	0.1835	-0.2191 to 0.5861	0.6530	ns
1 vs. 3	0.08903	-0.3135 to 0.4916	0.9722	ns
1 vs. 6	0.2592	-0.1433 to 0.6618	0.3204	ns
2 vs. 3	-0.09447	-0.4970 to 0.3081	0.9644	ns
2 vs. 6	0.07575	-0.3268 to 0.4783	0.9862	ns
3 vs. 6	0.1702	-0.2324 to 0.5728	0.7156	ns

Table S 9 Summary of multiple comparisons via one way ANOVA using Tukey's multiple comparisons test on spray dried Felix O1 encapsulated in LML when stored under protective packaging for 6 months under ambient conditions. Powders were packaged for stability study immediately after spray drying. One way ANOVA with multiple comparisons was performed, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, $0.001 \leq p < 0.01$ - **, $0.0001 \leq p < 0.001$ - ***, and $p \leq 0.0001$ - ****. Abbreviations: LML – leucine maltodextrin lactose.

Timepoint comparison (month)	Mean difference (log(PFU/mL))	95% Confidence interval	Adjusted *P* Value	Summary
0 vs. 0.5	0.1772	-0.1616 to 0.5160	0.5242	ns
0 vs. 1	0.5595	0.2207 to 0.8984	0.0014	**
0 vs. 2	0.7866	0.4478 to 1.125	<0.0001	****
0 vs. 3	1.073	0.7342 to 1.412	<0.0001	****
0 vs. 6	1.056	0.7172 to 1.395	<0.0001	****
0.5 vs. 1	0.3823	0.04352 to 0.7212	0.0242	*
0.5 vs. 2	0.6094	0.2706 to 0.9482	0.0006	***
0.5 vs. 3	0.8958	0.5570 to 1.235	<0.0001	****
0.5 vs. 6	0.8788	0.5400 to 1.218	<0.0001	****
1 vs. 2	0.2271	-0.1117 to 0.5659	0.2841	ns
1 vs. 3	0.5135	0.1747 to 0.8523	0.0028	**
1 vs. 6	0.4965	0.1576 to 0.8353	0.0037	**
2 vs. 3	0.2864	-0.05240 to 0.6252	0.1173	ns
2 vs. 6	0.2694	-0.06945 to 0.6082	0.1531	ns
3 vs. 6	-0.01705	-0.3559 to 0.3218	>0.9999	ns

Table S 10 Colour of LL and LML powder when freshly spray dried and after 6 months of ambient storage within protective packaging. Colour is given as *L*a*b** value, follwing CIELAB conventions. Number of associated pixels, and subsquent frequency, is given for each colour found within the respective powder sample. Abbreviations: LL – leucine lactose, LML – leucine maltodextrin lactose.

LL, t0			**LML, t0**			**LL, 6 month**			**LML, 6 month**		
*L*a*b** value	Pixel Count	Frequency (%)	*L*a*b** value	Pixel Count	Frequency (%)	*L*a*b** value	Pixel Count	Frequency (%)	*L*a*b** value	Pixel Count	Frequency (%)
[98,0,1]	1284	0.1%	[98,0,1]	11075	1.1%	[99,0,0]	97	<0.01%	[98,0,0]	7393	0.7%
[98,0,0]	416	<0.01%	[97,0,1]	601351	57.9%	[98,0,0]	5267	0.5%	[98,0,1]	1453	<0.01%
[97,0,1]	205871	19.8%	[97,0,0]	310213	29.9%	[97,0,0]	667922	65.4%	[97,0,1]	438665	42.9%
[97,0,0]	88841	8.6%	[96,0,0]	63097	6.1%	[96,0,0]	347787	34.0%	[97,0,0]	49333	4.8%
[96,0,0]	458930	44.2%	[96,0,1]	52600	5.1%	[95,0,0]	497	<0.01%	[96,0,0]	505564	49.5%
[96,0,1]	46179	4.4%				[94,0,1]	91	<0.01%	[96,0,1]	7829	0.8%
[95,0,0]	217856	21.0%				[93,0,0]	145	<0.01%	[95,0,0]	8605	0.8%
[94,0,0]	2228	0.2%				[91,0,0]	42	<0.01%	[94,0,0]	1339	<0.01%
[93,0,0]	1044	0.1%				[85,0,0]	104	<0.01%	[93,0,0]	1735	<0.01%
[92,0,0]	260	<0.01%							[92,0,0]	36	<0.01%
[91,0,0]	128	<0.01%									
[90,0,0]	32	<0.01%									

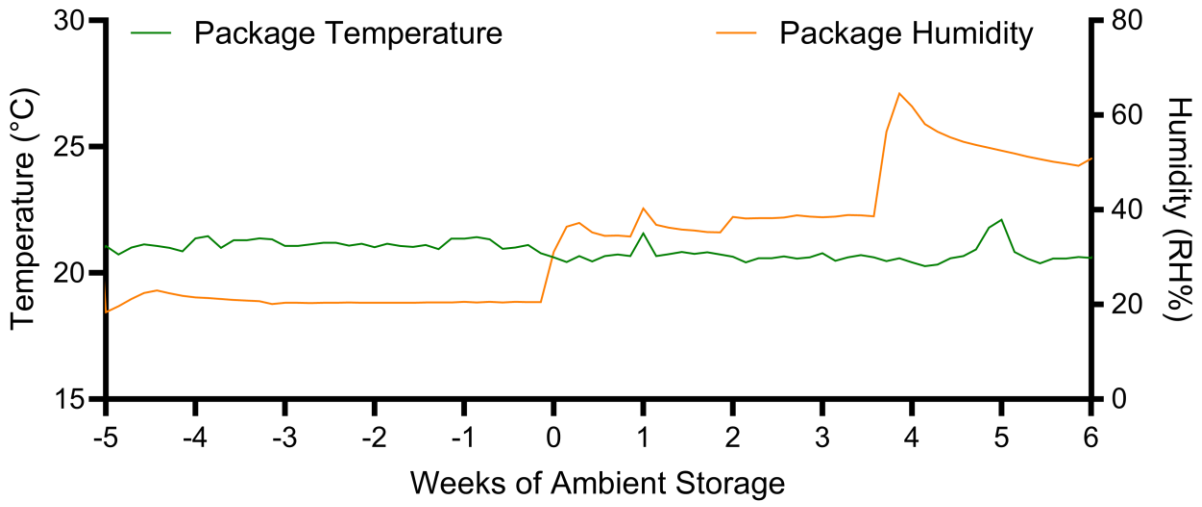

Figure S 5 Measured temperature and humidity within the package holding the LL and LML powder stored for the secondary stability study. Temperature and humidity was monitored over 5 weeks of protective storage (i.e. package contained desiccant and deoxidizers; vials were parafilmed) followed by 6 week of storage under ambient conditions wherein desiccant, deoxidizers, tape, and parafilm were removed from the package. Temperature remained stable over the course of storage at approximately 21 °C. Humidity within the package increased once the protective materials were removed, thus the phage powder was subjected to increasing humidity over the secondary stability study.

Table S 11 ANOVA comparison of LL timepoints over course of secondary stability study using Tukey's multiple comparisons test. Powders were packaged for stability study immediately after spray drying. One way ANOVA with multiple comparisons was performed, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, $0.001 \leq p < 0.01$ - **, $0.0001 \leq p < 0.001$ - ***, and $p \leq 0.0001$ - ****. Abbreviations: LL – leucine lactose.

Timepoint comparison (month)	Mean difference (log(PFU/mL))	95% Confidence interval	Adjusted *P* Value	Summary
-5 vs. 0	0.4148	0.1514 to 0.6781	0.0020	**
-5 vs. 1	0.5775	0.3141 to 0.8408	<0.0001	****
-5 vs. 2	0.4800	0.2167 to 0.7434	0.0006	***
-5 vs. 4	0.5155	0.2521 to 0.7788	0.0003	***
-5 vs. 6	0.4871	0.2238 to 0.7504	0.0005	***
0 vs. 1	0.1627	-0.1006 to 0.4260	0.3595	ns
0 vs. 2	0.06529	-0.1981 to 0.3286	0.9554	ns
0 vs. 4	0.1007	-0.1626 to 0.3641	0.7876	ns
0 vs. 6	0.07235	-0.1910 to 0.3357	0.9330	ns
1 vs. 2	-0.09742	-0.3608 to 0.1659	0.8086	ns
1 vs. 4	-0.06198	-0.3253 to 0.2014	0.9639	ns
1 vs. 6	-0.09036	-0.3537 to 0.1730	0.8502	ns
2 vs. 4	0.03544	-0.2279 to 0.2988	0.9970	ns
2 vs. 6	0.007063	-0.2563 to 0.2704	>0.9999	ns
4 vs. 6	-0.02838	-0.2917 to 0.2350	0.9990	ns

Table S 12 ANOVA comparison of LML timepoints over course of secondary stability study using Tukey's multiple comparisons test. Powders were packaged for stability study immediately after spray drying. One way ANOVA with multiple comparisons was performed, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, $0.001 \leq p < 0.01$ - **, $0.0001 \leq p < 0.001$ - ***, and $p \leq 0.0001$ - ****. Abbreviations: LML – leucine maltodextrin lactose.

Timepoint comparison (month)	Mean difference (log(PFU/mL))	95% Confidence interval	Adjusted *P* Value	Summary
-5 vs. 0	0.6830	0.4022 to 0.9637	<0.0001	****
-5 vs. 1	0.5970	0.3163 to 0.8777	0.0001	***
-5 vs. 2	0.5601	0.2794 to 0.8409	0.0002	***
-5 vs. 4	0.6388	0.3581 to 0.9196	<0.0001	****
-5 vs. 6	0.7714	0.4906 to 1.052	<0.0001	****
0 vs. 1	-0.08596	-0.3667 to 0.1948	0.8994	ns
0 vs. 2	-0.1228	-0.4036 to 0.1579	0.6880	ns
0 vs. 4	-0.04413	-0.3249 to 0.2366	0.9938	ns
0 vs. 6	0.08840	-0.1923 to 0.3691	0.8887	ns
1 vs. 2	-0.03687	-0.3176 to 0.2439	0.9973	ns
1 vs. 4	0.04183	-0.2389 to 0.3226	0.9952	ns
1 vs. 6	0.1744	-0.1064 to 0.4551	0.3545	ns
2 vs. 4	0.07870	-0.2020 to 0.3594	0.9276	ns
2 vs. 6	0.2112	-0.06950 to 0.4920	0.1905	ns
4 vs. 6	0.1325	-0.1482 to 0.4133	0.6216	ns

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Chapter 5: Concluding remarks

5.1. Summary of key findings

Ingesting food contaminated with bacteria contamination can cause food poisoning or foodborne illnesses, leading to hospitalization and even death. This threat can be mitigated through hygienic practices throughout food production and processing. However, the globalization of food distribution increases the risk of contamination during production and transit. Furthermore, rapid detection of contamination is not widely available. Voluntary product recalls often only occur after an outbreak has already begun. The inclusion of bactericides in food would reduce the extent and severity of outbreaks. Bactericide safety is an issue as broad-spectrum antibiotics cannot be included in food for fear of damaging the human biome and increasing the prevalence of resistant microbes. Other preservatives, such as sodium nitrate and citric acid, can only be used with certain types of food as they alter taste, or may be harmful when ingested in large quantities. Bacteriophages are a promising, taste-free, natural method of eliminating bacteria. Much research has been done on the isolation and characterization of new bacteriophages but the research on methods to integrate bacteriophages into commercial food applications is comparatively little. This thesis sought to bridge that gap in bacteriophage applications.

In Chapter 2, a commercial bacteriophage product was exposed to one of three stresses that are relevant to food production: desiccation, heat, and acidity. The bacteriophage populations were stressed and propagated to obtain stress-resistant populations, relative to the ancestral stock. The improved survival of these populations was maintained even when they were grown in the absence of a stressor. Genomic analysis found possible correlations between phenotypic changes and the changed encoded DNA in the stressed population. The improved, stress-resistant phage populations were created by vacuum desiccation, lysate exposure to heat in a water bath, and the addition of hydrochloric acid to lysate to create desiccation-, heat-, and acid-resistant populations, respectively. These populations also had the same infectivity as their ancestral counterparts. These higher survival populations were then tested under real-world applications, along with the stock product as a control. The tested conditions included air drying of phage droplets, representing the use of phage cleaning products, survival of phage in milk during batch pasteurization (63 °C for 30 minutes), and survival of phage when added to apple juice. In all cases, the phage populations failed to demonstrate statistically significant improved survivability relative to their ancestral counterpart.

The challenges encountered in evolving a phage population that would survive batch pasteurization in milk led to the creation of the pasteurization-protective matrix detailed in Chapter 3. In this work, a formulation composed of pullulan and trehalose was spray dried to encapsulate a commercial bacteriophage product within a powder. The powder was designed via particle engineering such that pullulan would accumulate at the exterior of the particle whereas the majority of the particle interior would be composed of trehalose. Spray-dried pullulan was demonstrated to have a much slower dissolution rate than spray-dried trehalose, due to the material properties of the polysaccharide as compared to the disaccharide. The structured powder was designed to protect phage during commonly used industry pasteurization (72 °C for 15 seconds). In its amorphous glass form, trehalose would protect the phage from degradation due to heat exposure whereas the

pullulan would slow the dissolution process, allowing the protective matrix to remain in place during pasteurization. Encapsulation of phage within the pullulan-trehalose powders protected the phage during simulated pasteurization, with no significant loss in dairy milks with different fat contents. Conversely, unprotected phage underwent significant loss by several orders of magnitude during simulated pasteurization. The application of this technology was demonstrated in commercial milk, wherein the protected phage reduced *listeria* growth as compared to a contaminated control.

The development of a protective matrix was also applied to the issue of contaminated powdered milk formula. In this application, the target is not to protect the phage from exposure to aqueous heat but rather to design a phage powder that is compatible with powdered infant formula in terms of composition and storage conditions. Chapter 4 covers the development of a spray-dried powder that can encapsulate phage for inclusion with powdered infant formula. In this case, the matrix was designed to only include ingredients found in powdered infant formula, namely leucine, lactose, and maltodextrin. The particle was designed such that lactose or a mixture of lactose and maltodextrin, was the primary stabilizer. Leucine was added to form a hydrophobic outer shell on the particle for improved storage stability. Two lead candidates were spray-dried with a model anti-*salmonella* phage. Powder yield was high and processing loss was low for laboratory-scale production. The powders did not significantly change the composition, pH, and dissolution when added to commercial infant formula at a mass ratio of ~4%. The phage powder, when stored in the absence of protective packaging for 6 weeks to simulate an opened package, underwent no significant loss. When stored for 6 months under protective packaging, the lead candidates stabilized after one or two months of storage, with no significant loss thereafter. The layered particle design likely contributed to the stability of the encapsulated phage.

5.2. Conclusions

The results in Chapter 2 illustrate the limitations of adaptational evolution to improve phage application in food. The phage populations evolved under certain stress conditions (desiccation, heat, low pH) demonstrated improved tolerance. However, the improved tolerance of the adapted phage was only demonstrated under laboratory conditions. The adapted phage did not show improved tolerance when tested in real world applications representative of the tested stress conditions. These results suggest that stress conditioning under laboratory conditions does not account for the complexities of real-world applications. Furthermore, the results are consistent with the literature showing that adaptational evolution is highly condition specific. The constraints of the adaptational evolution method means that the method cannot be used to generally improve phage integration with food and food processing.

The results of Chapter 2 provided inspiration for the remainder of this thesis. Both Chapter 3 and Chapter 4 approach the challenge of phage integration with commercial food through the development of protective matrices. The pullulan-trehalose spray-dried powder was designed to be compatible with industry pasteurization processes. Canadian government regulations limit additives for milk, where Vitamin A and D may be added prior to pasteurization. With this regulation in mind, the pullulan-trehalose powder was designed to be added to milk pre-pasteurization. The use of pullulan-trehalose powder allows for the inclusion of phage prior to

pasteurization as a preventative method of treating post-processing contamination of milk. The chosen ingredients, pullulan and trehalose, are GRAS sugars. The types of milk tested in this work were limited to dairy milks of different fat contents, however, the pullulan-trehalose formulation would be compatible with lactose-free milk alternatives such as oat milk and soy milk. The sugars will eventually dissolve, releasing phage into the surrounding milk for bacterial control. As sugars are already present in milk, the addition of pullulan and trehalose is not expected to affect taste at low concentrations. Pullulan-trehalose systems have been used to stabilize bacteriophage for long-term dry storage under ambient conditions[2,3]. The work presented here offers a novel application of the pullulan-trehalose system wherein the designed powder format can be used to protect biologics against short-term heat exposure in an aqueous environment.

Regarding phage intervention in infant formula, the field has been focused on the isolation of appropriate phages and testing infectivity in rehydrated formula. There has been less attention given to the issue of how to practically include phage in powdered infant formula. Only one study by Imm and Chang[4], which was published in the last year, also proposes converting phage into a dry format for inclusion in powdered infant formula. Their work focused on a lyophilized presentation composed of collagen peptides and trehalose. The bacteriophage platform investigated in Chapter 4 was developed for industry compatibility. The phage powders were produced via spray drying, the method by which powdered infant formula is also produced. The phage powders were also designed to be compatible with all dairy-based powdered infant formulas, based on WHO composition standards. The developed platform was able to encapsulate phage during the production process with high efficiency and showed excellent stability when exposed directly to ambient humidity and temperature. The developed phage powder may be intermixed with powdered infant formula as a failsafe that may activate upon reconstitution. Leucine and lactose formulations have been previously spray-dried for encapsulation of phage as an inhalable treatment. This is the first instance of leucine-lactose and a leucine-lactose-maltodextrin powder designed for the inclusion of phage in powdered infant formula.

The research undertaken in this thesis focuses on extending the applicability of bacteriophages in different food systems. The initial work on adaptational evolution found limitations on the ability of this method to prepare phage populations that would be effective under real-world conditions. The condition-specificity of this method also reduced the commercialization potential. Instead, protective matrices could be designed to allow the inclusion of phage in either pasteurized milk or in powdered infant formula. The design of these protective matrices would allow for more broad application. For instance, the developed pullulan-trehalose powder would be compatible with pasteurization of both dairy-based and non-dairy milk. Additionally, the developed leucine-lactose or leucine-lactose-maltodextrin powder would be compatible for inclusion with all dairy-based infant formulas as well as all dairy-based powdered milks. Through the use of these spray-dried protective matrices, phages may be added to foods where they would be either deactivated during processing or simply unable to be added as a liquid. The development of these spray-dried powders demonstrates that encapsulation of phages allows for direct integration with food for improved bacteria intervention.

5.3. Future Directions

The spray-dried powders developed in Chapter 3 and Chapter 4 may be characterized further and optimized for commercial applications.

Spray-dried pullulan-trehalose

Pullulan-trehalose spray dried powder is very effective in preserving encapsulated phage over the course of industrial pasteurization in dairy milk. Popular milk alternatives, including almond, soy, and oat, also must be sterilized via heat and are still at risk of bacterial contamination. The pullulan-trehalose phage powder system would likely be suitable for contamination intervention in these types of milk. Experiments are required to confirm this hypothesis.

The pullulan-trehalose powder was spray dried with no optimization. The processing loss of the commercial P100 phage was approximately 2 log loss. The target loss for a laboratory spray dryer would be under 1 log loss. The mechanism of P100 loss is currently unclear. Optimization may be achieved through modification of the processing parameters for less strenuous production or formulation adjustment for higher stability during spray drying. Formulation adjustment may include changing the overall solids content to adjust the particle size or adjusting the mass ratio of pullulan to trehalose. Optimization of the product may also extend past processing losses to changes in the format. Changes in particle size and distribution of pullulan relative to trehalose will affect the dissolution rate of the powder. The surface area available for dissolution can be further reduced by compressing the powder into an easy-to-use tablet form. The pullulan-trehalose powder is fully amorphous and a relatively sticky powder, making it well suited for granulation and tableting[5].

The system was designed to protect encapsulated biologics against aqueous short-term high-temperature exposure. Commercial anti-*listeria* phage was used as a model and the investigated application was milk pasteurization. Other phages, or even phage cocktails, may be tested for encapsulation within the pullulan-trehalose powder for pasteurization survival. The pullulan-trehalose system also has the potential to be used for food applications other than pasteurization survival. For example, protection against short term high temperature exposure may be useful when applied to the heat treatment of animal feed.

Spray-dried lactose-leucine or leucine-maltodextrin-lactose

Both lead candidates had low processing loss of phage and had similar stability study results. The lactose-leucine formulation would be more broadly applicable with powdered infant formulas as some formulas do not contain maltodextrin. However, the maltodextrin-containing formulation had consistently lower processing loss (insignificant loss). Consultation with industry experts would be needed to determine which of the two candidates would be better suited for commercialization. Stability experiments were completed up to 6 months of protected storage, however, the shelf life of unopened powdered infant formula can be longer than a year. An additional timepoint at 1 year of storage for both powders is currently planned.

Both powders were developed as a platform using Felix O1 as a model phage. However, this model phage is not able to effectively control bacteria growth in rehydrated powdered infant formula.

This platform should instead be tested with other phages, or even phage cocktails, that have demonstrated infectivity within infant formula. *Salmonella* phages suitable for use in infant formula were not readily available to our research group. Collaboration with other research groups or material transfer agreements for commercial products would be necessary. The ingredients used in the platform are very safe, and microbials are allowed to be added to infant formula to improve resemblance to breast milk. The phage powder is designed as a fail-safe food preservative, not as a treatment for bacterial infection in infants. This designation reduces the regulatory hurdles. However, a developed phage powder, particularly the phage or phage cocktail used, would need to be confirmed safe for infant ingestion.

The compatibility of the spray-dried platform with powdered infant formula also extends its use as a preservative outside of phage. This platform could be used to stabilize bacteria that are present in human milk for addition to the powdered infant formula. Additionally, this platform could also be used to stabilize formula nutrients that are temperature- or humidity-sensitive. The application potential of these formulations is very promising; however, assumptions have been made regarding the amount of leucine present in powdered infant formula. Confirmation of leucine concentration by manufacturers or consultation with an infant nutrition specialist would be useful for ensuring that the concentration of leucine in the spray-dried powders is suitable for infant growth.

5.4. References

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Appendix A: Encapsulation of Felix O1 phage within pullulan-trehalose particles

The pullulan-trehalose powder protection system may be useful to target foodborne pathogens other than *L. monocytogenes.* Felix O1 is an anti-*salmonella* phage that has been successfully spray dried in other formulations[1,2]. We spray dried Felix O1 phage at 10^8 PFU/ml to assess the versatility of the pullulan-trehalose system in stabilizing a different phage under water pasteurization. The initial titer of the phage feedstock was 1 log lower in this case than P100 due to a lower Felix O1 stock concentration. The same volume of phage stock solution between Felix O1 and P100 was prioritized to reduce the influence of the phage media background.

Felix O1 activity loss attributed to processing was less than P100, at 0.77 log loss (Figure 1a). Both the reconstituted and intact powder underwent an additional 0.64 and 0.83 log loss upon pasteurization in water only, respectively, after powder production (Figure 1b). The difference in titer loss between these two samples was statistically insignificant ($p \geq 0.05$). Two of the three pasteurized, pre-dissolved Felix O1 phage samples formed smaller plaques as compared to their protected counterparts and the unpasteurized sample (Figure 1c, Figure 2).

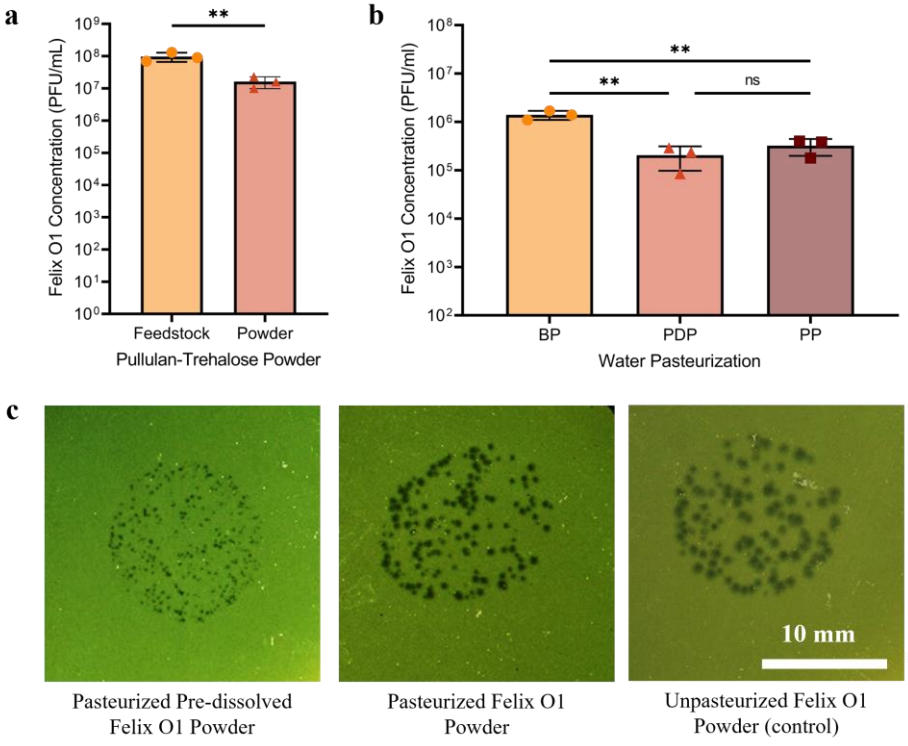

Figure 1. Anti-*salmonella* bacteriophage Felix O1 can be encapsulated within pullulan-trehalose powders for improved phage replication ability when pasteurized in water. a. Measurement of the feedstock concentration ("Feedstock" – orange circle) and the rehydrated powder ("Powder" – red triangle) indicates that Felix O1 undergoes less than 1 log loss on spray drying with the pullulan-trehalose formulation. **b.** Felix O1 sample underwent less than 1 log loss on pasteurization in water both when pre-dissolved ("PDP" – red triangle) and as an intact powder ("PP" – burgandy square). Experiments were conducted on separate samples in triplicate. There is no difference in loss between the two samples, statistically. Pre-pasteurization Felix O1 concentration is given for context ("BP" – orange circle). Triplicate measurements of pre-pasteurzation concentration were completed on a single batch. **c-d**. Comparison of Felix O1 plaque sizes when exposed to pasteurization. Two of three distinct pre-dissolved, pasteurized Felix O1 replicates showed reduced plaque size **(c)**. Felix O1 plaque size of the pasteurized powder

(d) was maintained with respect to the pre-pasteurization sample **(e)** for all samples. All plaque assays were incubated under the same conditions and timeframe. Scale bar represents 5 mm as the scale for all images. Statistical analysis was performed on the log-transformed values, where $p \geq 0.05$ – not significant, $0.01 \leq p < 0.05$ - *, and $0.001 \leq p < 0.01$ - **.

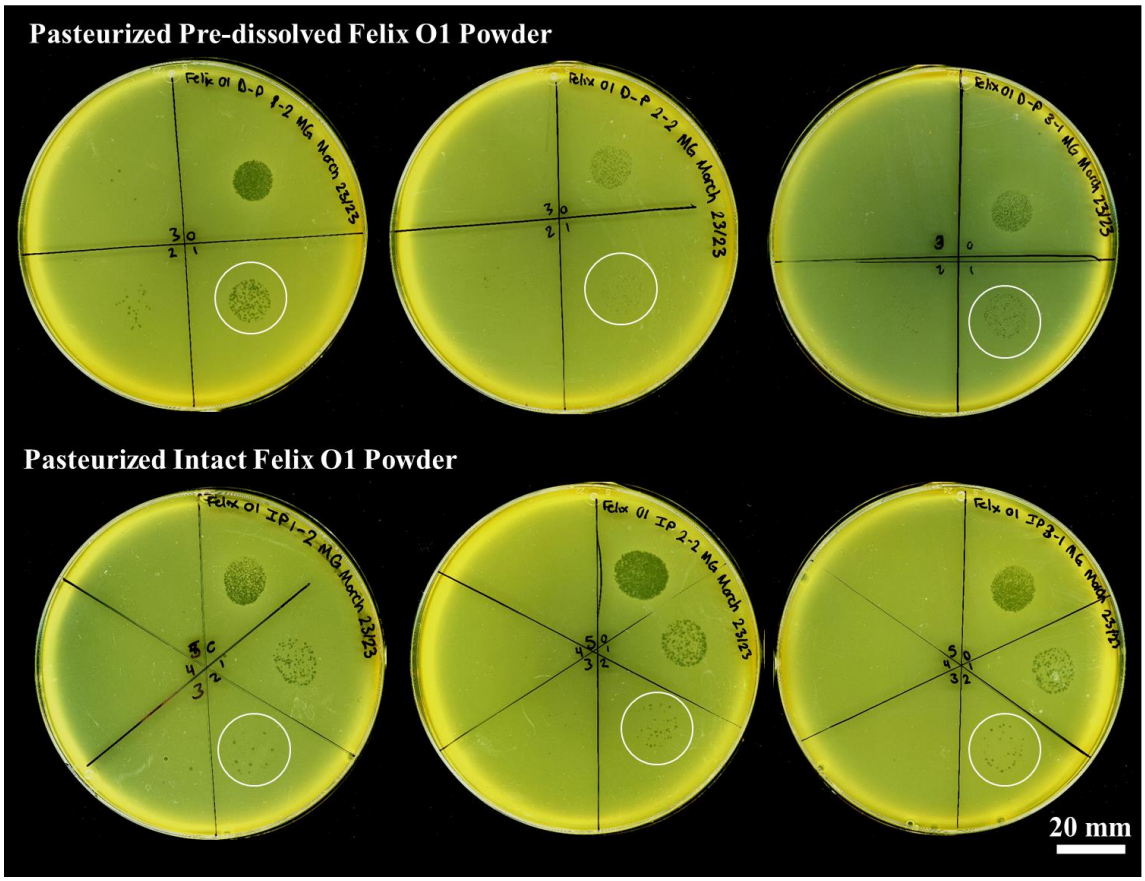

Figure 2. Spray dried anti-salmonella Felix O1 pullulan-trehalose samples' replication ability is affected by pasteurization in water. Plaque assays of Felix O1 pullulan-trehalose powder, where all samples were incubated under the same conditions and timeframe. Pasteurization experiments were completed in biological triplicate. Measurements pre-pasteurization were completed in triplicate on a single batch of powder. White circles indicate the dilution used for size comparison for a given plaque assay. Size of plaques in two of the three Felix O1 powders that were pre-dissolved prior to heating are qualitatively smaller than plaques from all Felix O1 powder samples where powder was intact during pasteurization and plaques from a sample that did not undergo heating. Scale bar for all samples is given.

Spray dried Felix O1 had approximately 40× retention compared to P100 under the same spray drying conditions. Additionally, pre-dissolved Felix O1 powder underwent <1 log loss when heated in water, with no difference compared to the encapsulated counterpart. Felix O1 is significantly more thermoresistant than phage A511[48], a P100 analogue, which may account for its increased survival during spray drying and heating. Critically, a few samples of pre-dissolved pasteurized Felix O1 had smaller plaques in comparison to the protected and the unpasteurized samples. The number of Felix O1 phage was preserved but its ability to replicate and subsequently lyse bacteria may have been impaired. Thus, the pullulan-trehalose system may be able to protect both overall phage numbers as well as their integrity when exposed to pasteurization. Plaque size comparison could not be completed on P100 as they were below the limit of quantification.

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