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ABSTRACT

Characterization of Bacteriophages Targeting *Bacillus licheniformis* in Milk Processes and Thermal Stability of Bacteriophage During HTST Pasteurization

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An array of *Bacillus licheniformis strains* were isolated from a commercial powdered milk process. Bacteriophages exhibiting activity against *B. licheniformis* were isolated from cattle manure and effluent samples destined for a lagoon at a dairy farm. After sequencing, 8 of the 10 phages were found to be novel and genetically differentiated. Transmission electron scanning microscopy (TSEM) was performed. All bacteriophages were of the family Herelleviridae with contractile tail sheaths ranging from 80µm to 150µm and, surprisingly, survived a common fluid milk processing treatment used to inactivate vegetative cells. The survival of the phage after high temperature short time pasteurization of 73℃ for 20 s shows that the use of bacteriophages in milk to control *B. licheniformis* could be applied as a potential quality control, retarding the germination of spores and reduction of final spore counts in products with long run times such as dairy powders.

Keywords: spore-forming bacteria, pasteurization, bacteriophage, thermal inactivation, biocontrol

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INTRODUCTION

Customers of the U.S. Dairy export council have set limitations on thermophilic spore-forming bacteria at <500 cfu/g and mesophilic spore-forming bacteria limits to <1000 cfu/g in milk powders (Watterson et al., 2014). These limits are established because spore-forming bacteria cause quality and safety concerns in all milk products, including but not limited to late bloating in cheese (Doyle et al., 2015; Boor et al., 2017), high spore counts (Bienvenue, 2014), the production of nitrites in infant formulas (Cho and Rhee, 2019), rapid spoilage of liquid milk (Doyle et al., 2015), and quality and safety concerns in canned condensed milk (Martinez et al., 2017).

Of the spore-forming bacteria found in milk, *Bacillus licheniformis*, *Anoxybacillus flavithermus*, and *Geobacillus stearothermophilus* are the most prevalent found in the powder milk industry (Dettling et al., 2020). Colonies of *B. licheniformis* have been shown to form biofilms (Jindal and Anand, 2018) and multiply at temperatures between 30-55℃, both of which lead to higher spore counts in final powdered products. Controlling these bacteria could have benefits for the milk powder industry, due to higher quality milk and increasing sales in the powder sector. There is currently high demand for low-spore count milk powders used in the production of infant formula and reconstituted powder for UHT milk. The largest producer of low-spore count milk powders is New Zealand, second Europe, and third the United States (Hoogwegt Group, 2019). An increased market share and long-term growth opportunity is available to the U.S. processors if they deliver against low spore specifications and other key quality standards.

In the U.S., production runs of 15 h or longer are increasing with vertical integration, increased pressure on commodity margins, and advances in automation. These long runs make maintaining low spore counts challenging because the bacteria have increased time to multiply in and out of biofilms before a cleaning cycle is performed. Minimizing downtime for cleaning during runs means less product output, leading to higher operating costs and an overall reduction of product output. In contrast, European manufacturers run for shorter times, clean more frequently and manage production with smaller operations, which keeps spore counts low. Therefore, finding new biofilm and spore forming bacteria mitigation strategies, has been the object of recent studies. Opportunities for increased operational efficiency and product quality in large facilities is of great interest to U.S. dairy processers desiring to compete globally.

Bacteriophages, or phages, may present a novel approach to the control of spore-forming bacteria, biofilms, and final counts of spores in dairy products. Bacteriophages are viruses that infect very specific bacteria through controlled attachment to the bacteria's outer cell wall via the bacteriophage tail fibers. Once attached, the phage inject their DNA into the cell, taking over the bacteria's cellular machinery and going through the subsequent process of production and assembly of more phage virions. In lytic phages, a signal is then given to lyse the cell, destroying it, and releasing more of the phage virions into the environment. These virions subsequently attach to other similar bacteria and the cycle repeats (Madigan et al., 2006).

Phages are currently sold for use in the food industry to target and kill pathogenic bacteria. Some of the companies at the forefront of this are, Intralytix, PhageGuard, and OmniLytics. FDA approval for phage P100 for use in food was given in 2006 (U.S. Food and Drug Administration, 2006). In subsequent years several phage cocktails, combinations of different phages that all target the same organism, and individual phages have been granted

GRAS status (U.S. Food and Drug Administration, 2016; U.S. Food and Drug Administration, 2018; U.S. Food and Drug Administration, 2021). These phage cocktails target pathogenic bacteria such as *Salmonella*, *E. coli* O157:H7, *Listeria,* and *Bacillus cereus*. Currently, *Listeria* phage cocktails can be applied to cheese, produce, smoked meats, and other frozen foods (Perera et al., 2015). Despite the current use of phages on dairy and other food products, almost no research has been done on the use of lytic bacteriophage to control the growth of non-pathogenic spore-forming bacteria in milk.

One of the main outgrowth sources of spore-forming bacteria in milk powder production, is through their proliferation of, and integration into biofilms; Phage are effective at controlling biofilms (Burgess et al., 2014; Gopal et al., 2015). Knowing this, the use of phage in milk products might be useful in controlling spore-forming bacterial outgrowth and has been mentioned in review articles as an area of needed research (Pujato et al., 2019; O'Sullivan et al., 2020). This study focuses on addressing the following knowledge gaps concerning the use of phage in milk products to control spore-forming bacterial growth during long commercial processing runs.

The objectives of this study were to:

1. Characterize and identify spore-forming bacteria found in the milk fractionation process by using 16S RNA sequencing.
2. Hunt for and isolate lytic phages that can target the most prevalent bacteria found in the process (i.e., *B. licheniformis, A. flavithermus,* and *G. stearothermophilus*).
3. Sequence the isolated phage genomes to characterize; Screen for pathogenic and undesirable genes.

4. Classify phages by how effective they are against the target bacteria and test host range.
5. Test, within the parameters of pasteurization, to determine survivability and resistance of the selected phages.

MATERIALS AND METHODS

Isolation of Spore-forming Bacteria

Samples from a milk fractionation process were received shipped overnight and analyzed the next day over the course of 2 years. Included in those samples are the locations in Table 1.

Table 1. Places that samples were taken from in the milk fractionation process

NO	SAMPLE
1	*Raw Milk (Lab Pasteurized)*
2	*Pasteurized Cream - Beginning of Run*
3	*Pasteurized Cream - Before Second Mid-run*
4	*Pasteurized Cream - End of Run*
5	*Skim Milk to HTST - Beginning of Run*
6	*Skim Milk to HTST - Before Second Mid-run*
7	*Skim Milk to HTST - End of Run*
8	*UO* Concentrate to dryer preheat - Beginning of Run*
9	*UO* Concentrate to dryer preheat - End of Run*
10	*Powder Beginning of Lot*
11	*Powder Mid-run*
12	*Powder End of Run*
13	*LPC***

**UO stands for ultra-filtration ** LPC stands for lactose permeate concentrate, all sampling locations were determined by our industry partner to search for spore-forming bacteria proliferation and problem areas in their process*

After receiving, samples were reconstituted, in the case of powders, and diluted and enumerated following the method outlined in the Standard Methods for the Examination of Dairy Products 17th edition, with modifications (2004). Those modifications included the use of TSB (trypticase soy agar) instead of SMA (standard methods agar) and an anerobic gas chamber for enumeration of anerobic spore-forming bacteria. Random samples were taken from the plates with a total of 127 viable isolates for cryogenic storage. Samples were grown in TSB and when

turbid added to a 30% glycerol solution and aliquoted into 2.5 ml cryogenic vials for storage at -80℃ until DNA extraction could occur.

Characterization of Bacteria Through 16S rDNA Sequencing

Spore-forming bacteria were obtained from a milk fractionation process in the manner stated above. DNA was extracted using a DNeasy UltraClean Microbial Kit (Qiagen). DNA was amplified using PCR (Table 2) and amplifications cleaned using a PCR clean kit (Qiagen). Gel electrophoresis was performed after DNA amplification and after DNA cleaning to check purity of the DNA and that PCR reactions had occurred. A NanoDrop spectrophotometer (Thermo Fisher Scientific) was then used to check DNA concentrations. 16S rDNA was sequenced in the BYU Sequencing lab using (Big Dye Cycle Sequencing). Various primers were used to get the full sequence (Table 2). In total 71 isolates were processed, and viable DNA obtained. After sequencing, DNA was trimmed and aligned with Geneious Prime 2021.1.1 then assembled using the Geneious de novo assembler. The resulting consensus sequences were then searched for and compared to the NCBI nt/nr database to identify the organism on the genus level (NCBI, 2016).

Table 2. Primer sequences

Sequence Name	Sequence 5’ to 3’
8F	AGAGTTTGATCCTGGCTCAG
337F	GACTCCTACGGGAGGCWGCAG
1100F	YAACGAGCGCAACCC
1492R	CGGTTACCTTGTTACGACTT
1100R	GGGTTGCGCTCGTTG
518R	GTATTACCGCGGCTGCTGG
336R	ACTGCTGCSYCCCGTAGGAGTCT

Phage Isolation

Following methods outlined in the SEA-Phages manual (Poxleitner et al., 2018) by the University of Pittsburg. Samples from various probable bacterial harborage areas were taken and screened for phages (e.g. manure samples, water runoff from a dairy farm, raw milk, whey samples, etc.).

Two different isolation methods were used. First, samples were cleaned of bacteria and particles that might interfere with phage attachment via centrifugation and sterile filtration using a .45µm sterile filter (Millipore Sigma). An .5 mL aliquot of *B. licheniformis* culture was added along with 50µL of the filtrate from the sample. A period of attachment followed (15 min) followed by plating with a soft top agar. Lytic phages were determined by clear zones of inhibition, or plaques, present on the plate after 24 h of incubation at 37°C.

The second method utilized an enrichment which involved adding concentrated growth medium (TSB) to the samples along with a 1ml aliquot of log phase *B. licheniformis* culture. Incubation overnight at 37°C was followed by the steps in the first procedure. This procedure allowed for amplification of the phages for easier isolation.

Characterization of Phages

A DNA phage kit (Qiagen) was utilized to extract phage DNA. Samples were then sequenced at Brigham Young University (Provo, Utah). Phage sequences were analyzed using the BLASTn tool (Altschul et al., 1990) and further compared with the Virulence Factor Database (Kong et al., 2019). In addition, scanning transmission electron microscopy (STEM) images were taken of the phage using a negative stain with 2% uranyl acetate (Ackermann, 2009) and a Helios NanoLab 600 (Thermo Fisher Scientific).

Annotation of the phage genomes was performed by using DNA Master (Pitt) which auto annotated the open reading frames using Glimmer (TIGR) and GeneMark (GATech). Each gene was checked to verify that the open reading frames were being called. Identification of the proteins was done using the BLASTn tool from the National Center for Biotechnology Information (Bethesda, MD) in concert with analysis of the product region using the HHpred tool (Tübingen). Identified genes were compared against similar phage and were assigned functions. If a gene was not identified, it was assigned NKF or no known function. This was following protocol found in the sea phage hunters bioinformatics guide (Pope et al., 2017).

Phage host range. Phage host range was assessed by the double agar overlay method where a pre-poured agar plate has a mixture of molten agar, bacteria, and phage poured on top to see phage plaque formation. We made modifications to this method by spotting an aliquot of phage lysate on top of the agar that contained the bacteria instead of including the phages in the molten top layer (Jensen et al., 2015). Different spotting zones were set up in a pinwheel pattern, and 6 phage were spotted per plate. Each *B. licheniformis* isolate was tested against each phage. When left to incubate the bacterial lawn will have clear or slightly clear zones of inhibition if the phage is active against that bacterial strain. Various isolates of *B. licheniformis* were used including 22 wild type and one ATCC strain. This experiment was replicated four times to ensure the lytic nature of the phage against the bacterial isolates.

Testing phage heat resistance and process survivability. Using a UHT/HTST pasteurizer (Lab25 EHVH; MicroThermics), 300mL high titer lysate was added to 2.7 L of raw skim milk (at 8°C). Less than 10 min after inoculation, pasteurization occurred just above the HTST standard of 72°C for 20 s Preheat was set to 50°C and the hold tube at 73°C, the hold tube exit temperatures were an average of 72.3 ± 0.25°C.

Preliminary batch pasteurization data at 63°C for 30 min showed no reduction in phage titer in milk. Subsequent experiments showed that the phage could be inactivated in broth at 70°C in 50 min but a reduction in PFU/ml of 2 logs was shown after 10 min. Lab-scale pasteurization was chosen for the final experiment because it highly mimics the heat exchange of plate heat exchangers used in production facilities, as opposed to a tube in a water bath model which has less efficient heat exchange (Wagner et al., 2018).

Pasteurized milk containing phage lysates were plated in triplicate using the double agar method directly after pasteurization. Samples were taken at 30, 60, and 90 s from the time milk began to exit the pasteurizer. A non-pasteurized control was used to compare phage titer with and without pasteurization. Each phage was run through in duplicate in a randomized order to help prevent bias. After each phage in milk pasteurization run, the lab scale pasteurizer was sanitized using procedures that were outlined in the instruction manual. Samples were taken of the output water after sanitization and tested for phage to confirm that there were none left in the pasteurizer and subsequent experiments would not be contaminated.

RESULTS AND DISCUSSION

Bacterial Isolation

Our techniques identified 71 isolates from the commercial milk powder process as seen in Table 3 below.

Table 3. List of all 71 bacteria identified using 16S RNA sequencing

Organism	Number Identified	Percent total
Bacillus aerius	1	1.41%
Bacillus amyloliquefaciens	1	1.41%
Bacillus atrophaeus	2	2.82%
Bacillus australimaris	2	2.82%
Bacillus cereus	4	5.63%
Bacillus coagulans	3	4.23%
Bacillus halotolerans	2	2.82%
Bacillus haynesii	4	5.63%
Bacillus licheniformis	31	43.66%
Bacillus paralicheniformis	1	1.41%
Bacillus paramycoides	2	2.82%
Bacillus piscis	2	2.82%
Bacillus subtilis	1	1.41%
Bacillus velezensis	3	4.23%
Bacillus wiedmannii	1	1.41%
Bacillus xiamenensis	1	1.41%
Bacillus zhangzhouensis	4	5.63%
Enterococcus faecalis	2	2.82%
Kurthia gibsonii	1	1.41%
Staphylococcus hominis	1	1.41%
Staphylococcus warneri	2	2.82%

Genus distribution: 93% belong to Bacillus, 3% to Enterococcus, 3% to Staphylococcus, and 1% to Kurthia genera

Of the spore-forming bacteria isolated from a commercial dairy ingredient supplier 31 of the 65 *Bacillus* isolates or 47.7% were *Bacillus licheniformis* (Table 3). We estimated that, 42% of total spore-forming bacteria identified in 600 samples of milk were *B. licheniformis*. However, based on the findings of other studies, we were surprised when *A. flavithermus* and *G.*

stearothermophilus were not identified, as these bacteria have been known to form spores in milk powder processes (Dettling et al., 2020). The other species endemic to milk powders from the supplier included: *Bacillus cereus*, *Bacillus haynesii*, *Bacillus zhangzhouensis* and *Enterococcus faecalis*. These results were similar to what was expected as these have been reportedly found in raw milk and are survivors of high temperatures when exposed for brief durations (Porcellato et al., 2018).This percentage of *B. licheniformis* correlates with the studies done by (Li et al., 2020), 31% in raw milk, (Scheldeman et al., 2005), 22% in raw milk, (Miller et al., 2015), 44.9% in nonfat dry milk, 31.9% in WPC-80, 73.5% in acid whey, and 47% in raw milk. Comparing these studies shows that the predominance of an organism as a percentage of the total spore presence is variable and can be impacted by processing conditions at a single location.

Along with location, seasonal environment and climate conditions are a key factor in the spore-forming bacterial populations present in milk and milk powders. According to Ortuzar and others (2018), these two factors account for 56.35% of the heterogeneity. These, however, are not the only known factors. Dettling et al. (2020) suggested that the processing equipment itself, can harbor bacteria causing outgrowth. For example, they reported that the most predominant organism in a skim milk powder (SMP) was *Anoxybacillus flavithermus* at 100% of strains identified. The organism was discovered in sufficient numbers to be enumerated only after a 16 h processing run. This thermophilic spore-forming bacterium was not found in the bulk milk tank raw milk in their study utilizing the same processing plant. Surprisingly, the raw milk contained 74% *B. licheniformis* at hour 0 (Dettling et al., 2020). This strongly suggests that the population of spore-forming bacteria shifts during production, making identifying the bacteria more complex. This is very similar to what we saw in our study. Although we did not test in the same

plant every hour of the day during a production run, we saw differences in the bacteria identified depending on the sampling location. This shows that in many cases one process cannot be directly compared with another. Another factor in the microbial diversity of a process may be regionality, as Dettling took samples from a German milk powder plant while other studies conducted or reviewed during the course of this project, sampled plants in the Americas, including our own study. This lack of *A. flavithermus* and *G. stearothermophilus* could also be attributed to this regional difference.

Phage Isolation

Locations. Based on predominance, we chose *B. licheniformis* as the target organism to search for phages. Despite having taken samples from three dairy farms, a cheese making operation, various milk powder samples from different suppliers, and various silage, grain, and environmental samples; the only location that we isolated *B. licheniformis* phages was on a single dairy farm in central Utah. Of the 25 samples taken from the farm 5 contained phages that lysed *B. licheniformis.*

Table 4. Location of samples where bacteriophages were found

Location	Phage
Exit Alley Manure	A20, C20
Calf Manure	P13
Effluent runoff	P11
Lagoon	A11, C11A, C11B, C11C
Dry-Lot Dirt/Manure	A13, C13

Phage isolates in this study were successfully sourced exclusively in manure or manure byproducts (Table 4). In a different study, Wagner was able to find cheese starter culture bacteriophage in whey powders (Wagner et al., 2017a). In cheese plants, phage contamination is common as phage can become harbored in the processing equipment causing slow ripening and

dead vats of cheese. This necessitates the rotation of various starter cultures to prevent failure. It is not surprising that Wagner found lactic acid bacteria (LAB) phage in whey powders because of their proliferation of the cheese making process. In contrast, this study found no phage in the milk fractionation process where spore-forming bacterial isolates were originally sourced. Phages were also not found in silage, or in raw and pasteurized milk despite our spore-forming bacterial isolates originating from milk. This is because, in contrast with cheese, spore-forming bacteria are not placed in an environment where their growth is encouraged, or even engineered to reach high concentrations to enable milk acidification for cheesemaking. Generally speaking, spore-forming bacteria are taking advantage of the heat shock presented by pre-heating and pasteurization. This enables their growth in milk, a medium that is rich in nutrients but their concentrations starting out are very low (<100 CFU/mL). These low concentrations mean phage present have a very small chance of replicating utilizing these bacteria. Whereas in cheese, the presence of more starter bacteria will result in an increase in probability that a single phage could infect and impact the production process. These phage would then be drained with the whey and survive spray drying to be isolated. That is why phage for *B. licheniformis* were not found in the milk, powders, and silage we tested. In our work, we discovered phages exclusively in manure and manure byproducts most likely due to high bacterial concentrations in those locations.

Phage Characterization

Phage Bioinformatics.

Phage A11 "Holstein", A13 "Guernsey", and A20 were able to be annotated and compared to similar phages in the NCBI database (Appendix 2, Table i). Phage Guernsey and phage A20 were similar enough not to be considered genetically different (similarity >99%). Bacillus phage Holstein and Bacillus phage Guernsey did not have any virulence factors associated with them as found in the virulence factor database (VFDB). They were also found to be of high similarity to both Bacillus phage SIOphi, MAWWA, and TimeGriffin (Table 5); The

Table 5. Average nucleotide identity comparison between phage (%)

Average nucleotide identity	SIOphi	Mawwa	TimeGriffin	Guernsey	Holstein
SIOphi	1				
Mawwa	0.9684	1			
TimeGriffin	0.9689	0.9785	1		
Guernsey	0.964	0.9675	0.9709	1	
Holstein	0.9659	0.9743	0.9735	0.9821	1

lineage for both phage was found to be Viruses; Duplodnaviria; Heunggongvirae; Uroviricota; Caudoviricetes; Caudovirales; Herelleviridae; Bastillevirinae; Siophivirus as compared to the nearest relative through the major capsid protein gene. Table 6 shows a general comparison of between the phages their hosts, and other properties.

Table 6. Phage comparison table

Phage name	Host	Genome Length	GC(%)	ORFs	Known (%)*	Unknown (%)*	Accession #
Holstein	*Bacillus licheniformis*	149932	39.06	232	26	74	NA
Guernsey	*Bacillus licheniformis*	151527	39.07	238	24	76	NA
Mawwa	*Bacillus spp.*	149014	38.98	230	30	70	MW749002
TimeGriffin	*Bacillus spp.*	148525	39.06	235	27	73	MW749007
SIOphi	*Bacillus subtilis*	146698	39.02	206	20	80	KC699836

*known and unknown functions assigned to open reading frames (ORFs) from the analysis

Phage Imaging. TSEM and phage size are shown on Table 7 with the eight phage that were imaged of the ten. Phage C20 and C11B were not able to be imaged. The size of the phage tails varied from 118nm to 199.07nm in length which helps determine its phylogeny. Some of that variation may be due to two of the phage samples only containing phages with retracted tails, thus shortening the tail by approximately 70nm as compared to the other phages (Table 7). The head size of the phages also varied from 87.21nm at the smallest to 98.40nm at the largest. These differences in phage size help to distinguish the phage as having Siphovirus morphology, as shown by the icosahedral heads but larger head size of a Herelleviridae phage (See Images 1-8).

Table 7. Average phage head and tail size (nm)

Phage	Head Size	Tail Length
A11 “Holstein”	97.44 ± 2.5	199.07 ± 3.2
A13 “Guernsey”	94.34 ± 2.9	118.87 ± 3.7*
A20	87.7 ± 6.9	192.57 ± 21
C11A	90.14 ± 10	120.47 ± 18*
C11C	91.70 ± 5.6	179.68 ± 10
C13	94.01 ± 2.7	192.89 ± 10
P11	98.40 ± 2.9	192.34 ± 2.9
P13	87.21 ± 3.9	193.00 ± 10

*indicates no non-retracted tails were found for measurement

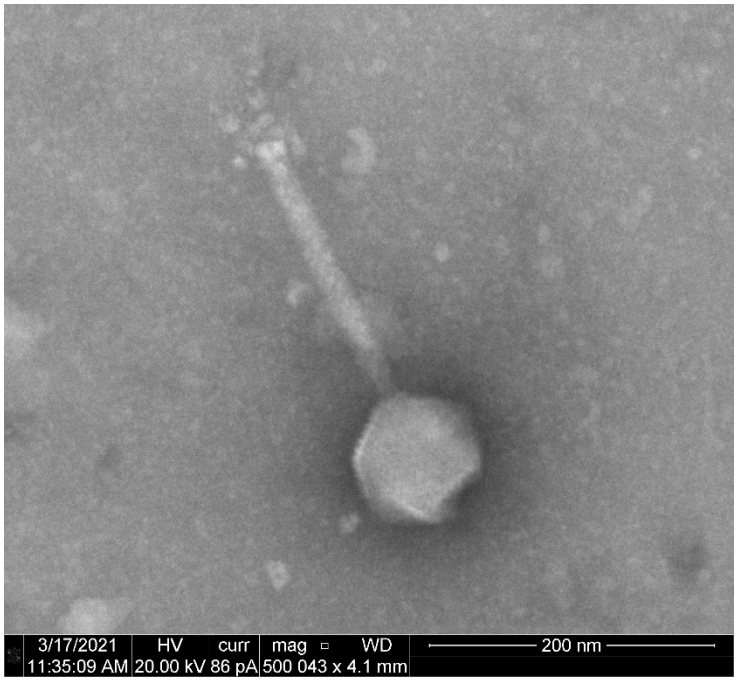

Image 1. Phage A11 “Holstein”

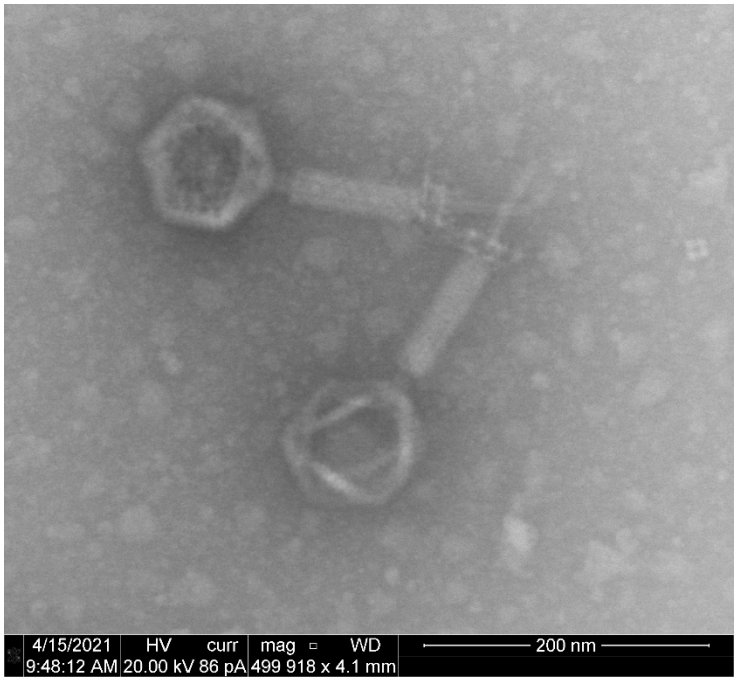

Image 2. Phage A13 “Guernsey”

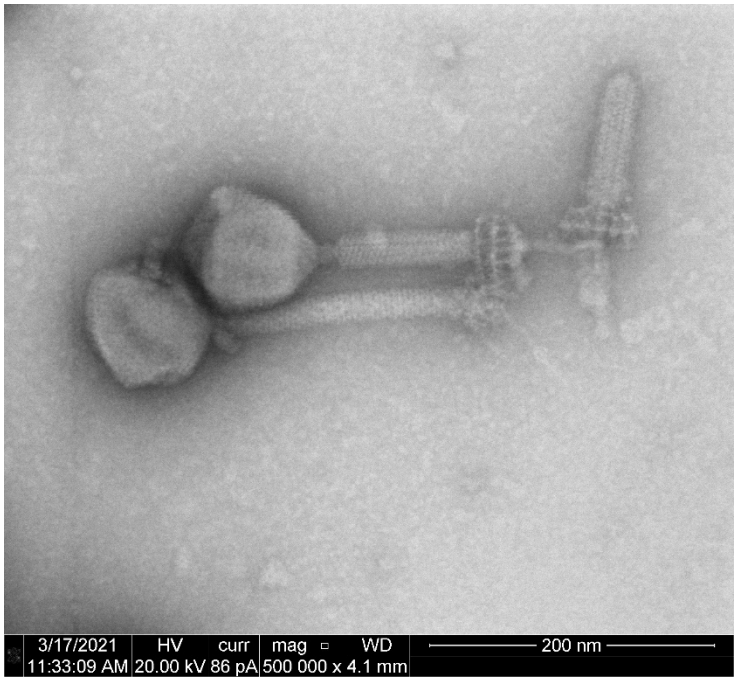

Image 3. Phage A20

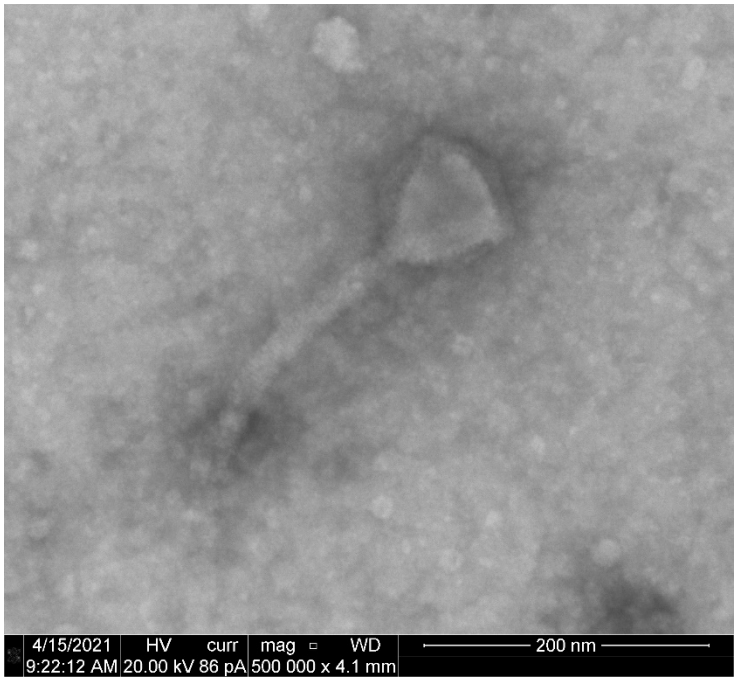

Image 4. Phage C11A

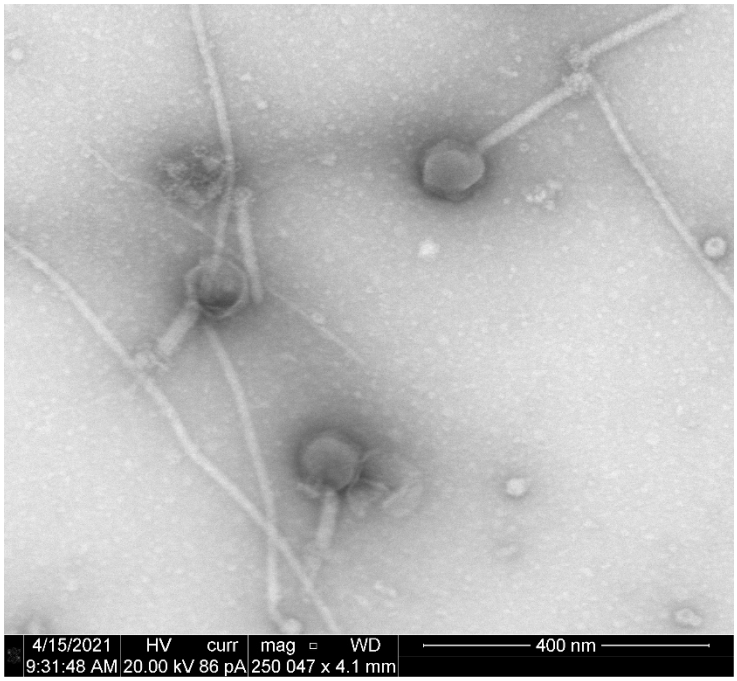

Image 5. Phage C11C

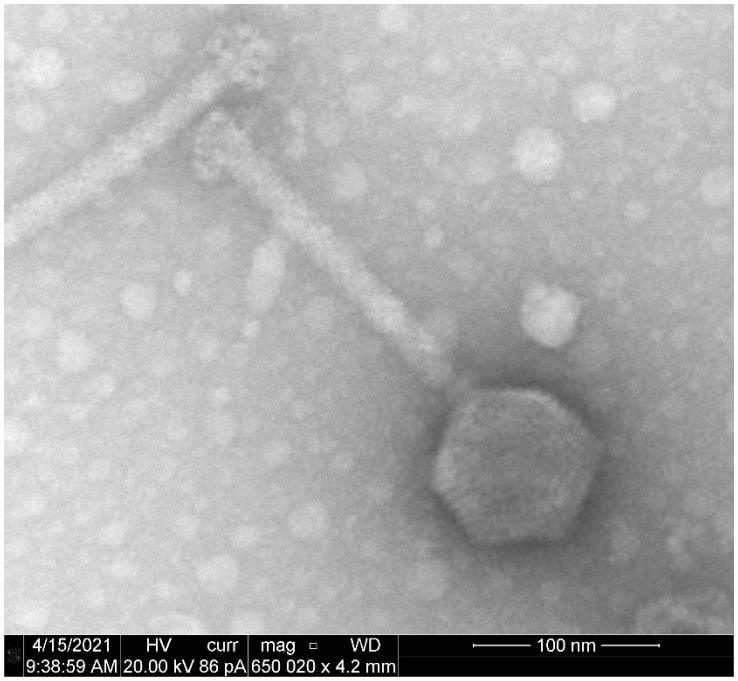

Image 6. Phage C13

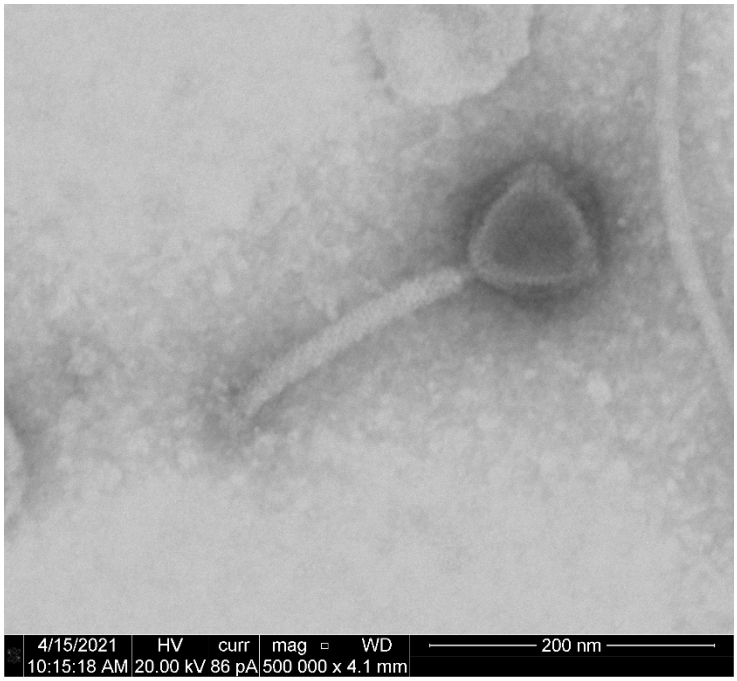

Image 7. Phage P11

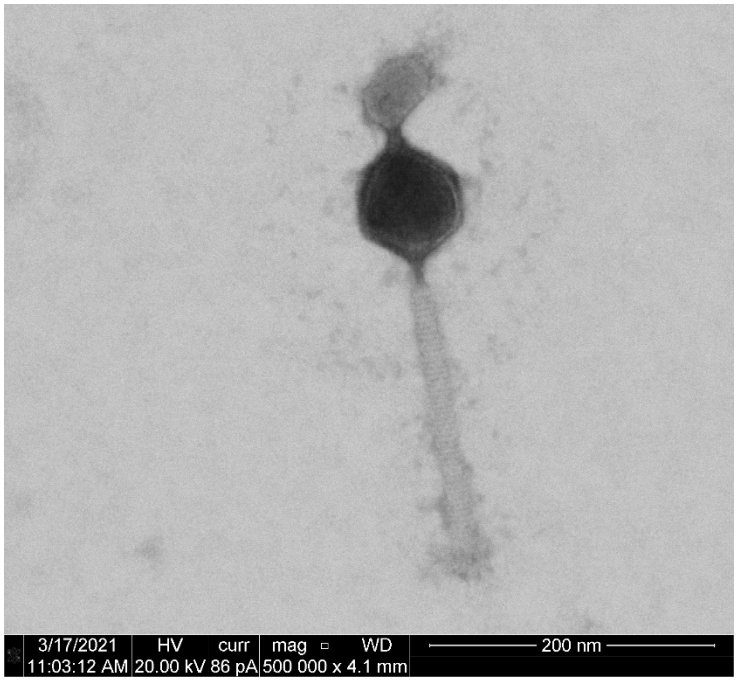

Image 8. Phage P13

Phage Host Range. The host range of the phages was either complete lysis or no lysis at all with the *B. licheniformis* isolates. Only bacterial isolates 2, 30, 35, and 42 exhibited variance in their susceptibility to the different phage isolates. Phage 20A and phage C11A exhibited the highest host range infecting 15 of the 23 isolates. It is interesting to note that ATCC strain 14809 was lysed by all phage as it was our standard *B. licheniformis* reference.

Table 8. Phage host range on Bacillus licheniformis wild type and ATCC strain

	Phage							
Bacterial Isolate number	P11	P13	11A	20A	C11A	C11C	C13	C20
2	+	-	+	+	+	-	-	+-
9	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+
14	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-
21	+	+	+	+	+	+	+	+
25	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-
30	+	-	+	+	+	+	+	+
32	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-
35	+	-	+	+	+	+	-	+
42	-	-	-	+	+	+	-	-
43	+	+	+	+	+	+	+	+
44	+	+	+	+	+	+	+	+
45	+	+	+	+	+	+	+	+
47	+	+	+	+	+	+	+	+
2-22	+	+	+	+	+	+	+	+
2-24	-	-	-	-	-	-	-	-
2-36	-	-	-	-	-	-	-	-
ATCC 14809	+	+	+	+	+	+	+	+

+ indicates that the phage lysed the *Bacillus licheniformis,* - indicates there was no lytic activity on the plate, replicated 4 times

Phage Survival During Milk Pasteurization. Significance was set at a 2-log decrease or more in PFU/ml. All phages showed no significant decrease in titer from plaque counts taken before and after pasteurization. Because of significant variance in the inoculation concentrations of the phage statistical analysis not done. However, the data showed that the phages did not decrease significantly due to pasteurization.

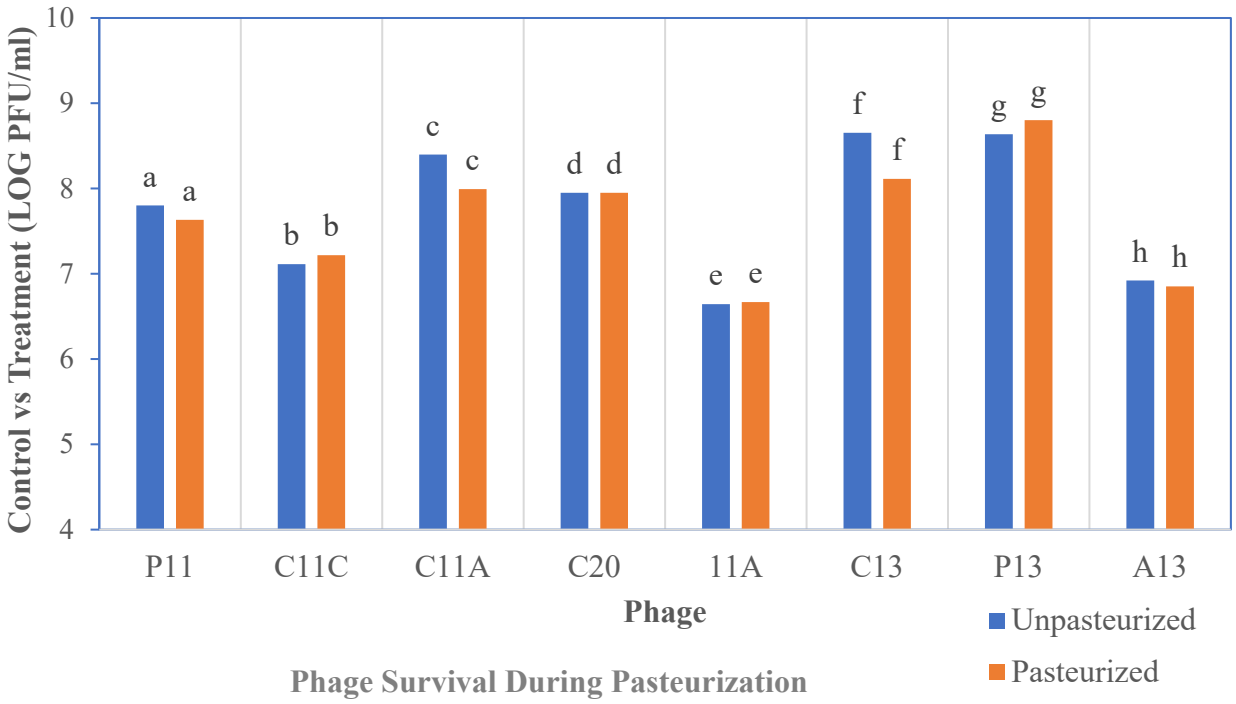

Figure 1. Survival of bacteriophages in unpasteurized and pasteurized milk. The bars represent the average of the runs of unpasteurized vs pasteurized milk inoculated with the different phages which was performed in duplicate. Different letters indicate significant differences only between the same samples.

The main results of our study indicate that the phages of *B. licheniformis* are not inactivated by pasteurization. These results are comparable with those of Chen et al. (2018) who found that *Lactobacillus* virulent phage P2 could survive at 72°C for 20 min in a tube-in bath model before complete inactivation. We expected greater thermal conductivity with use of a lab-scale pasteurizer instead of a tube-in bath method based on the learning from a study by Wanger

and others in 2018. In addition, Wagner's study found that the inoculation medium containing milk may help prevent thermal inactivation of phages (Wagner et al., 2017b; Wagner et al., 2018). For this reason, we used raw skim milk, to simulate the processing environment and test the phage under real world conditions patterned after observed dairy processing practices.

CONCLUSIONS

This was a very important step towards using phages in milk to control spore-former outgrowth. If the phages of *B. licheniformis* did not survive pasteurization, they could not inhibit spore outgrowth. However, because they survive pasteurization, there is a possibility that they can continue to inhibit spore outgrowth during the process. This would not mean that processors that have high spore-count incoming raw milk would be able to process it and sell it as low spore count powders. This means that spore counts could remain steady throughout the process instead of increasing thus becoming out of spec. Many pieces still need to be put together to make this a reality, including: testing the spore-forming bacteria with phages in milk, testing the phages against starter cultures, higher pasteurization temperatures, and a plethora of other tests to receive GRAS status from the FDA. Preliminary data we collected shows promise with a 2.5 log reduction of *B. licheniformis* when held at 50℃ with phages in milk. To continue this research, higher concentrations of phages would need to be assessed, the use of vegetative vs sporulated bacteria, as well as the shear and centrifugal forces the phages could survive before inactivation. Overall, results indicate that the phages would not be inactivated by the heat and would continue to be active in the system.

The heat resistance of the *Bacillus* phage in this study gives hope to the phage of *A. flavithermus* and *G. stearothermophilus* to be able to survive the same conditions. More work

would need to be done to isolate and identify those phages to see if they exhibit the same type of high heat tolerance that the *Bacillus* phages in this study exhibited.

REFERENCES

Ackermann, H. 2009. Basic phage electron microscopy. Pages 113-126 in Bacteriophages. Vol. 501. Basic phage electron microscopy. Humana Press, Totowa, NJ.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology. 215:403-410.

Boor, K. J., M. Wiedmann, S. Murphy and S. Alcaine. 2017. A 100-year review: Microbiology and safety of milk handling. J. Dairy Sci. 100:9933-9951.

Burgess, S. A., S. H. Flint and D. Lindsay. 2014. Characterization of thermophilic bacilli from a milk powder processing plant. J. Appl. Microbiol. 116:350-359.

Chen, X., Y. Liu, S. Chai, J. Guo and W. Wu. 2018. Inactivation of *Lactobacillus* virulent bacteriophage by thermal and chemical treatments. J. Food Prot. 81:1673+.

Cho, T. J. and M. S. Rhee. 2019. Underrecognized niche of spore-forming bacilli as a nitrite-producer isolated from the processing lines and end-products of powdered infant formula. Food Microbiol. 80:50-61.

Dettling, A., C. Wedel, C. Huptas, J. Hinrichs, S. Scherer and M. Wenning. 2020. High counts of thermophilic spore formers in dairy powders originate from persisting strains in processing lines. Int. J. Food Microbiol. 335:108888.

Doyle, C. J., D. Gleeson, K. Jordan, T. P. Beresford, R. P. Ross, G. F. Fitzgerald and P. D. Cotter. 2015. Anaerobic sporeformers and their significance with respect to milk and dairy products. Int. J. Food Microbiol. 197:77-87.

Gopal, N., C. Hill, P. R. Ross, T. P. Beresford, M. A. Fenelon and P. D. Cotter. 2015. The prevalence and control of bacillus and related spore-forming bacteria in the dairy industry. Front. Microbiol. 6:1418.

Hoogwegt Group. 2019. Demand for low-spore milk powders erupts. Horizons. 1-2.

Jensen, K. C., B. B. Hair, T. M. Wienclaw, M. H. Murdock, J. B. Hatch, A. T. Trent, T. D. White, K. J. Haskell and B. K. Berges. 2015. Isolation and host range of bacteriophage with lytic activity against methicillin-resistant *Staphylococcus aureus* and potential use as a fomite decontaminant. PLoS One. 10:e0131714.

Jindal, S. and S. Anand. 2018. Comparison of adhesion characteristics of common dairy sporeformers and their spores on unmodified and modified stainless steel contact surfaces. J. Dairy Sci. 101:5799-5808.

Kong, L., Y. Ding, Q. Wu, J. Wang, J. Zhang, H. Li, S. Yu, P. Yu, T. Gao, H. Zeng, M. Yang, Y. Liang, Z. Wang, Z. Xie and Q. Wang. 2019. Genome sequencing and characterization of three B*acillus cereus*-specific phages, DK1, DK2, and DK3. Arch. Virol. 164:1927-1929.

Li, F., K. Hunt, A. K. Buggy, K. M. Murphy, Q. T. Ho, T. F. O'Callaghan, F. Butler, K. Jordan and J. T. Tobin. 2020. The effects of sequential heat treatment on microbial reduction and spore inactivation during milk processing. Int. Dairy J. 104:104648.

Madigan, M. T., J. M. Martinko and T. D. Brock. 2006. Brock Biology of Microorganisms. 11. ed., internat. ed. ed. Pearson Prentice Hall, Upper Saddle River, NJ.

Martinez, B. A., J. Stratton and A. Bianchini. 2017. Isolation and genetic identification of spore-forming bacteria associated with concentrated-milk processing in Nebraska. J. Dairy Sci. 100:919-932.

Miller, R. A., D. J. Kent, M. J. Watterson, K. J. Boor, N. H. Martin and M. Wiedmann. 2015. Spore populations among bulk tank raw milk and dairy powders are significantly different. J. Dairy Sci. 98:8492-8504.

NCBI, R. C. 2016. Database resources of the national center for biotechnology information. Nucleic Acids Res. 44:D7-D19.

Ortuzar, J., B. Martinez, A. Bianchini, J. Stratton, J. Rupnow and B. Wang. 2018. Quantifying changes in spore-forming bacteria contamination along the milk production chain from farm to packaged pasteurized milk using systematic review and meta-analysis. Food Control. 86:319-331.

O'Sullivan, L., D. Bolton, O. McAuliffe and A. Coffey. 2020. The use of bacteriophages to control and detect pathogens in the dairy industry. Int. J. Dairy Technol. 73:1-11.

Perera, M. N., T. Abuladze, M. Li, J. Woolston and A. Sulakvelidze. 2015. Bacteriophage cocktail significantly reduces or eliminates L*isteria monocytogenes* contamination on lettuce, apples, cheese, smoked salmon and frozen foods. Food Microbiol. 52:42-48.

Pope, W. H., D. Jacobs-Sera, D. A. Russell, S. G. Cresawn and G. F. Hatfull. 2017. SEA-PHAGES Bioinformatics Guide. Howard Huges Medical Institute, Maryland.

Porcellato, D., M. Aspholm, S. B. Skeie, M. Monshaugen, J. Brendehaug and H. Mellegård. 2018. Microbial diversity of consumption milk during processing and storage. Int. J. Food Microbiol. 266:21-30.

Poxleitner, M., W. Pope, D. Jacobs-Sera and V. Sivanathan. 2018. Phage Discovery Guide. Howard Hughes Medical Institute. Howard Hughes Medical Institute, Maryland.

Pujato, S. A., A. Quiberoni and D. J. Mercanti. 2019. Bacteriophages on dairy foods. J. Appl. Microbiol. 126:14-30.

Scheldeman, P., A. Pil, L. Herman, P. De Vos and M. Heyndrickx. 2005. Incidence and diversity of potentially highly heat-resistant spores isolated at dairy farms. Appl. Environ. Microbiol. 71:1480-1494.

Standard Methods for the Examination of Dairy Products. 2004. 17th ed. ed. American Public Health Association, Washington, DC,

U.S. Food and Drug Administration. 2021. Agency response letter GRAS notice no. GRN 000966.2021:.

U.S. Food and Drug Administration. 2018. Agency response letter GRAS notice no. GRN 000757.2020:.

U.S. Food and Drug Administration. 2016. Agency response letter GRAS notice no. GRN 000603.2020:.

U.S. Food and Drug Administration. 2006. Agency response letter GRAS notice no. GRN 000198.2021:.

Wagner, N., E. Brinks, M. Samtlebe, J. Hinrichs, Z. Atamer, W. Kot, Franz, Charles M. A. P., H. Neve and K. J. Heller. 2017a. Whey powders are a rich source and excellent storage matrix for dairy bacteriophages. Int. J. Food Microbiol. 241:308-317.

Wagner, N., S. Matzen, H. Walte, H. Neve, Franz, Charles M. A. P., K. J. Heller and P. Hammer. 2018. Extreme thermal stability of L*actococcus lactis* bacteriophages: Evaluation of phage inactivation in a pilot-plant pasteurizer. LWT. 92:412-415.

Wagner, N., M. Samtlebe, Franz, Charles M. A. P., H. Neve, K. J. Heller, J. Hinrichs and Z. Atamer. 2017b. Dairy bacteriophages isolated from whey powder: Thermal inactivation and kinetic characterization. Int. Dairy J. 68:95-104.

Watterson, M. J., D. J. Kent, K. J. Boor, M. Wiedmann and N. H. Martin. 2014. Evaluation of dairy powder products implicates thermophilic sporeformers as the primary organisms of interest. J. Dairy Sci. 97:2487-2497.

APPENDIX 1

LITERATURE REVIEW

Spore Formation and Germination

The lifecycle of a spore forming cell typically starts in a vegetative state. As the cell prepares for cellular division, if stressed, it might send out a signal to form a spore instead of replicating (sporulation). Asymmetric division of the cell occurs with the smaller side making up the spore core. It contains all the spore needs to germinate later, and the larger side, or the "mother cell," creates protective layers that make the spore resilient to the environment. The next step is the formation of the cortex around the core, which aids in the dehydration and protection of the cell. The production of coat proteins by the mother cell follows cortex formation. This step creates the differences seen in spore resistance properties of different species because of the specific proteins created by the mother cell. Following the protein coating, the cell will mature and become more resistant to the environment (UV radiation, heat, desiccation, etc.). Lysis of the mother cell is the last step; the spore is set free. If it is in favorable conditions it will begin germination, and undergo symmetric cell division after a period of outgrowth to become a vegetative cell again. After symmetric division, if a cell is stressed, the cycle of spore sporulation will repeat (Slepecky and Hemphill, 2006).

Occurrence of Spores in Liquid Milk

Spore-forming bacteria are all around us. They can easily contaminate our food, and they are hard to get rid of because of their extreme resilience to heat, chemical, UV, and pressure treatments (Slepecky and Hemphill, 2006). In liquid milk, many species of bacteria can survive pasteurization, including thermotolerant bacteria that do not form spores (*Streptococcus,*

Lactobacillus, Micrococcus, and Microbacterium) and spore formers such as Bacillus, Paenibacillus, and Clostridia species (Murphy et al., 2016; Boor et al., 2017).

Multiple studies found that spore formers of the genera *Bacillus* and *Paenibacillus* made up the majority of the total thermoduric spore-forming bacteria that survived pasteurization (Huck et al., 2007; Ranieri et al., 2009; Ivy et al., 2012; Ribeiro Junior, J. C. et al., 2018). In addition to surviving pasteurization, biofilms can be created that contaminate liquid milk even further. Biofilms will be discussed in a later section.

In the majority of studies on spore-forming bacteria in raw milk, *Bacillus licheniformis* was the predominant spore found (Scheldeman et al., 2005; Ribeiro Junior, J. C. et al., 2018). Post-pasteurization, spores of the Bacillus spp. dominate, yet, following prolonged refrigeration, Paenibacillus spp. make up the majority of the spore-forming bacteria found in spoiled milk (Ranieri et al., 2009). These are the two species that make up most of the contamination, although there are many more.

Occurrence of Spores in Powdered Milk

The spores commonly associated with powdered milk vary slightly due to regional variation and changes in spore testing parameters (Kent et al., 2016). Most prevalent within milk powders are the thermophilic spore formers, Anoxybacillus flavithermus and Geobacillus stearothermophilus, and the mesophilic *Bacillus licheniformis* (Rückert et al., 2004; Dettling et al., 2019). The origins of these bacteria are thought to be from raw milk (*B. licheniformis*) and the processing environment (A. flavithermus and G. stearothermophilus (Kent et al., 2016; Dettling et al., 2019)). In a study that sampled from 18 different countries, 27 of 28 milk powder samples contained the same strain of *Bacillus licheniformis*, indicating the ubiquity of the

bacteria in the soil and their ability to contaminate milk. In the same study, over 43% of bacteria identified were A. flavithermus, 39% *B. licheniformis*, and 10.8% G. stearothermophilus (Rückert et al., 2004). As with raw milk, milk powders have many more spore-forming bacteria associated with them. Despite this, these are the most prevalent worldwide and were searched for in this study.

Biofilm Presence and Consequences in Dairy Processes

As stated before, biofilms can cause more spore-forming bacteria to be present in the final products of milk processes. Spores are more likely to attach to stainless steel surfaces used in milk processing (Gopal et al., 2015; Ribeiro et al., 2017; Jindal and Anand, 2018). After attachment, they will germinate and continue to reproduce on the surface. Further contamination of dairy products occurs when bacteria slough off the biofilm. In dairy processing, the regeneration section of the pasteurizer and the evaporator are places that have been identified as biofilm harborage sites. The outgrowth of spore-forming bacteria can occur here (Scott et al., 2007; Gopal et al., 2015; Sadiq et al., 2017; Ortuzar et al., 2018). The separator could also be one of those harborage sites, as it is directly after preheating for pasteurization and it has a very large surface area with optimal temperatures for thermophilic outgrowth. Bacteria that can form biofilms in the dairy process are not fully understood, and more research should be conducted to see the effect of co-culture biofilm formation. As stated before, *Bacillus licheniformis* is very prevalent in the biofilms created in milk processes; however, it is debated as to whether it forms biofilms or takes advantage of the biofilms built by other bacteria (Gopal et al., 2015; Sadiq et al., 2017). Thermophilic spore-forming bacteria in the genus Geobacillus and A. flavithermus should also be a focus in the control of biofilms because of their ability to contaminate milk powders at high levels (Sadiq et al., 2017).

Defects Caused by Spores in Milk Products

Some defects attributed to spoilage bacteria that form endospores in liquid milk are fruity, bitter, rancid, or yeasty flavors (Samarzija et al., 2007; Boor et al., 2017). Another defect is sweet coagulation or hydrolysis of the casein micelle and chymosin-like coagulation typically seen in the cheese industry (Samarzija et al., 2007) but occurs because of spore-forming bacteria present in the milk. When butyric acid bacteria spores are in cheese, they can cause undesirable gas production, breakage, off-flavors, and other problems that cause economic losses for cheese manufacturers (Ribeiro Junior, J. C. et al., 2018). The infant formula industry demands high-quality low-spore count skim milk powders (SMP) to produce safe-to-consume infant formula. Currently, tests are done to verify that there are no pathogenic spore formers (Clostridium botulinum, perfringens, Bacillus cereus, etc.). A generalized count of spore-forming bacteria is also taken to gauge the powder quality to prevent unwanted bacterial contamination of infants' digestive tracts. It is safe to say that overall spore-forming bacteria need to be controlled in dairy processes.

Current Control Methods for Spores in Milk on the Farm

Current milking practices include cleaning udders with an iodine solution before and after milking. This helps to prevent infection in the udders and to keep microbial loads in milk low. This practice has proven to lower the bacterial contamination in milk, but inadequate cleaning can occur, which leads to contamination (Galton et al., 1984). Silage fed to cows may impact spore counts and because of increased spore counts in silage, increased counts are then seen in cow feces which can soil the teats and enter the milking process (Scheldeman et al., 2005). Because of this, there have been recent pushes to have low microbial counts in silage, not only for the health of the cows but also for the microbial impact that it has later in the process.

Spore Control Methods in the Plant

Bactofugation is another commonly used practice in the milk industry using centrifugal force to remove vegetative and spore-forming bacteria. Centrifuging the milk helps lower spore counts because the spores are denser than the proteins and sugars present in milk. As the milk is spun, the bacteria are separated from "cleaned milk." This method has proven to lower microbial loads in milk up to 1 log pre-pasteurization (Ribeiro Junior, Jose C. et al., 2019). Refrigeration is the most commonly used control method, and when milk is stored at 8℃ for no more than 72 h pre-pasteurization, it has been found to prevent the increase of *B. licheniformis* by 1 log CFU/ml (Awasti et al., 2019). The overall design of the manufacturing plant directly impacts the control of the spore-forming bacteria. Minimizing places that can harbor bacteria is essential to preventing biofilms that can grow after pasteurization due to spore-forming bacteria. Sanitization practices help decrease the number of spore-forming bacteria but will probably not kill the bacteria if they have already formed a spore. Bacteriophage have been suggested as a control measure for biofilms (Cappitelli et al., 2014; Gopal et al., 2015; Yuan et al., 2018) and for reducing psychotropic organisms during refrigeration pre-pasteurization (O'Sullivan et al., 2020), which could help prevent the formation of spore-forming bacteria before and after pasteurization. A novel approach to controlling spore-forming bacteria in the plant is through "disruptive technology". Thermal cycling has shown in lab models to inhibit the growth of Streptococcus thermophilus when applied in the plate heat exchanger, fluctuating temperature above the pasteurization level to "disrupt" the bacteria's replication cycle. However, this approach needs more study to see if it would also disrupt the formation of biofilms on such equipment (Knight, 2015).

Overview of Bacteriophage

Bacteriophage (phage) are all around us. They are the most prevalent organism on the earth because they are found wherever bacteria are. They are viruses that are specific to bacteria and have both intentional uses and unintentional effects. Some of their desirable uses have included treating bacterial infections and controlling bacterial contamination in food. Some of the most undesirable effects are dead vats – where bacterial cultures are completely wiped out by phage – in food fermentation operations and the genetic transduction of virulent genes to non-virulent bacteria (Parviz M. Sabour, Mansel W. Griffiths, 2010).

Bacteriophage Biology and Life Cycles. The life cycle of phage follows one of two routes, the first being lytic and the second being lysogenic. In the lytic cycle, the phage finds a bacterial host and via very specified receptors, attaches to the cell. This attachment is permanent, and the phage shell or "ghost" will remain there throughout the infection process and after. The step following attachment is penetration, where the phage inserts its sheath into the bacterial cell followed by the injection of the phage DNA. Transcription of the host's DNA follows penetration and is when the phage DNA takes over the host and gets ready for production inside the cell. Biosynthesis follows, which produces the different parts of the phage structure. Maturation occurs as the phage self-assembles the various parts produced by biosynthesis (i.e. the head, tail, and filaments). Lysis occurs when the phage has reproduced to the cell's capacity and sends a signal to release an endolysin – a chemical created to explode the cell – which ensures cell death and phage release. The subsequent burst from the cell propels the phage to seek new hosts so that the process can continue. Lysogenic phage follows this same pattern up until biosynthesis, which does not happen until later in the lysogenic cycle. Instead, the lysogenic or temperate phage transcribe their DNA into the host cell, creating a prophage. This becomes part of the cell and is

copied each time the cell undergoes mitosis. The prophage can exit this cycle and begin biosynthesis when DNA-damaging agents are present or even at random, then the rest of the lytic cycle is followed (Madigan et al., 2006).

Taxonomy. Bacteriophage in the order of Caudovirales all have non-enveloped icosahedron heads, selectively infect bacteria, and can be categorized into families by morphology which includes but is not limited to Siphoviridae, Myoviridae, and Podoviridae. Siphoviridae phage are the most common type of phage isolated at 60%. They have a flexible, non-contractile tail that extends out more than 2x the length of their head. Myoviridae phage are characterized by a contractile tail and make up the 25% of phage studied in literature. Podoviridae are characterized by their short non-contractile tail with a large head and make up 15% of isolations (Parviz M. Sabour, Mansel W. Griffiths, 2010).

Bacteriophage as Biocontrol Tools

The use of phage has recently become a popular subject due to its ability to target and control different types of bacteria. Phage can do many things ranging from therapeutic use on infections, preventing food and waterborne pathogens, or even increasing food and plant yields and quantity. There is very little research for using this ubiquitous organism in food control other than how phage can target specific pathogens. LISTEX (PhageGuard) was approved by the FDA for use on cheese to prevent Listeria contamination. It consists of a 5-phage cocktail that specifically targets *Listeria monocytogenes* (U.S. Food and Drug Administration, 2019). It has also been further approved for use in RTE products across the spectrum that might be susceptible to *L. monocytogenes* infection. A review done by Moye, Woolston, and Sulakvelidze (2018) and another by Lewis and Hill (2020) summarized studies that have applied phage in food. Studies that specifically apply to the dairy industry are summarized in a review by O'Sullivan and others

(O'Sullivan et al., 2020). While both of these reviews focus on the theoretical, there are many approved uses of phage in food, namely, phage that targets pathogenic bacteria like *L. monocytogenes* (U.S. Food and Drug Administration, 2019), E. coli 0157:H7 (U.S. Food and Drug Administration, 2018), and *Salmonella spp.* (U.S. Food and Drug Administration, 2016) that have received GRAS status in the United States.

Milk-specific studies include control of *Pseudomonas lactis* in raw milk, which reduced the microbial load of the milk by approximately 3 logs as compared to a control (Tanaka et al., 2018). It has been suggested that the use of phage in liquid mediums requires less phage and can be more effective because phage is more motile in liquid mediums. Using phage has also been shown to reduce the amounts of *E-Coli spp.* in ultra-pasteurized milk (Moye, et al., 2018). Phage used to reduce *Cronobacter sakazakii* showed significant inhibition of the organism in reconstituted infant formula, although further research would be needed to test the feasibility of phage used in powdered infant formula production (Kim et al., 2007). All these studies suggest that the use of phage to control spore forming bacteria in milk is very possible.

Feasibility of Application of Phages to Control Spores in Dairy Processing

Requirements for a phage to be used in food should be strict. Since phage can only be used to specifically target one pathogenic bacteria, putting together cocktails of phage allows us to eradicate multiple pathogenic bacteria in foods; while leaving the natural flora of bacteria that is beneficial. If possible, cocktails of phages should be used to decrease bacterial phage resistance and target a broader spectrum of bacteria. In addition, purely lytic phage should be used to prevent the transfer of virulence genes or antibiotic resistance (Lewis and Hill, 2020). Phage should also be able to resist the environment it is subjected to. Lactic acid bacteria (LAB) phages have shown to be very resilient to the pasteurization process and have even survived

HTST pasteurization temperatures (Binetti and Reinheimer, 2000; Marcó et al., 2009; Mercanti et al., 2012; Chen et al., 2018; Wagner et al., 2018). Phage can be used on both liquid and solid foods. Liquids can have phage added, while solids need to be sprayed or dipped in phage. The survivability of LAB phage shows promise in phage control of spore forming bacteria because phage could be added in the bulk tank pre-pasteurization and continue to control bacteria throughout the process. Even after spray drying, there is evidence of phage survival in whey powders, which could further control the bacteria after reconstitution and germination of spores (Wagner et al., 2017).

REFERENCES

Awasti, N., S. Anand and G. Djira. 2019. Sporulating behavior of *Bacillus licheniformis* strains influences their population dynamics during raw milk holding. J. Dairy Sci. 102:6001-6012.

Binetti, A. G. and J. A. Reinheimer. 2000. Thermal and chemical inactivation of indigenous S*treptococcus thermophilus* bacteriophages isolated from Argentinian dairy plants. J. Food Prot. 63:509-515.

Boor, K. J., M. Wiedmann, S. Murphy and S. Alcaine. 2017. A 100-year review: Microbiology and safety of milk handling. J. Dairy Sci. 100:9933-9951.

Cappitelli, F., A. Polo and F. Villa. 2014. Biofilm formation in food processing environments is still poorly understood and controlled. Food Engineering Reviews. 6:29-42.

Chen, X., Y. Liu, S. Chai, J. Guo and W. Wu. 2018. Inactivation of lactobacillus virulent bacteriophage by thermal and chemical treatments. J. Food Prot. 81:1673+.

Dettling, A., E. Doll, C. Wedel, J. Hinrichs, S. Scherer and M. Wenning. 2019. Accurate quantification of thermophilic spores in dairy powders. International Dairy Journal. 98:64-71.

Galton, D. M., L. G. Petersson, W. G. Merrill, D. K. Bandler and D. E. Shuster. 1984. Effects of premilking udder preparation on bacterial population, sediment, and iodine residue in milk. J. Dairy Sci. 67:2580-2589.

Gopal, N., C. Hill, P. R. Ross, T. P. Beresford, M. A. Fenelon and P. D. Cotter. 2015. The prevalence and control of bacillus and related spore-forming bacteria in the dairy industry. Front. Microbiol. 6:1418.

Huck, J. R., B. H. Hammond, S. C. Murphy, N. H. Woodcock and K. J. Boor. 2007. Tracking spore-forming bacterial contaminants in fluid milk-processing systems. J. Dairy Sci. 90:4872-4883.

Ivy, R. A., M. L. Ranieri, N. H. Martin, H. C. den Bakker, B. M. Xavier, M. Wiedmann and K. J. Boor. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. Appl. Environ. Microbiol. 78:1853-1864.

Jindal, S. and S. Anand. 2018. Comparison of adhesion characteristics of common dairy sporeformers and their spores on unmodified and modified stainless steel contact surfaces. J. Dairy Sci. 101:5799-5808.

Kent, D. J., K. Chauhan, K. J. Boor, M. Wiedmann and N. H. Martin. 2016. Spore test parameters matter: Mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. J. Dairy Sci. 99:5180-5191.

Kim, K., J. Klumpp and M. J. Loessner. 2007. *Enterobacter sakazakii* bacteriophages can prevent bacterial growth in reconstituted infant formula. Int. J. Food Microbiol. 115:195-203.

Lewis, R. and C. Hill. 2020. Overcoming barriers to phage application in food and feed. Curr. Opin. Biotechnol. 61:38-44.

Madigan, M. T., Brock, Thomas D. Biology of microorganisms and J. M. Martinko. 2006. Brock biology of microorganisms. Brock Biology of Microorganisms.

Marcó, M. B., G. L. De Antoni, J. A. Reinheimer and A. Quiberoni. 2009. Thermal, chemical, and photocatalytic inactivation of *Lactobacillus plantarum* bacteriophages. J. Food Prot. 72:1012-1019.

Mercanti, D. J., D. M. Guglielmotti, F. Patrignani, J. A. Reinheimer and A. Quiberoni. 2012. Resistance of two temperate *Lactobacillus paracasei* bacteriophages to high pressure homogenization, thermal treatments and chemical biocides of industrial application. Food Microbiol. 29:99-104.

Moye, Z. D., J. Woolston and A. Sulakvelidze. 2018. Bacteriophage applications for food production and processing. Viruses. 10:10.3390/v10040205.

Murphy, S. C., N. H. Martin, D. M. Barbano and M. Wiedmann. 2016. Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield? J. Dairy Sci. 99:10128-10149.

Ortuzar, J., B. Martinez, A. Bianchini, J. Stratton, J. Rupnow and B. Wang. 2018. Quantifying changes in spore-forming bacteria contamination along the milk production chain from farm to packaged pasteurized milk using systematic review and meta-analysis. Food Control. 86:319-331.

O'Sullivan, L., D. Bolton, O. McAuliffe and A. Coffey. 2020. The use of bacteriophages to control and detect pathogens in the dairy industry. Int. J. Dairy Technol. 73:1-11.

Parviz M. Sabour, Mansel W. Griffiths. 2010. Bacteriophages in the control of food- and waterborne pathogens.

Ranieri, M. L., J. R. Huck, M. Sonnen, D. M. Barbano and K. J. Boor. 2009. High temperature, short time pasteurization temperatures inversely affect bacterial numbers during refrigerated storage of pasteurized fluid milk. J. Dairy Sci. 92:4823-4832.

Ribeiro Junior, J. C., R. Tamanini, de Oliveira, A. L. M., A. A. Alfieri and V. Beloti. 2018. Genetic diversity of thermoduric spoilage microorganisms of milk from Brazilian dairy farms. J. Dairy Sci. 101:6927-6936.

Ribeiro Junior, J. C., G. A. S. Peruzi, S. R. Bruzaroski, R. Tamanini, C. M. O. Lobo, B. Alexandrino, A. C. M. Conti, A. A. Alfieri and V. Beloti. 2019. Short communication: Effect of bactofugation of raw milk on counts and microbial diversity of psychrotrophs. J. Dairy Sci. 102:7794-7799.

Ribeiro, M. C., M. d. S. Fernandes, A. Y. Kuaye, R. Jimenez-Flores and M. Gigante. 2017. Preconditioning of the stainless steel surface affects the adhesion of bacillus cereus spores. Int. Dairy J. 66:108-114.

Rückert, A., R. S. Ronimus and H. W. Morgan. 2004. A RAPD-based survey of thermophilic bacilli in milk powders from different countries. Int. J. Food Microbiol. 96:263-272.

Sadiq, F. A., S. Flint, L. Yuan, Y. Li, T. Liu and G. He. 2017. Propensity for biofilm formation by aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. Int. J. Food Microbiol. 262:89-98.

Samarzija, D., M. Podoreski, S. Sikora, A. Skelin and T. Pogacic. 2007. Spoilage microorganisms in milk and dairy products. Mljekarstvo. 57:251-273.

Scheldeman, P., A. Pil, L. Herman, P. De Vos and M. Heyndrickx. 2005. Incidence and diversity of potentially highly heat-resistant spores isolated at dairy farms. Appl. Environ. Microbiol. 71:1480-1494.

Scott, S. A., J. D. Brooks, J. Rakonjac, K. M. R. Walker and S. H. Flint. 2007. The formation of thermophilic spores during the manufacture of whole milk powder. Int. J. Dairy Technol. 60:109-117.

Slepecky, R. A. and H. E. Hemphill. 2006. The genus *Bacillus*—Nonmedical. Pages 530-562 in The Prokaryotes: Volume 4: Bacteria: Firmicutes, Cyanobacteria. M. Dworkin, S. Falkow, E. Rosenberg, K. Schleifer and E. Stackebrandt eds. Springer US, New York, NY.

Tanaka, C., K. Yamada, H. Takeuchi, Y. Inokuchi, A. Kashiwagi and T. Toba. 2018. A lytic bacteriophage for controlling *Pseudomonas lactis* in raw cow's milk. Appl. Environ. Microbiol. 84:111.

U.S. Food and Drug Administration. 2018. Agency response letter GRAS notice no. GRN 000757.2020:.

U.S. Food and Drug Administration. 2016. Agency response letter GRAS notice no. GRN 000603.2020:.

US Food and, Drug Administration. 2019. *Listeria*-specific bacteriophage preparation.21 C.F.R. § 172.785:.

Wagner, N., S. Matzen, H. Walte, H. Neve, Franz, Charles M. A. P., K. J. Heller and P. Hammer. 2018. Extreme thermal stability of *Lactococcus lactis* bacteriophages: Evaluation of phage inactivation in a pilot-plant pasteurizer. LWT. 92:412-415.

Wagner, N., M. Samtlebe, Franz, Charles M. A. P., H. Neve, K. J. Heller, J. Hinrichs and Z. Atamer. 2017. Dairy bacteriophages isolated from whey powder: Thermal inactivation and kinetic characterization. Int. Dairy J. 68:95-104.

Yuan, L., F. A. Sadiq, M. Burmolle, T. Liu and G. He. 2018. Insights into bacterial milk spoilage with particular emphasis on the roles of heat-stable enzymes, biofilms, and quorum sensing. J. Food Prot. 81:1651+.

APPENDIX 2

ADDITIONAL TABLES

Table 9 Comparison of identified phage genomes* of phage targeting *Bacillus licheniformis*

Domain Function	Holstein	Guernsey	Mawwa	TimeGriffin	SIOphi
Tail tape measure protein	gp001	gp001	gp001	gp001	gp001
Repressor of ComK	gp002		gp002	gp002	
Peptidoglycan binding protein	gp004	gp004	gp004	gp004	
Tail tube protein	gp005	gp005	gp005	gp005, gp152	gp005
Tail sheath protein	gp006	gp006	gp006	gp006	gp006
Head-to-tail adaptor protein	gp011	gp011	gp011	gp011	
Major capsid protein	gp013	gp013	gp013	gp013	gp013
Prohead protease	gp015	gp015	gp015	gp015	gp015
Portal protein	gp016	gp016	gp017, gp018	gp017	gp016
Helix-turn-helix domain protein	gp020, gp125, gp213, gp214	gp021, gp128, gp131	gp125, gp161, gp211	gp023, gp126, gp215	gp019, gp047, gp112, gp188
Terminase large subunit	gp029, gp032	gp030, gp032	gp032, gp034, gp036	gp033, gp034, gp184	gp029, gp030, gp031, gp032
Group I intron protein	gp031	gp031	gp033		
Acetyltransferase	gp033	gp033	gp037		
N-acetylmuramoyl-L-alanine amidase	gp037	gp037	gp041	gp039	gp036
PhoH family protein	gp038	gp038	gp042	gp040	gp037
3'-5' exoribonuclease	gp040				gp039
Nucleotidyltransferase domain-containing protein	gp041	gp041	gp045	gp043	gp040
Thyamidylate synthase	gp043	gp042, gp043	gp046	gp044, gp045	gp041
Dephospho-coA kinase dephosphocoenzyme A kinase	gp044	gp044	gp047	gp046	gp042
Dihydrofolate reductase subunit	gp045	gp045	gp048	gp047	gp043
Minor tail protein	gp048	gp048	gp051		
Poly-gamma-glutamate hydrolase family protein	gp049	gp049	gp052	gp051	gp048
tRNA His guanylyltransferase	gp062	gp061	gp064	gp066	
Lysis protein	gp128		gp091, gp128		
DNA translocase	gp136	gp137	gp135	gp136	

Domain Function	Holstein	Guernsey	Mawwa	TimeGriffin	SIOphi
Membrane protein	gp137		gp136		
Metalloendopeptidase	gp139			gp140	gp123
Plasmid segregation protein	gp141		gp140	gp142	
tRNA-splicing ligase	gp148		gp147		
Tail spike protein	gp152	gp158	gp151	gp050, gp154	
Holin	gp177	gp167		gp179	
RNA polymerase sigma factor	gp178	gp135, gp137, gp166	gp175, gp207	gp180, gp211	
RNA polymerase	gp178, gp226	gp112, gp135, gp137, gp166	gp174, gp230	gp179, gp235	gp206
RecA-like protein	gp180	gp164	gp177	gp182	gp159
Zinc ribbon containing protein	gp183	gp161	gp180		
UvsY-like recombination mediator	gp184	gp160	gp181		
DNA polymerase I	gp185, gp187, gp188, gp189	gp158, gp159	gp182, gp183, gp184	gp190	gp164, gp165, gp167
HNH endonuclease	gp186	gp143	gp194	gp188	
HU family DNA-binding protein	gp191	gp155			gp169
MLB fold metallo-hydrolase	gp196	gp150	gp192	gp197	
Thioredoxin family protein	gp197	gp149	gp193	gp198	gp174
Ribonucleotide-diphosphate reductase subunit beta	gp198	gp148	gp195	gp199	gp175
Ribonucleotide-diphosphate reductase subunit alpha	gp202	gp142, gp144	gp199, gp200	gp203, gp204	gp177
Ribonucleotide reductase	gp203				
Holiday junction resolvase	gp204	gp140	gp202		
Deoxyuridine 5	gp207		gp205		gp182
DNA primase	gp208	gp136	gp206	gp210	gp183
Recombination endonuclease	gp210	gp134	gp208	gp212	
Metallophosphoesterase	gp211	gp133	gp209	gp175, gp213	gp186
DNAB-like helicase protein	gp212		gp210		
Minor capsid protein	gp215	gp125, gp129	gp213, gp217	gp217, gp221	
Tail protein	gp217, gp228	gp116, gp127	gp215, gp226	gp219	
Baseplate protein	gp218	gp126	gp216	gp222	
Baseplate j-like protein	gp221	gp123	gp219	gp223	gp195
Tail lysosome	gp222	gp122	gp220	gp224	
Peptidase	gp225	gp119	gp223	gp227	
Glycosaminidase domain-containing protein	gp227	gp113		gp234	

Secreted cell wall DL-endopeptidase	gp228	gp114			
Tail fiber 2	gp229			gp231	
Domain Function	Holstein	Guernsey	Mawwa	TimeGriffin	SIOphi
Alcohol dehydrogenase				gp016	
Membrane bound protein			gp025, gp027	gp026	
Half transporter ABCB family				gp064	
Response regulator protein				gp092	
RtcB family protein				gp148	
Exopolyphosphotase				gp177	
Fibronectin type III domain-containing protein			gp173	gp178	
DNA polymerase III subunits gamma and tau				gp183	
DNA polymerase II				gp187	
DNA polymerase				gp189	
Integration host factor like protein				gp193	
Class Ib ribonucleoside-diphosphate reductase assembly flavoprotein Nrdl		gp141	gp201	gp205	gp178
dUTPase				gp209	
Helicase DNAb-like protein		gp132		gp214	
Intein containing helicase/endonuclease protein				gp216	
DUF859 family minor structural protein				gp230	gp201
Tail lysin				gp232	
Intron-encoded nuclease				gp233	
AAA family ATPase					gp185, gp187
Baseplate wedge protein		gp124	gp218		
C40 family peptidase					gp203
DEAD/DEAH box helicase family protein		gp130	gp212		gp189
DNA-binding protein			gp187		
DUF4376 domain-containing protein					gp200
Endo-beta-N-acetylglucosaminidase LytB					gp204
Endonuclease		gp174, gp175	gp167		
Glycosyltransferase			gp203		
Ig-like domain-containing protein					gp053
KID repeat family protein					gp190

Metallopeptidase		gp141	gp138		
Metallophophatase		gp171	gp170		
Domain Function	Holstein	Guernsey	Mawwa	TimeGriffin	SIOphi
Metallophosphoesterase family protein					gp152
Minor tail fiber		gp115			
ParM/StbA family protein					gp125
Phosphodiesterase		gp169	gp172		
Secreted membrane protein			gp118		
ssDNA binding protein			gp178		
XRE family transcriptional regulator			gp022		
YopX family protein					gp139, gp140
Zinc DNA binding protein			gp124		

**All phage numbered genes were adjusted to compare side by side and in the same direction starting with the the tape measure protein and were specific to this analysis*