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Isolation and Characterization of Bacteriophages from Laban Jameed

By

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In Partial Fulfillment of the Requirements for the Degree

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES FROM LABAN JAMEED

By
Murad Mohammad Ishnaiwer

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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Isolation and Characterization of Bacteriophages from Laban Jameed

ABSTRACT

Laban jameed is a dried salty dairy product obtained by fermentation of milk using a complex population of lactic acid bacteria. Jameed is considered a traditional food product in most eastern Mediterranean countries and is usually made from sheep or cow milk. The aim of this study was to assess phage contamination of jameed dairy product. Two phages were isolated; one from sheep milk jameed (PPUDV) and the other from cow milk jameed (PPURV). Each of the two bacteriophages was partially characterized. The PPUDV phage was identified as a single stranded DNA virus with an approximately 20 kb genome that was resistant to RNase, whereas PPURV phage possessed a double stranded RNA genome of approximately 20 kb and was resistant to DNase. The phage bacterial strain hosts were identified as *Lactobacillus helveticus* and *Bacillus amyloliquefaciens* for PPUDV and PPURV, respectively.

One step growth curve using a double layer plaque assay test was carried out to monitor the phage life cycle after host infection. PPUDV showed a latent period of about 36 h, burst period of 70 h and a burst size of about 600 Plaque Forming Units (PFU) per infected cell. PPURV phage showed latent period of about 24 h, burst period of 47 h and a burst size of about 700 PFU per infected cell. Unlike their host strains, both phages could not, however, lyse avian pathogenic *E. coli* that cause respiratory diseases among birds.

SDS-PAGE analysis of total viral proteins showed at least three major bands (27, 40, and 45 kDa) for PPUDV, and two major bands (6 and 46 kDa) for PPURV phage.

This is the first study to report the isolation of both DNA and RNA bacteriophages from Laban Jameed. This study adds new insights into the complexity of dairy contamination and fermentation microbiology of the Laban Jameed revealing the existence of two viral genomes in this dried and salty dairy product.

ملخص بالعربية

عزل وتحديد خصائص نوعين من الفيروسات اكلة البكتيريا من اللبن الجميد

لبن الجميد في فلسطين هو لبن محلي الصنع يصنع عن طريق عملية التخمر للحليب وانتاج حمض اللبن باستخدام بكتيريا (Lactic Acid).

تم عزل نوعان من الفيروسات الاكلة للبكتيريا وتسمى "Bacteriophages". الأول اسمه "PPURV"من منطقة طولكرم شمال فلسطين، والآخر "PPUDV" من منطقة الظاهرية جنوب فلسطين. بعد ذلك تم معرفة نوع البكتيريا المدمرة من قبل الفيروسات وكانت "*Bacillus amyloliquefaciens*" للفيروس "PPURV" والأخرى "*Lactobacillus helveticus*" للفيروس "PPUDV". أيضاً تم دراسة الأحماض النووية للفيروسات وكانت "DNA" للفيروس "PPUDV" نتيجة حساسيتها للأنزيمات الهاضمة "DNase" بحجم (٢٠) ألف قاعدة نيتروجينية، أما الفيروس "PPURV" فله حمض نووي "RNA" نتيجة حساسيته للإنزيمات الهاضمة "RNase" أيضاً بحجم (٢٠) ألف قاعدة نيتروجينية.

تم أيضاً دراسة مراحل اقتحام الفيروسات للبكتيريا، فكانت للفيروسات "PPURV" "PPUDV" على الترتيب؛ المرحلة الأولى -مرحلة الكمون- "Latent Period" استغرقت (24) ساعة، (36) ساعة، المرحلة الثانية –مرحلة الانفجار- "Burst Period" وكانت (47) ساعة، (70) ساعة، أما بالنسبة لحجم الانفجار الناتج من تدمير خلية بكتيرية واحدة فكان (700)، (600) فيروسات لكل من PPURV و PPUDV على التوالي .

علاوة على ذلك تم التعرف على بعض أحجام البروتينات الموجودة على السطح الخارجي للفيروسات باستخدام تقنية "SDS-PAGE"، وكان عددها ثلاثة بروتينات بأحجام (٢٧، ٤٠، ٤٥) كيلودالتون للفيروس "PPUDV"، أما الفيروس "PPURV" فكان له بروتينان رئيسيان بأحجام (٦و٤٦) كيلودالتون.

في النهاية تم اختبار أثر الفيروسات المعزولة على بعض بكتيريا مسببة لأمراض الجهاز التنفسي للطيور ولكن لم يكن لها أي قدرة على تدميرها.

تعتبر هذه الدراسة الأولى التي تعزل فيروسات آكلة للبكتيريا حاملة الأحماض النووية "DNA"، "RNA" من اللبن الجميد الفلسطيني، والتي بدورها تساهم في زيادة المعرفة العلمية تجاه هذه العلاقة المعقدة بين البكتيريا والفيروسات وخصوصاً مع زيادة سلالات البكتيريا المقاومة للمضادات الحيوية، أصبح هناك إمكانية للقضاء على هذه السلالات من خلال فيروسات غير ضارة للإنسان ومتخصصة في قتل البكتيريا.

DECLARATION

I declare that the Master Thesis entitled " **Isolation and Characterization of Bacteriophages from Laban Jameed** " is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Murad Mohammad Ishnaiwer

Date: 15th July 2013

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Murad Mohammad Ishnaiwer

Date: 15th of July 2013

DEDICATION

I would like to dedicate my thesis and express my deepest gratitude and utmost appreciation to my beloved parents, brothers and sisters who gave me supportive words of encouragement and push for tenacity, they gave me whatever I needed, they always pray to Allah for my success.

Also I dedicate this work to my deceased grandfather who always had encouraged me during my study, he wanted me to keep going in my study until getting PhD degree, I wish I could do this later.

Not to forget to give special thanks to my friends, particularly those who joined the master program, they were a lovely and cooperative group, I wish for them all the best.

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ABBREVIATIONS

a. a	amino acid
BLAST	Basic Local Alignment Search Tool
BRC	Biotechnology Research Center
$CaCl_2$	Calcium Chloride
°C	Celsius degree
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides triphosphate
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic Acid
EtBr	ethidium bromide
Fig	Figure
g	Gram
ICTV	International Committee on Taxonomy of Viruses
Kb	Kilo base
KDa	Kilodalton
LAB	Lactic acid bacteria
LB	Luria broth
M	Molarity
µg	Microgram
mg	milligram
min	Minute
µl	Microliter
ml	Milliliter
mM	Millimolar
mw	Molecular weight
NaCl	Sodium chloride
NAD	Nicorinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
ng	Nanogram
OD	Optical density
O/N	Overnight
PCR	Polymerase Chain Reaction
PH	Potentia Hydrogen
PPU	Palestine Polytechnic University
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate

SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
sec	second
SM	Skim milk
ssDNA	Single stranded DNA
ssRNA	Single stranded RNA
TBE	Tris base EDTA buffer
TE	Tetracycline
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
Tm	Annealing Temperature
UV	Ultra Violet
%	percentage
w/v	Weight per volume
-	Negative result
+	Positive result
rDNA	Ribosomal gene

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CHAPTER 1.0 INTRODUCTION

1.1 Laban Jameed Food Products

Laban Jameed is an ancient traditional diet and a form of dried dairy product common in the Middle East, particularly in Syria, Lebanon, Jordan, Saudi Arabia and Palestine. It is also common and favored by Bedouin communities because of the ease of storing this dairy product for a long period of time. It is highly dried and contains high salt concentration, which makes it stable and resistant to pathogenic bacteria **(Alomari et al. 2008; Mazahreh et al. 2008; Al-Saed et al. 2012)**.

Jameed is used in many Arab food dishes, such as Mansaf and is traditionally made by first fermenting milk to yogurt then fording it in special containers made of goatskin. It is mostly made from sheep and/or cow milk. The main biochemical changes that occur in Jameed manufacture is the production of lactic acid from lactose in a fermentation process that relies on the conversion of the milk sugar lactose into lactic acid. It occurs mostly through the action of a highly complex microflora of lactic acid bacteria (LAB), and through continuous shaking, leading to a highly acidic product, formation of butter (curd) and expulsion of the milk serum liquid (whey). The curd is formed by the action of the milk protein, mainly casein. The curd is then slightly heated to accelerate the onset of fermentation process and to further ensure the full separation of whey. The whey is decanted and the curd is usually kept into a cheesecloth container to remove the excess water. When it becomes a thick paste, called Labaneh in Palestine, it is eventually kneaded or sprinkled with sodium chloride salt and placed to dry for few days in the sun to ensure no dampness remain, which could spoil the product **(Alomari et al. 2008).**

1.2 Laban Jameed Phage Contaminations

In most processed dairy products, the fermentation of milk is facilitated by using a mixture of LAB. They are mostly normal flora, but a few show pathogenicity for humans such as, *Streptococcus pneumonia* that cause human sepsis, meningitis, and pneumonia **(Cunningham 2000; Bogaert et al. 2004)**, while *Streptococcus pyogenes* represent the major causative for scarlet fever and impetigo diseases **(Cunningham, 2000)**. *Lactobacilli* species are the most

common microbes comprising 65–80% of the microbial population in dairy industries **(De Antoni et al. 2010)**. Examples of *Lactobacilli* species isolated from dairy products include: *L. acidophilus*, *L. brevis*, *L. casei*, *L. fermentum*, *L. kefir*, *L. parakefir*, *L. plantarum*, and *L. Helviticus* **(Bosch et al. 2006; De Antoni et al. 2010).** Lactic acid bacteria are generally gram positive and acid tolerant and produce lactic acid as a final product of carbohydrate fermentation **(De Antoni et al. 2010; Marco et al. 2012)**. Similar to many other bacterial species, the LAB are affected by several types of bacteriophages and several phage particles have already been isolated and characterized **(Del Rio et al. 2007; De Antoni et al. 2010)**. With the extremely wide dairy product industries, phage contamination is extensively increasing. This contamination is believed to affect the fermentation process and the quality of dairy product **(Haq et al. 2012; Marco et al. 2012)**. Contamination could have originated from different sources, such as water, soil, air, cattle feces, cattle udder and milk equipment **(Alomari et al. 2008; Haq et al. 2012)**. The phage contamination in dairy products becomes problematic to dairy industries even with a minute amount of phage particles due to phage ability to rapidly increase its numbers once a bacterial host is available. To add to this problem, phages usually tolerate high acidity and high temperatures during the pasteurization process. Phages can cause a collapse of the lactose-lactic acid pathway and decreases the overall efficiency of the fermentation process. These problems are detrimental to dairy product quality and often make the food products susceptible to more pathogenic and spoilage bacterial invasion, which can further hurt the fermentation process (**Alomari et al. 2008; Marco et al. 2012)**.

Several phages have been found to parasitize on LAB strains (**Mills et al. 2011; Marco et al. 2012)**. Most LAB known phages are tailed and members of the Caudovirales order **(Mills et al. 2011)**.

1.3 Bacteriophages

Bacteriophages are viruses that specifically parasitize bacteria; they are small obligatory agents that carry out their replication and metabolism only in their bacterial host cells. In 1915 Frederick W. Twort reported degenerative transparent changes in *Staphylococcus* cultures from calf lymph. In 1917, Felix d'Herelle named these particles bacteriophages (derived from the Greek word "phage", which means eat i.e., bacteria eaters). Microscopically, phages were

invisible and with ambiguous nature, thought to be an infection that killed bacteria **(Duckworth, 1976)**. In 1971, the International Committee on Taxonomy of Viruses (ICTV) classified bacteriophages according to their entities under the electron microscope to six phage groups: 1) T-even phages, 2) λ phages, 3) lipid phages PM2, 4) the fX group, 5) filamentous phages, and the 6) ribophages group **(Ackerman, 2011; ictvonline.org)**. Nowadays, 12 families were described by the ICTV according to phage morphology and nucleic acid composition **(Ackerman, 2011; ictvonline.org)**. They comprise either double strand (ds) or single strand (ss) DNA or RNA genomes, though mostly they belong to Caudovirales order with dsDNA nucleic acid genomes (**Table 1.1**) **(ictvonline.org)**.

Bacteriophages exist wherever bacteria could grow like in water, soil, feces, etc., and represent the most abundant particles on earth **(Deschavanne et al. 2010)**. Diversity is due in part to the dynamic ability of phages to cope with extreme conditions with highly competent mechanisms **(Deschavanne et al. 2010)**. Although bacteriophages are quite diverse in their genome size, host range, and nucleic acid and protein composition, in general, there are two main categories recognized relative to their growth cycle. Bacteriophages can be either virulent (lytic phages), which usually rapidly burst the host cell and kill it, or temperate (lysogenic phages) that can coexist with the host genome, or directly shift to lytic cycle and kill the host cell **(Kowalczykowski, 1991; Turn and Trempy 2003; Sturino and Klanhammer 2006)**.

Table 1.1. List of the most common bacteriophage families and their shapes as shown by electron microscopy studies and approved by the ICTV. The PPUDV and PPURV bacteriophages are possibly belong to the Microviridae (circular ssDNA nucleic acid with possible non-enveloped and isometric shapes) and/or Inoviridae (circular ssDNA nucleic acid with possible non-enveloped and filamentous shapes) for PPUDV and Cystoviridae for the dsRNA (segmented nucleic acids with possible enveloped and spherical shapes) for the PPURV. The three families are highlighted with red **(Ackerman, 2011; ictvonline. org)**.

Shape	Order / Family	Morphology	Nucleic acid
	Caudovirales	Talied ,nonenveloped	dsDNA
	*Myoviridae*	Nonenveloped, contractile tail	Linear dsDNA
	*Siphoviridae*	Nonenveloped, noncontractile tail (long)	Linear dsDNA
	*Podoviridae*	Nonenveloped, noncontractile tail (short)	Linear dsDNA
	Clavaviridae	Nonenveloped, rod-shaped	Circular dsDNA
	*Corticoviridae*	Nonenveloped, isometric	Circular dsDNA
	*Cystoviridae*	Enveloped, spherical	Segmented dsRNA
	Guttavirus	Nonenveloped, ovoid	Circular dsDNA
	Inoviridae	Nonenveloped, filamentous	Circular ssDNA
	Leviviridae	Nonenveloped, isometric	Linear ssRNA
	*Microviridae*	Nonenveloped, isometric	Circular ssDNA
	*Plasmaviridae*	Enveloped, pleomorphic	Circular dsDNA
	*Tectiviridae*	Nonenveloped, isometric	Linear dsDNA

1.3.1 Bacteriophage basic structure

Bacteriophage genome sizes are extremely variable ranging between 2,435 bp (*Leuconostoc* phage L5) and 497,513 bp (*Bacillus* phage G) **(Deschavanne et al. 2010; Hatfull, 2010)**.

Bacteriophage genomes are enclosed in a protein or lipoprotein coat called capsid (Head) that could be either linear or circular **(Turn and Trempy 2003)** (**Table 1.1**). Bacteriophage basic structure is shown in **(Figure 1.1)**. Some phages are enveloped and contain lipid bilayers surrounding their capsids that can be spike-like shapes and are considered important for the virus penetration into the host cell. It is believed that no infections could occur for enveloped viruses without those layers. It helps in phage attachment to their host cell surface. The non-enveloped viruses do not have any layers surrounding their capsids and their capsids are solely responsible for phage–host attachment **(Turn and Trempy 2003)**. Some bacteriophages contain hair like structures called spikes that are used for phage attachment (**Figure 1.1**). In addition, they contain tails that might have specific receptors used for phage

attachment to the host and collar-like structures through which the genomes are inserted into the host cells **(Turn and Trempy 2003)**.

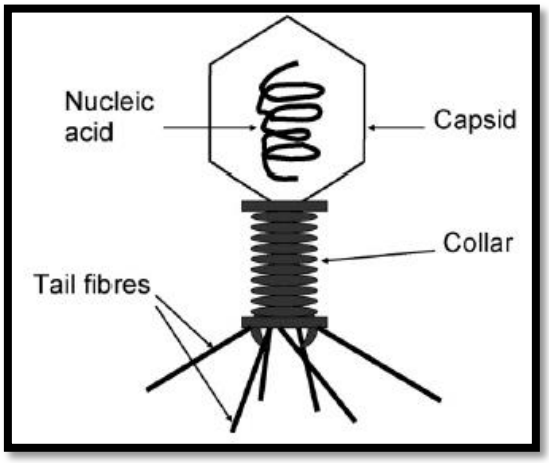

Figure 1.1 The basic structure of bacteriophages. The tail contains specific receptors used for phage attachment to the host and the collar is used to insert the viral genome into the host cells **(Turn and Trempy 2003)**.

1.3.2 Bacteriophage growth curves

To monitor bacteriophage life cycles following infection, the one step growth curve is usually constructed (**Moce-Llivina et al. 2004**).

It comprises an agar layer at the plate surface, overlaid with another soft layer (0.7%) of agar containing phage-bacterial suspension. The phage-bacterial suspension is first incubated at 37 ^{o}C to ensure phage adsorption to bacteria, then the soft agar is added in a sterility conditions to avoid any other microorganism contaminations. The latent period, burst period and burst size are then determined.

The latent period illustrates the time period from the adsorption of phage to the host bacteria, until the onset of cell lysis and burst. Host cells rapidly lyse and release infective phage at burst period, occurring after the latent period. The average number estimated for phage progeny to be formed per infected bacterial host cell symbolizes the burst size (**Figure 1.2**).

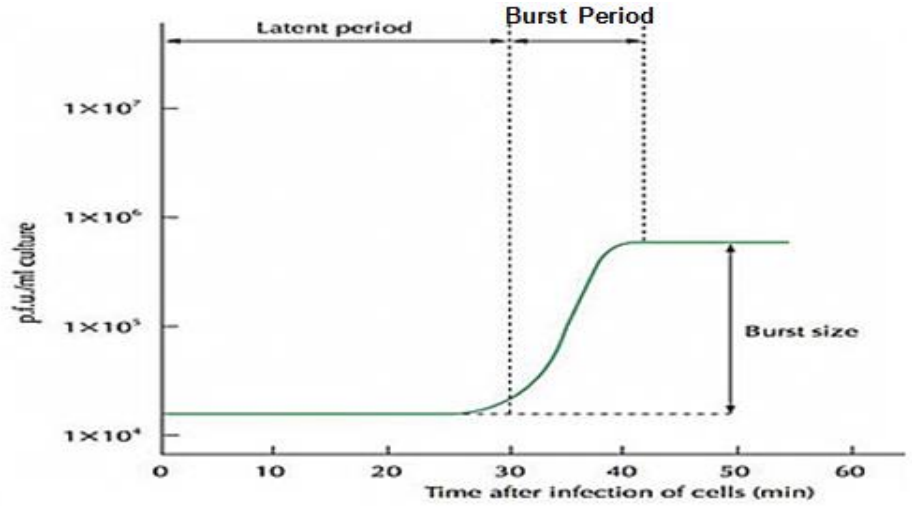

Figure 1.2. **An example of one step growth curve**. It shows the different phases of the bacteriophage infection to host bacteria. The X axis represents the number of plaque forming units against time after infection on the Y axis (**Cann, 2005).**

1.3.3 RNA / DNA bacteriophages

In relative to nucleic acid composition, phages can be double stranded (ds) or single stranded (ss) DNA or RNA genomes enclosed in their capsids **(Turn and Trempy 2003)**. Treatment of purified phage genomes with DNase and RNase allows the differentiation between RNA and DNA bacteriophages based on their genome sensitivity to digestion by these enzymes. This method has allowed the identification of both RNA and DNA bacteriophages in dairy products. In addition, there are protocols available to differentiate between ds and ss genomes. For RNA genomes, the most widely used method to differentiate between ds or ss RNA genomes is by treating them with the Ribonuclease A **(Edy et al. 1976; Morris and Dodds 1979; Chu and Westaway 1985; Westaway et al. 1999; Kuznetsov and Mcpherson 2006; Targett et al. 2008; Ablasser et al. 2009; Dayer et al. 2012)**. At low salt concentration, Ribonucleases A break down both single and double stranded RNA, while they degrade only ssRNA at high salt concentration **(Edy et al. 1976; Morris and Dodds, 1979; Chu and Westaway 1985; Westaway et al. 1999; Kuznetsov and Mcpherson 2006; Targett et al. 2008; Ablasser et al. 2009; Dayer et al. 2012)**.

The Ribonuclease A is a pancreatic enzyme that is frequently used to catalyze the degradation of both ssRNA and dsRNA at low salt concentrations **(Dayer et al. 2012)**. It was shown that the Ribonuclease activity is affected by the concentration of some salts, like NaCl,

reaching an optimum activity at 0.1 M concentration **(Dayer et al. 2012)**. The Ribonuclease activity declines under extremely high or low salt concentrations. It is proposed that a conformational change in the enzyme structure enables the Ribonucleases to act on double-stranded RNA at low salt concentration, or protect them from degradation at high salt concentration **(Edy et al. 1976; Nosek et al. 1993)**. The mechanism by which salt affects RNase A activity is still not completely understood, but it is speculated that it occurs through affecting RNA binding to the RNase active site and active site arrangement, or by reducing the disulfide bonds at very high salt concentration of 0.4 M. At low salt concentration, a conformational change occurs affecting the active site normal folding, thus causing a decline in the susceptibility of the enzyme-substrate interactions **(Edy et al. 1976; Dayer et al. 2012)**.

In contrast, ds and ssDNA could simply be differentiated by heating genome above melting point, the temperature at which the dsDNA dissociate into ssDNA. Heating causes unwinding of the dsDNA and denaturing of the ds to ssDNA through the breaking of hydrogen bonding between the bases and their structure became more fragile. The ssDNA migrate in agorose gels faster than dsDNA due to their small size. Thus, through monitoring genome migration in gel after and before heating, it is possible to detect the entity of DNA genomes whether ds or ss **(Thomas, 1993)**. Also sets of dsDNA restriction enzymes like *EcoR*I and *BamH*I could be used to confirm the ssDNA resistance and dsDNA sensitivity to them **(De Antoni et al. 2010; Yang et al. 2010; Ownes et al. 2012)**. Another assay that could be used to identify the entity of genomes is the Hyperchromicity test. It demonstrates that ssDNA will heighten the UV absorbance (Hyperchromic), while dsDNA tighten it (hypothermic). The explanation is that hydrogen bonds that attract dsDNA bases become un-stacked and the aromatic ring of their bases declines after denaturation, which leads to a decrease in the UV absorption, while in ssDNA the bases freely exist and no need for hydrogen bonds and therefore the UV absorption is increased **(Edy et al. 1976; Morris and Dodds 1979; en. wikipedia. org)**.

1.3.4 Lytic and lysogenic cycles of bacteriophages

Bacteriophages are viruses that attack their hosts once they detect specific surface receptors. Following attachment, they inject their genomes inside the host cells and then pursue either lytic or lysogenic life cycles **(Figure 1.3).**

In the lytic cycle, phages rapidly lyse the host cell, producing multi phage progeny that, once they are out of the cell, infect other cells. Typically, phages that follow this route are called virulent viruses. If the phages follow the second route, i.e., the lysogenic cycle, they incorporate their genomes within the bacterial chromosome forming what is called a prophage. In this route, the phage has the advantage of replicating its genome whenever the bacterial cell divides, although they might suddenly shift to the lytic cycle and regenerate a new progeny **(Kowalczykowski, 1991; Mills et al. 2011)**.

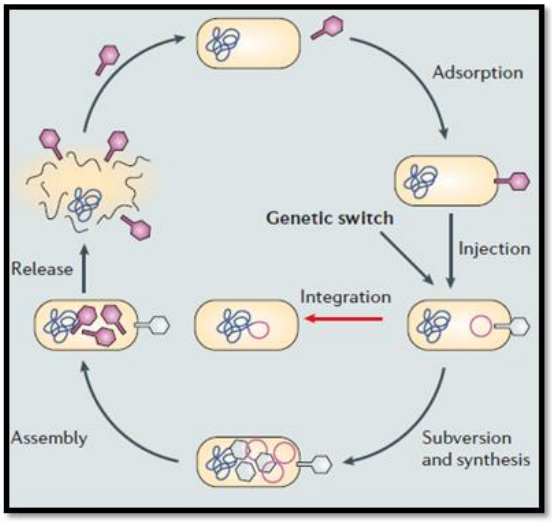

Figure 1.3. Mechanism of bacteriophage lytic and lysogenic cycles. After bacterial infections, bacteriophages either replicate and kill bacteria through lytic cycle or incorporate their genomes as prophage within bacterial chromosome **(Sturino and Klanhammer 2006)**.

1.3.4.1 Lytic cycle

In the lytic cycle, various steps are maintained during phage propagation. Once phages detect their host receptors, they adsorb to the cell surface and inject their genomes **(Kowalczykowski, 1991; Mills et al. 2011)**. Inside the cell, sets of early proteins encoded by the phage genome and needed for phage replication begin to be expressed using bacterial ribosomes, tRNA, amino acids, and other needed metabolites, yielding large number of copies of phage genomes **(Kowalczykowski, 1991; Mills et al. 2011)**. A second round of proteins is then expressed and forms the main phage components, such as capsids and tails that allow the assembly of hundreds of new progeny. Eventually, during the burst period, phages escape the host cells by weakening the cell wall via specific phage lysozymes encoded by their genome

(**Figure 1.3**). Bacterial lysis results in circular plaques that can be counted in order to assay for host cell burst. The average yield of phages per infected bacterial cell is known as burst size **(Figure 1.2) (Kowalczykowski, 1991; Sturino and Klanhammer 2006; Mills et al. 2011)**.

1.3.4.2 Lysogenic cycle

In contrast to phages following the lytic route, temperate phages could multiply in two directions once they infect their host. They can be either lysogenic or, as previously discussed, follow the lytic cycle **(Figure 1.3)**. In lysogenic cycle, phages reproduce their genetic material without killing the host cell. This requires that the lytic genes being repressed and the phage genomes integrate into the bacterial DNA within specific host chromosome sites and called a prophage. Phage genome then replicates with the subsequent propagation cycles of the host bacteria. At any time, the prophage may leave the host chromosome and enter a lytic cycle breaking down the host chromosome and killing the bacteria **(Figure 1.3)** (**Kowalczykowski, 1991; Sturino and Klanhammer 2006; Mills et al. 2011**).

Temperate phages, once in prophage inside the host cell, are capable of shifting mechanisms and entering the lytic cycle. This mechanism was clearly demonstrated in λ phage model **(Turn and Trempy 2003)**. When the genome enters the host cytoplasm, it becomes circularized and supercoiled to be protected from the host's degradative enzymes. The phage genome encodes integrase enzyme (*int*) that catalyzes the recombination between the phage and the bacterial genome attachment sites *attp* and *attB*, respectively. It also uses the bacterial integrase host factor enzyme (*IHF*) to maintain recombination (**Turn and Trempy 2003**). As a result, the phage genome is bounded to the chromosome by hybrid of phage *attp* and bacterial *attB* sites called *attL* and *attR* sites and forms a linear prophage **(Turn and Trempy 2003)**.

Temperate genomes could suddenly enter the lytic cycle, causing excision of prophage from the bacterial chromosome before destroying it. The directionality between prophage integration or excision is maintained through the phage encoded excision enzyme (*Xis*) **(Figure 1.4)**. In the presence of *Xis*, the phage and bacterial integrase enzymes, (*int*) and (*IHF*) are only able to perform phage genome excision from bacterial chromosome, while in the absence of *Xis*, they catalyze the phage genome integration **(Turn and Trempy 2003)**.

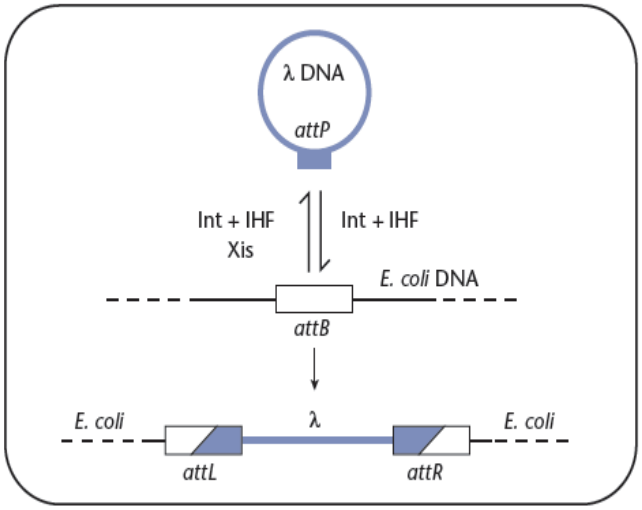

Figure 1.4. Directionality of temperate phage genomes. The integration of phage genomes in chromosomal DNA or excision from the chromosomal DNA following is maintained by specific enzymes **(Turn and Trempy 2003)**.

1.3.4.3 Decision between Lytic or lysogenic cycles

When temperate phages inject their genomes inside the host cell, they have choices between lytic and/or lysogenic cycles. The mechanism by which decision is taken has been extensively studied, particularly on λ phage model **(Bakk et al. 2004) (Figure 1.4)**. Once the genome is integrated into the host cell, it becomes supercoiled and enhances the onset of transcription of early genes from either Promoter Rightward (*PR*), or Promoter for Repressor Maintenance (*PRM*). The *PR* and *PRM* are two major promoters located at the phage genome. *PR* leads to lytic while *PRM* to lysogenic cycle. Briefly, it depends on the adhesion of specific phage gene regulatory proteins (CI) and (Cro) to a cascade of specific genome promoters. The Cro leads to lytic and the CI leads to lysogenic route and the alteration between the two cycles depends on the activation or repression of each other. Genome promoters contain a 17 base pair sequence operators (*OR1, OR2,* and *OR3*), in which the Cro protein binds to these operators leading to the activation of the lytic *PR* promoter and inhibition of lysogenic *PRM* **(Bakk et al. 2004).** If the CI protein binds to these operators, it leads to the activation of the lysogenic *PRM* promoter and the repression of the lytic *PR* promoter, leading to the lysogenic cycle (**Figure 1.5**) **(Bakk et al. 2004)**.

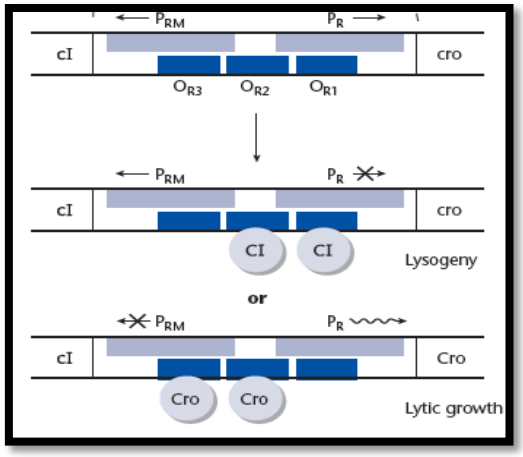

Figure 1.5. Pathways that determine bacteriophage decision for lytic or lysogenic cycles. The attachment of Cro and CI to *PR* lytic promoter or *PRM* lysogenic promoter plays a significant role in determining the route bacteriophages will take **(Bakk et al. 2004)**.

1.4 Bacteriophage replication

Phage replication flows through a series of steps, beginning with phage adsorption (attachment), genome injection, and phage assembly. During phage attachment, specific phage structures or receptors at their tails or capsids recognize specific host receptors that usually contribute to other cell functions. Examples include receptors, such as lipopolysaccharides and peptidoglycans (**Mills et al. 2011**). The λ bacteriophage recognizes a specific host surface protein called LamB through specific J protein at their tails **(Turn and Trempy 2003).** Some phages encode hair-like spikes at their capsids for their attachment. Thereafter, if a specific receptor is successfully detected, phages integrate their nucleic acids inside the cell leaving the empty protein coat outside. Once they are inside, they use the host machinery to reproduce their own particles needed for assembly of new phage progeny (**Crighton, 1999; Leclerc, 2002)**. Accordingly, Phage replication is slightly different between ss, ds RNA or DNA phage genomes (**Crighton, 1999; Leclerc, 2002; Haq et al. 2012**).

In ssDNA, as in M13 bacteriophages, they recognize specific target at the bacterial hair like F pilus, and often contain positive strand genomes which act as a substrate for the synthesis of complementary negative strands (-ve) (**Crighton, 1999; Leclerc, 2002; Haq et al. 2012**). The -ve strand acts as a template for mRNA, the virus replication is carried out via host enzymes and replication then precedes using specific RNA primers that enhance the negative complementary chain. Elongation is carried out using bacterial DNA polymerase.

The ligation of duplex circular dsDNA, termed as replicative form, is then maintained by bacterial repair enzymes through stabilizing the phosphodiester bonds between strands. The dsDNA is translated into phage coat proteins, tails and other structural proteins when sufficient levels of phage proteins are produced. Specific phage proteins block the positive strand and inhibit the complementary strand formation, the dsDNA replicative form is then converted into positive ssDNA which then incorporates into the phage during assembly **(Crighton, 1999; Leclerc, 2002; Turn and Trempy 2003)**.

In the RNA phage replication mechanism, dsRNA is first transcribed to mRNA by RNA polymerase followed by translation using the host ribosomes. It produces early phage structural proteins that enhance the formation of the replicative form of the dsRNA (**Crighton, 1999; Leclerc, 2002**). Transcription is enhanced using host RNA-dependent RNA polymerase (RdRp), which yields multiple copies of +ve strands using -ve strands as templates before being translated into early structural proteins (**Crighton, 1999; Leclerc, 2002**). Finally, a mature dsRNA phage progeny is formed through forming other +ve strands that are translated to late phage assembly proteins **(Leclerc, 2002).**

The ssRNA phages mostly infect F pilus strains of bacteria since their recognition site is at the pilus **(Crighton, 1999; Leclerc, 2002)**. The +ve strand acts typically as mRNA, which is translated to phage structural proteins, such as capsid, tail and RNA polymerase or RNA replicase which is used to replicate more +ve strands through copying the ssRNA strands **(Crighton, 1999; Leclerc, 2002)**. Finally the polymerase and the major capsid proteins assemble phage progeny surrounding the +ve ssRNA strand. The -ve ssRNA cannot serve as an mRNA template. It has to be copied into +ve strand and then proceed similar to the +ve strand pathway.

1.5 Bacteriophage resistance mechanism

Lactic acid bacteria have adopted and modified a variety of anti-phage defense mechanisms to avoid phage infections. Such mechanisms are mostly observed against dsDNA phages **(Labrie et al. 2010; Garneau and Moineau 2011; Mills et al. 2011)**. Phages, however, utilize several circumventions that are reported to suppress those mechanisms **(Labrie et al. 2010; Garneau and Moineau 2011; Mills et al. 2011)**. The most common bacterial anti-phage defense mechanisms are developed to suppress phage adsorption, DNA injection and

recruitment of restriction modification systems. Accordingly, bacteria use specific proteins that mask the phage recognition receptor site located on the bacterial cell surface or even invoke a conformational change, which mislead phages from their attachment point **(Labrie et al. 2010; Garneau and Moineau 2011; Mills et al. 2011)**. In addition, bacteria can alternatively mask their receptors through secreting specific sugar residues called exopolysaccharides, which help in inhibiting phage adsorption. Phages, however, can secrete specific polysaccharide degrading enzymes called lyases that degrade those bacterial exopolysaccharides **(Labrie et al. 2010; Garneau and Moineau 2011; Mills et al. 2011)**.

Moreover, some LAB generate bacteriophage-insensitive mutants (BIMs) in which a cascade of phage mutations results in the alteration of recognition sites that inhibit bacteriophage adsorption **(Labrie et al. 2010; Garneau and Moineau 2011; Mills et al. 2011)**. Such point mutations reported in chromosomal genes coding for *Lactococcus* cell receptors **(Labrie et al. 2010; Garneau and Moineau 2011; Mills et al. 2011)**. Some mutant phages could, however, overcome this modification and infect those resistant bacteria **(Mills et al. 2011; Labrie et al. 2010)**. In some cases, if bacteria couldn't inhibit phage adsorption, they instead secret other specific proteins that affect genome translocations to the cytoplasm through changing the injection site and blocking the cell wall degradation **(Labrie et al. 2010; Garneau and Moineau 2011; Mills et al. 2011)**. Otherwise, if phage genomes are able to adapt to these challenges and successfully pass through the cytoplasm, bacteria recruit other further defense mechanisms termed as restriction–modification systems to degrade unmethylated genomes at specific sites **(Labrie et al. 2010; Mills et al. 2011)**.

1.6 Bacteriophages as alternatives to antibiotics

Bacteriophages are viruses that specifically kill bacteria. During the 1920s, researchers from Britain and the former Soviet Union tried to find out the possibility of using phages in therapy for the treatment of some bacterial infections. It was first tested on *Vibrio cholera* (bacteria that cause cholera disease), followed by other trials to treat dysentery bacteria **(**cited in **Sulakvelidze et al. 2001; Mathur et al. 2003)**. Early studies concluded that phage therapy was not sufficiently effective to treat bacterial infections. Thereafter, the idea of phage therapy received little attention due to the discovery of antibiotics and also because phage biology and phage quality control preparations were poorly understood.

In 1982, however, William Smith described effective trials to treat *E. coli* infections in mice using phages **(**cited in **Sulakvelidze et al. 2001; Mathur et al. 2003)**. Several other studies also described the efficiency of phages in the treatment of human bacterial diseases. For example, 74% phage treatment success was reported in treating human skin ulcers that are caused by *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Proteus*, and *E. coli* bacteria (cited in **Sulakvelidze et al. 2001; Mathur et al. 2003),** phages were also found effective in treating newborn cerebrospinal meningitis **(**cited in **Sulakvelidze et al. 2001)**.Furthermore, a study reported the ability of raw sewage bacteriophage (φNK5) in lysing and killing gram negative *Klebsiella pneumonia*e bacteria that causes liver necrosis in mice **(Hung et al. 2011)**.

More recently**, Narasimhaiah et al. 2013** described the efficiency of two virulent phages to lyze 85% of clinical *Staphylococcus aureus* isolates that caused several human infections as septicemia and endocarditis.

Nowadays, with multiple antibiotic-resistant bacteria, the use of bacterial viruses, i.e. bacteriophages therapy as an alternative to conventional antibiotics is rapidly increasing. Bacteriophages can be more specific than antibiotics. One advantage of phage therapy is the specificity of targeting only the host bacterial cells, while antibiotics could also kill a wide range of bacteria in addition to the targeted harmful one **(Haq et al. 2012)**. In addition, there is no reported cases of side effects following the use of LAB phages, unlike most antibiotics that may cause side effects and resistance **(Haq et al. 2012)**.

Consequently, the aim of this study was to isolate and characterize Laban Jameed bacteriophages and to test their lysis potential against some available strains of pathogenic bacteria. To our knowledge, this study represents the first isolation of ssDNA and dsRNA bacteriophages from dairy sources.

CHAPTER 2.0 PROBLEM STATEMENT AND OBJECTIVES

2.1 Problem statement

Dairy products are essential components in food industries and individual nutrition in Palestine. The quality of dairy products is quite critical and any contamination is considered detrimental and usually results in major economic losses. Contaminations caused by bacteriophages generally decrease the quality of food products and is considered undesirable by diary industries. Bacteriophages, however, can have useful uses as alternatives to antibiotics, in what is widely known as phage therapy. The notion of using phage therapy to treat pathogenic bacteria has been increasing, especially with the high increase in antibiotic-resistance among bacteria. Unlike antibiotics, phages can be more specific, possess potentially lower side effects, and possibly can be available in a cost-effective manner. Phage therapy is now well established, however, only few phages have been isolated with potential lysis effect on pathogenic bacteria. It is, therefore, a rich area of interest to discover and isolate new phages and test their abilities in lysing pathogenic bacteria.

It is believed that this study may provide further information on the complex interactions between phages and their hosts, and promote studies on phage therapy.

2.2 Specific objectives

1. To isolate phage filtrates from Laban Jameed.
2. To identify the host cells of the isolated phages.
3. To determine the nucleic acid composition of the isolated phage genomes and partially characterize them.
4. To test the ability of the isolated phages in lysing some selected avian pathogenic bacteria.

CHAPTER 3.0 MATERIALS AND METHODS

3.1 Materials

All materials used in this study and their sources are included in **Appendix 1**. Samples of Laban Jameed used in this study were obtained from the cities of Tulkarm (north) and Dahriya (south) of Palestine. Jameed from Tulkarm was made from cow milk, whereas Jameed from Dahriya was made from sheep milk. The isolated bacteriophages were named after the Palestine Polytechnic University (PPU) and its nucleic acid composition: PPUDV (PPU DNA Virus) for phage isolated from Dahriya Jameed and PPURV (PPU RNA Virus) for phage isolated from Jameed obtained from the city of Tulkarm. Samples were stored at -80 °C to avoid cross contamination until they were cultured on agar plates.

3.2 Methods

3.2.1 Marker genes and PCR conditions

The primer sequences for the two markers used to identify the host strains isolated from the Jameed are included in (**Table 3.1**). The two markers were the *recA* gene, which is considered specific for LAB and the 16S rRNA. Sequence results of the two genes were compared with available GenBank sequences and were approximately 800 bp and 330 bp for the 16S rRNA *and recA* sequences, respectively.

The 16S rRNA, with its 1542 bp conserved sequence, lying at the small subunit of prokaryotic ribosomes, codes for *rDNA* which is involved in making the prokaryotic ribosomes **(Stiegler et al. 1981)**.

The *recA* (Recombination Protein A) comprises 352 amino acids in *E.coli*, with a well-studied role. It helps in homologous DNA recombination, a process that contributes to enhanced genetic diversity within bacteria. Briefly, it includes commutative DNA strands between complementary DNA substrates, which results in forming gaps and breaks within DNA strands, therefore, *recA* protein plays effective role in DNA maintenance and repair of these strands through a cascade of pathways **(Kowalczykowski, 1991; Torriani et al. 2001)**.

The *recA* primers were designed according to **(Torriani et al. 2001)**, while the 16S rRNA primers were kindly provided by Dr, Robin Abu Ghazaleh **(BRC/PPU) (Table 3.1).**

Table 3.1. Primer sequences used to amplify the marker genes, *recA* and 16S *rDNA* partial sequences. Information in relative to their sequence and size of amplified fragment are included. Y for (C or T), H for (A or C or T), R for (A or G), W for (A or T), N for (A or C or G or T), M for (A or C), K for (G or T)

Name	Sequence	Length of amplified fragment
***recA* gene Reverse partial sequence**	**5`- T TY ATHGAY GCN GAR CAY GC -3`**	**340 bp**
***recA* gene Forward partial sequence**	**5`- CCW CCW GKWGTHGTYTCNGG -3`**	**340 bp**
***16S rDNA* gene Reverse partial sequence**	**5`- GGACTACCAGGGTATCTAAT -3`**	**780 bp**
***16S rDNA* gene Forward partial sequence**	**5'- AGTTTGATCCTGGCTCAG -3`**	**780 bp**

The two *recA* and *16S rDNA genes* were used as markers to confirm the identity of the host bacteria. For both *recA* and *16S rDNA* genes, the same reaction PCR mix conditions were obtained and contained 1 µl of template DNA from each host colony, 2.5 µl of 10x PCR reaction buffer, 0.5 µM of each primer (10 pmol concentration), 2.5 µl of 20 mM $MgSO_4$, 0.5 µl of 20 mM dNTPs and 1.25 U of thermostable Taq polymerase. The mixture volume was completed with ultrapure water to a final volume of 25 µl.

Amplification of the *recA* specific product was conducted in 30 cycles using the following conditions: initial denaturation was performed at 94°C for 3 min and for 30 sec for the subsequent cycles, followed by 30 sec for primer annealing at 54 °C, elongation of the target gene with taq polymerase at 72°C for 30 sec. A final extension of 5 min at 72 °C was followed by cooling down to a temperature of 4 °C to store as in refrigerator conditions.

The 16S rRNA PCR condition was also for 30 cycles as follows: 95 °C for 5 min for the initial denaturation, 1 min denaturation for the subsequent cycles, primer annealing at 51 °C for 1 min, target elongation at 72 °C for 1.30 min. A final extension of 10 min at 72 °C was followed by cooling down to 4 °C.

For each amplicon product, an extra 25 µl PCR tube was prepared for the purpose of purification and sequencing. PCR amplicon purification was achieved by following instructions provided in the AccuPrep PCR Purification Kit (Bioneer K-3035). Briefly, 5

volumes of PCR Binding Buffer were mixed with PCR sample, after which it was transferred onto DNA binding columns and centrifuged at 13,000 rpm for 1 min. the supernatant decanted, salts and impurities were removed by washing the column with 500 µl of washing buffer, and samples were centrifuge at 13,000 rpm (Bench-top Microtube Refrigerated Centrifuge/ # 0031526) for 1 min, the supernatant was decanted then and re-centrifuged was to ensure complete dry, supernatant was decanted. Finally, the DNA binding column was transferred to a 1.5 ml Eppendorf tube, amplicon was eluted by the addition of elution buffer and collected by centrifugation at 13.000 rpm for 1 min. The elute were checked on 1.5% agarose **(Appendix 2)** for amplicon purity.

3.2.2 Laban Jameed bacterial culture

From each Jameed, a small piece of Jameed (1 gram) was incubated for 2 days at 37 °C with continuous shaking at 200 rpm in 100 ml skim milk (SM) broth (**Appendix 2**) until the O.D at 600 nm reached 0.2. This was followed by plating 200 µl from each culture on SM agar plates (**Appendix 2**) using a sterile glass pasture pipette. Individual colonies were then picked and subcultured separately in new 100 ml SM broth to examine the presence of the bacteriophage.

All bacterial stock cultures were stored at -80°C in SM containing 16% (v/v) glycerol. When needed, frozen cultures were allowed to thaw before plating onto Skim milk agar through overnight culture in Skim milk broth.

3.2.3 Phage isolation

Fifty ml of two subcultures of skimed milk broth inoculated with a piece of Jameed milk was tested for the presence of phages. To ensure purified phage filtration from bacteria, 5 drops of chloroform were added to each sample, stored for 15 min at room temperature before centrifugation at 13,000 rpm for 5 min. This step was repeated twice to ensure sufficient phage particles were purified.

The supernatant was then transferred to a new microfuge tube and re-centrifuged again. It was finally filtered through 0.45 µm sterile filters and filtrates were labled according to Palestine Polytechnic University.

3.2.4 Bacteriophage lysis

Phage filtrates were examined upon overnight cultured bacterial lawns in skimed milk plates to confirm the phage resistance (no lysis) or sensitivity (lysis) of strains called plaques. Through which phage filtrates (30 µL) were added to each bacterial colony separately in skim milk broth. This means that PPUDV phage filtrate was tested on both Tulkarm and Dahriya bacterial colonies, the same was done with filtrate PPURV. As a control, each time a tube containing only bacteria without phage filtrates was used in each manipulation, plates were kept for 15 min under laminar flow to dry, before being incubated at 37 °C for 3 days until bacterial lysis was detected. Each bacteriophage lysis was carried out for at least three consecutive rounds until a presumptive pure phage was obtained.

Stab culture was prepared by cutting a small piece of each lysis and then stored in a 1.5-ml Eppendorf tube in -80 °C freezer.

The effect of the isolated bacteriophages was tested upon three avian pathogenic *E. coli* (APEC) strains 1, 2, 3 **(available at Biotechnology Research Center, BRC)** in addition to the control bacteriophage host. Briefly, the *E. coli* APEC strains were cultured overnight on LB plates (**Appendix 2)** before they were infected with 3 drops from each bacteriophage. Following phage addition, plates were incubated at 37 °C, and plaque formation was monitored from 1-4 days. Finally, 100 µl of each filtrate was stored at -80 °C in 50 % glycerol.

3.2.5 Plaque titer assay

The double layer Plaque assay method was used according to **(Lu et al. 2003; Moce-Llivina et al. 2004)** as follows: A previously prepared concentrated titer filtrates were used, a single plaque was picked up from agar plate, mixed with log phase bacteria $O.D_{600}$ nm of 0.2 in SM suspension, and then incubated for 3 hours at 37°C. Samples were then purified as in section 3.2.3, and then used for one step growth curve and genome isolation. To determine phage titers, 10-fold serial dilution was performed with each dilution prepared through mixing 0.1 ml of stock phage suspension in 0.9 ml water (tube labeled as tube 1). The 0.1 ml from tube 1 sample was transferred into tube 2, containing 0.9 ml sterile water; the same was done for the other remaining dilution. From each titer 0.1 ml bacteriophage suspension was inoculated to 0.5 ml of O. D_{600} nm = 0.2 bacterial culture

SM, incubated at 37 °C for 40 min, and then added to a tube containing 3 ml of 0.7% soft agar heated at 49 °C and gently mixed. Finally it was poured onto a prepared monolayer SM plate. Plates were then incubated upside down at 37 °C. Plaque formation was monitored and data were recorded.

3.2.6 Characterization of bacterial host

3.2.6.1 Extraction of bacterial genomic DNA

Genomic DNA for each bacterial host was extracted according to EZ-DNA Kit protocol (**Biological Industries, Cat# 20-600-50**) with some modifications as follows; 0.5 ml from each overnight bacterial skim milk broth was pelleted by centrifugation at 13,000 rpm for 2 min, and then gentle mixing with 0.5 ml EZ-DNA solution for 60 min at 60 °C. DNA was then precipitated by the addition of 0.5 ml of absolute ethanol, the mixture was stored at room temperature for 5 min, centrifuged for 5 min at 10,000 rpm, supernatant was decanted, and the pellet was washed twice with 95% ethanol and finally suspended with 50 µl TE buffer. Bacterial DNA concentration was measured using a spectrophotometer; the blanking of spectrophotometer was done in 500 µl distilled water, then 5µl from each bacterial colony genome were mixed with 495 µl distilled water and measurements have been done in a quartz cuvette. Also genome was detected by running 10 µl of each sample plugged with µl 6X loading dye before loading on 1% gel electrophoresis (**Appendix 2**) . Separation was carried out in 1X TBE buffer at 99 V for 25 min. The amplicon was visualized using a gel documentation system (Alpha Inotech /100,240V).

3.2.7 Characterization of Bacteriophage

3.2.7.1 Bacteriophage genome isolation

Bacteriophage genome was extracted according to a protocol developed by (**Manasrah and Barghouthi 2012**) with minor modifications. Two volumes (1000 µl) of saturated ammonium sulfate containing 0.1% of 2-mercaptoethanol, a chemical compound that protect genomes from degradation with nucleases through reducing their disulfide bonds which lower their functional stability structure (**Manasrah and Barghouthi 2012**), were mixed with the phage sample (500 µl) for 5 min. Supernatant was removed after centrifugation at 13,000 rpm for 5 min at 4 °C. The pellet was then dissolved in (0.2 ml) 1% SDS and (0.2 ml) 0.5 N NaOH and centrifuged at 13,000 rpm for 5 min. To the clear

supernatant 0.4 ml of 3N Sodium Acetate (PH=5) was added in addition to 0.6 volume of isopropanol to precipitate the genome, and then held for 15 min at room temp. The mixture was then centrifuged at 13,000 rpm for 10 min and the resulting pellet was incubated with 100 µg/µl of proteinase K at 37 °C for 30 min. Finally, the phage genome was precipitated using 70% ethanol and the pellet was collected in 0.2 ml TE buffer after 2 min centrifugation at 10,000 rpm.

3.2.7.2 DNase and RNase phage genome susceptibility

The entity of nucleic acids was determined via the treatment of phage genome with DNase **(Endonuclease, Promega / Z358A)** and RNase **(Endonuclease, Sigma /R6148)**. 10 µl of each genome sample was incubated with 3 µl of DNase and the same with RNase for 35 min at 37 °C. The mixture was then loaded on 0.7% agarose gel using undigested genome as a control, and lambda phage genome treated with *Hind*III restriction enzyme was used as a high molecular weight ladder, and then detected under ultraviolet light.

3.2.7.3 State of strandedness of phage genome

The RNase A treatment at low and high concentrations was used to determine ss/ds RNA genomes according to the following references (**Morris and Dodds 1979; Chu and Westaway 1985; Westaway et al. 1999; Targett-Adams et al. 2008; Ablasser et al. 2009**). It breaks down both single and double stranded RNA, while they degrade only ssRNA at high salt concentration. Briefly, 10 µl of PPURV genome were incubated with 3 µl RNase A (**Sigma, 70 U/mg, R 6513**) either with low (0.1M) or high (0.4M) NaCl concentration for 1h at 37 °C. Following treatment, samples were mixed with 6x loading dye and electrophoresed on 0.7% agarose gel as prepared in section 3.2.6.1.

To determine whether phage genomes belong to dsDNA or ssDNA, 10 µl of genome was boiled for 6 min in water bath, plugged on ice, before rapidly loaded on 0.7 % gel electrophoresis with unboiled genome as a control and *Hind*II-digested lambda phage genome as a ladder. Furthermore, genome were treated with sets of specific dsDNA restriction enzymes including, *Mlu*I (**Thermo/ ER0561**), *BamHI (**New England Biolabs/ R0136S**), EcoRI (**New England Biolabs/R010S**), *Hind*III (**Fermentas/ ER0501**), *Pvu*I (**Thermo scientific/ ER0621**), according to manufacture instructions. Briefly, the same reaction mixture of 20 µl was prepared for all restriction enzymes, through which 16 µl nuclease-free water was mixed with 2 µl 10x buffer, 1 µl of each restriction enzyme and 1

µl of DNA template. Samples were then incubated at 37 °C for 8 and 16 h periods, and reaction mixtures were loaded on 1% agarose gel.

3.2.7.4 Bacteriophage total protein analysis

The SDS-PAGE was carried out according to **(Sambrook and Russel 2001**; **Al-Manasra and Al-Razem)**. Total phage proteins were separated on a 10% SDS-PAGE, stained with 0.1% (w/v) Coomassie Brilliant Blue (**Applichem/ A3480, 0010**). The gel glass plates were cleaned and assembled; the stacking and separation gels were prepared using fresh reagents (**Appendix 2**) and were polymerized for 2 h. Sample of total phage proteins wasprepared by mixing 20 µl of each phage filtrate with 10 µl 2x SDS Gel-loading buffer (**Appendix 2**) and then heated for 3 min at 100 °C. The gel comb was removed, and each well was washed with running buffer to avoid any gel impurities, before apparatus reservoir was filled with 1200 ml 10x running buffer (**Appendix 2**). Following sample loading, the gel electrophoresis was allowed to run first at 80 V for 2 h until the protein passed the stacking gel, then increased to 150 V until the run completed. The gel orientation was emphasized by cutting one of the gel corners. Low and high protein molecular weight marker lanes were used at both ends of gel. The gel was then placed in Coomassie Brilliant Blue stain solution (**Appendix 2**) for 3h with continuous shaking. Following staining, the gel was transferred to distaining solution and fixing solution (**Appendix 2**) for 1-2 hour. The destainig solution was renewed twice for each 15 min.

CHAPTER 4.0 RESULTS

4.1 Isolation of bacterial strains and phage filtrates

Three bacterial colonies from each skim milk agar plate representing different Jameed samples (see M&M section 3.2.2) were selected for further testing against phage filtrates. Each colony was labeled in reference to the Bacterium (B) and colony number (1-6), (i.e., BC1 for bacterial colony 1, BC2 for bacterial colony 2, etc.). Thus, the bacterial colonies BC1, BC2, BC3 were selected from Dahriya samples, whereas BC4, BC5, BC6 were selected from Tulkarm samples. Phage filtrates were prepared from Jameed samples as described in section 3.2.3. Filtrates were tested against all bacterial colonies from both cities, PPUDV was tested on Tulkarm and Dahriya, the same was done for the other filtrate PPURV.

Screening of phage filtrate effects on the bacterial lawns showed two clear round plaques, each with approximately 1.5 cm diameter (**Figure 4.1**). The viruses which could cause the plaques were named Palestine Polytechnic University DNA Virus (PPUDV) (**Figure 4.1. A**) and Palestine Polytechnic University RNA virus (PPURV) (**Figure 4.1. B**).

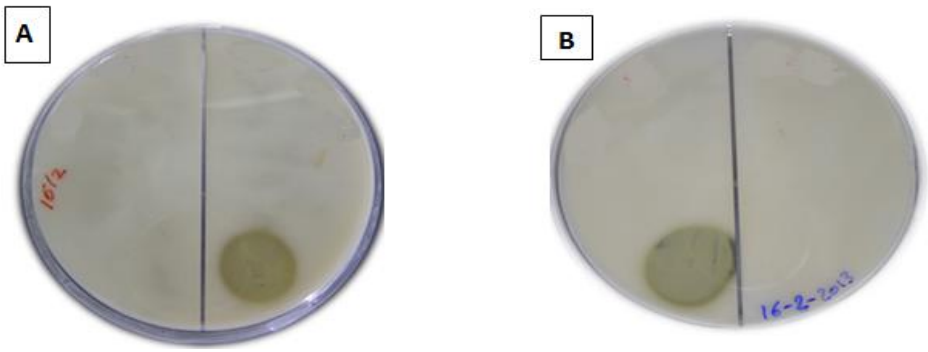

Figure 4.1. Bacterial cultures on skim milk plates showing bacteriophage lysis. Phage filtrate (30µL) dropped onto each bacterial colony**. A.** The PPUDV phage was able to lyse the bacterial host (BC1) isolated from Jameed. Lysis appeared as a clear yellow circle at the right side. The left side is a control with the host bacteria, but with no phage added. **B.** the PPURV phage lysed the bacterial host (BC4) isolated from Jameed with clear yellow circle at the left side. The right side represents the control host bacteria showed no lysis.

The PPUDV bacteriophage propagated on Bacterial colony 1 (BC1), while PPURV bacteriophage lysis Bacterial colony 4 (BC4) bacterial hosts, the results were confirmed by triplicate experiments (**Figure 4.1**). In each case, a control side in a separated plate was used, for example bacterial colony 1 cultured on both sides of the central line, then 30 µL

drops of phage filtrate was spotted on one half, while keeping the other half as a control with bacteria only. The overall trials are summarized in **(Table 4.1)**.

Table 4.1. **Effect of phage filtrates (PPUDV and PPURV) on bacteria isolated from Jameed**. Positive results (+) indicate phage lysis, while negative results (-) showed no lysis. The experiments were carried out in triplicate.

No.	Host Bacteria	PPUDV	PPURV
1	Bacterial colony 1 (BC1)	+	-
2	Bacterial colony 2 (BC2)	-	-
3	Bacterial colony 3 (BC3)	-	-
4	Bacterial colony 4 (BC4)	-	+
5	Bacterial colony 5 (BC5)	-	-
6	Bacterial colony 6 (BC6)	-	-

4.2 Effect of isolated bacteriophages on selected strains of avian pathogenic bacteria

The two bacteriophages (PPUDV and PPURV), which were able to cause cell lysis were further tested on three Avian Pathogenic Strains of *E. coli* (APEC) (**Table 4.2**). These APEC strains cause respiratory diseases known as colibacillosis among birds, which can lead to high mortality and economical loses, particularly to poultry industries **(Qabajah and Ashhab 2012)**. No lysis was observed with any of the APEC pathogenic strains (**Table 4.2**).

Table 4.2. Effect of phage filtrates (PPUDV and PPURV) isolated from Jameed bacterial colonies on avian pathogenic *E. coli*, three different strains (APEC1, APEC2, and APEC3) (Nos. 1-3). Positive results (+) indicate phage lysis, while negative results (-) showed no lysis. Numbers 4-5 show the phage host bacteria as a control. The experiments were carried out in triplicate.

No.	Host Bacteria	PPUDV	PPURV
1	Avian pathogenic *E. coli* strain 1 (APEC1)	-	-
2	Avian pathogenic *E. coli* strain 2 (APEC2)	-	-
3	Avian pathogenic *E. coli* strain 3 (APEC3)	-	-
4	Bacteria colony 4 (BC4)	-	+
5	Bacteria colony 1 (BC1)	+	-

4.3 Characterization of phage host bacteria

To identify the bacteria which were susceptible to phage lysis, two marker genes: *16S rDNA and* r*ecA*, were amplified from genomic DNA isolated from BC4 or BC1 host bacteria. The PCR amplification results showed clear bands matching the expected sizes of the two target genes (**Figure 4.2A, B**). PCR amplification for the 16S rRNA *and* r*ecA* were approximately (780 bp and 340 bp), respectively for both BC1 and BC4.

The purified gel products for the two genes, *16S rDNA and recA* were sequenced in the Hereditary Research Laboratory/ Life Science Department/ Bethlehem University. Sequences are included in (**Appendices 3&4**). The identity for each sequence was determined and confirmed by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLAST N program. Both host bacteria were identified by matching the sequence with the highest maximum identity score. BC4 was 97% identical to *Bacillus amyloliquefaciens*, while it was 97% identical to *Lactobacillus helveticus* for BC1. The overall results obtained from *recA* and 16S rRNA alignments are included in (**Appendix 5**) for BC4 and in (**Appendix 6**) for BC1 bacteria.

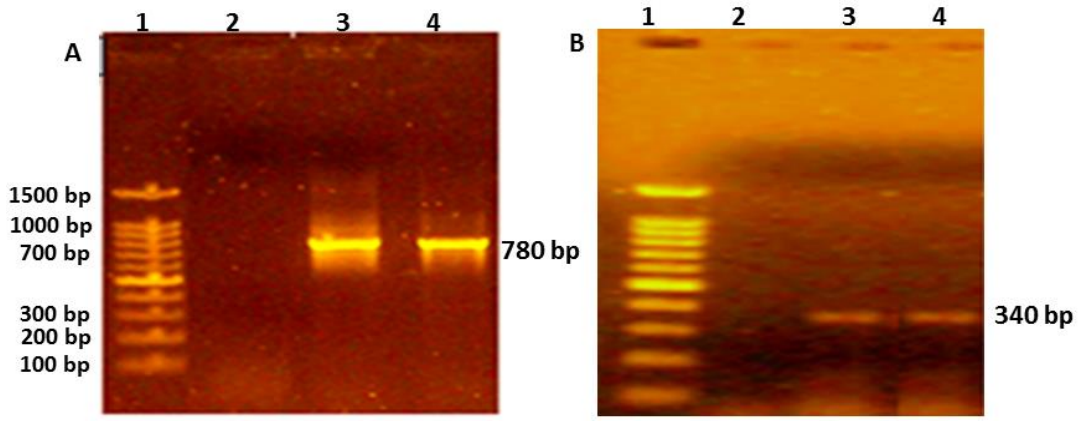

Figure 4.2. Amplification of two marker genes from the BC4 and BC1 host cells. A. PCR results of the 16S rRNA partial sequence shows the expected fragment size of 780 bp. Lane 1: 1kb ladder (Promega/G5711), lane 2 is a negative control H_2O (master mix with no DNA template). Lanes 3&4 show the 16S rRNA *amplicons* for BC4 and BC1, respectively. **B.** PCR results of the *recA* gene partial sequence. Lane 1 shows the 1 kb ladder (Promega/G5711) Lane 2 is a negative control H_2O (master mix with no DNA template). Lanes 3&4 show the *recA* gene amplicon for BC4 and BC1, respectively. Amplicons of the two genes were separated on 1.5% agarose gel and visualized by ethidium bromide staining according to standard protocols **(Sambrook et al. 1989)** using 1 Kb DNA Ladder.

To determine the relationship of the host strains with lactic acid bacteria that are mostly found in dairy products, the multiple sequence alignments for both *recA* and 16S rRNA sequences were constructed using the CLUSTAL W software. Then phylogenetic trees were constructed comprising all highly similar alignments, most common dairy bacteria and BC4, BC1 *recA* and 16S rRNA sequences, to better illustrate the homology with available bacterial strains (**Figures 4.3&4.4**). All closely related sequences were obtained from the National Center for Biotechnology Information **(NCBI)** databases. Phylogenetic trees were constructed using UPGMA method with MEGA5 software. In addition, the 16S rRNA and *recA* partial sequences of the *Streptococcus thermophilus* (a gram positive bacteria that play a role in milk fermentation), several phylogenetic studies recommended its use as an out-group, since other lactic acid bacterial strains like *Listeria* are more closely related and therefore not being suitable as an out-group **(Felis and Dellaglio 2005; Canchaya et al. 2006)**. From the marker analyses of the two genes, 16S rRNA and *recA*, results showed that the BC4 host was a close relative to the *Bacillus amyloliquefaciens* (**Figure 4.3A, B**), whereas the BC1 was a close relative to the *Lactobacillus helveticus* strain (**Figure 4.4A, B**).

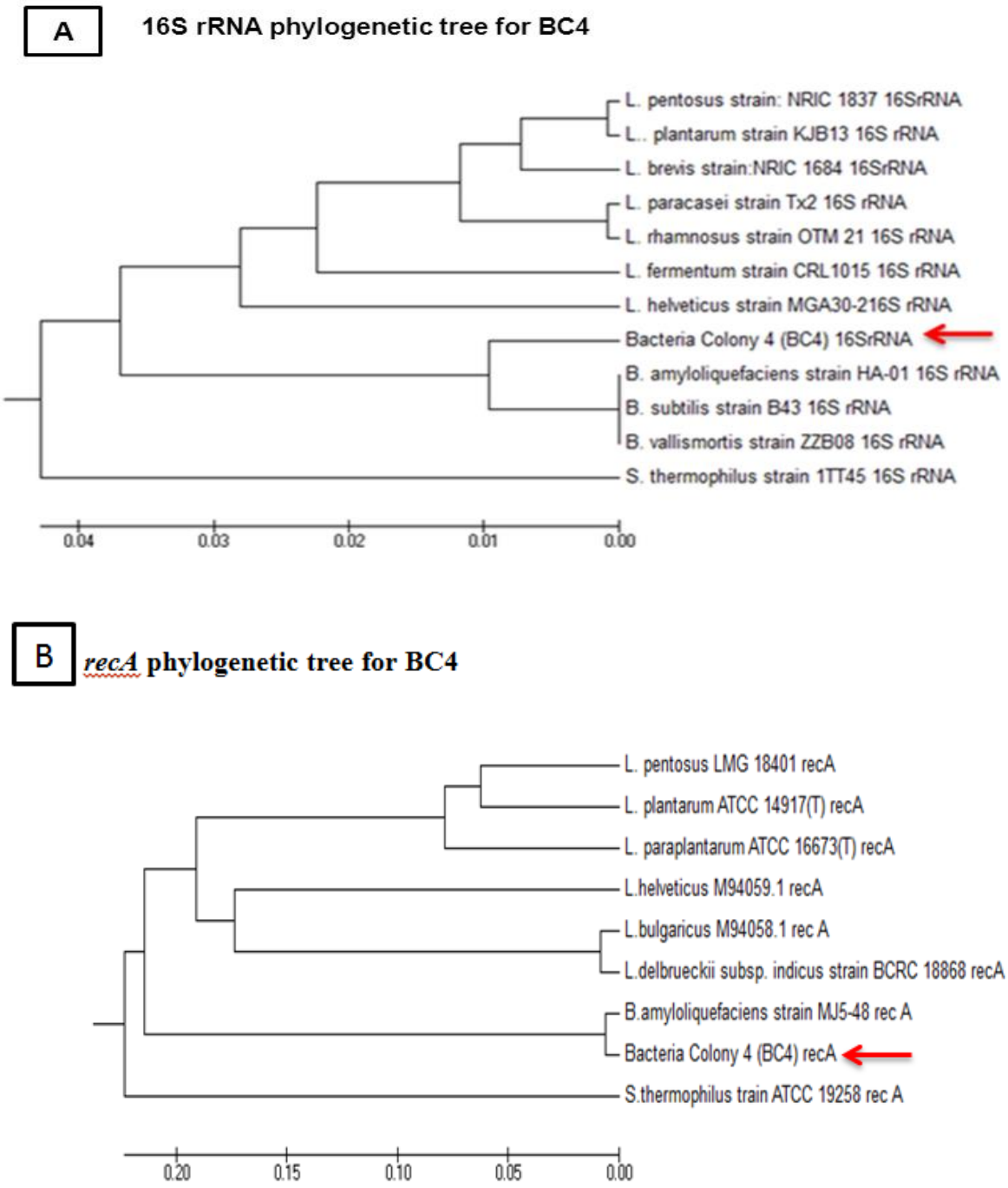

Figure 4.3. The phylogenetic trees for BC4 using 16S rRNA (A) and *recA* gene (B). Trees were constructed using the UPGMA software. Trees were built using high sequence similarity from the alignment with the most common Lactic acid bacterial sequences form GenBank. *Streptococcus thermophilus* was used as an out-group as shown. The *recA* gene of BC4 clustered with *Bacillus amyloliquefaciens*, whereas in 16S rRNA *tree*, it was difficult to determine whether BC4 did actually belonged to *Bacillus amyloliquefaciens*.

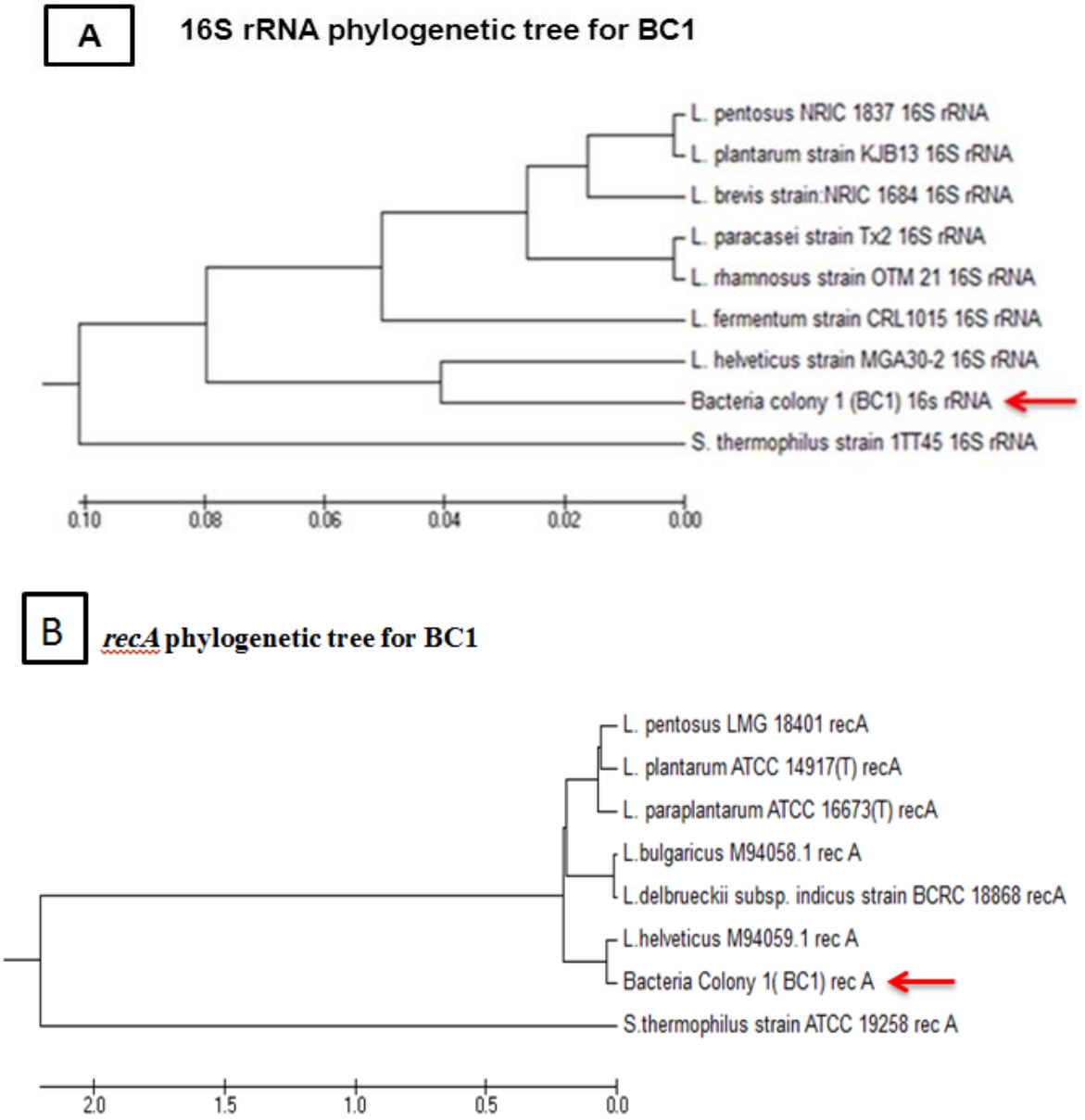

Figure 4.4. The phylogenetic trees for BC1 using 16S rRNA (A) and *recA* gene (B). Trees were constructed using the UPGMA software and high sequence similarity from the alignments with the most common lactic acid bacteria sequences form GenBank. *Streptococcus thermophilus* was used as an out-group as shown. The *recA* and 16S rRNA phylogenetic trees of the BC1 clustered with the *Lactobacillus helveticus*, one of the common lactic acid bacteria.

4.4 Characterization of isolated bacteriophages

Two phages representing RNA and DNA genomes were isolated. The PPURV bacteriophage that was able to cause lysis to BC4; (*Bacillus amyloliquefaciens)* was found to be an RNA bacteriophage as it was sensitive to RNase and resistant to DNase digestion (**Figure 4.5A**). The PPUDV, on the other hand, was a DNA bacteriophage sensitive to

DNase, but resistant to RNase digestion (**Figure 4.5B**). Genome sizes, however, seemed to be very similar, approximately 20 kb each (**Figure 4.5**).

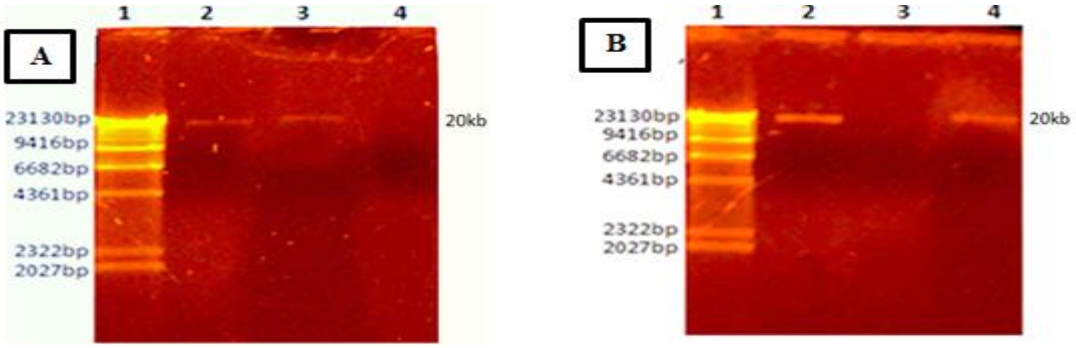

Figure 4.5. Identity and sizes of PPUDV and PPURV bacteriophages. Both genomes were treated with DNase and RNase before they were loaded on 0.7% agarose gel electrophoresis. **A.** the PPURV genome is an RNA phage. Lane1 contains 23 kb ladder and lane 2 contains undigested PPURV genome. Lane 3 contains PPURV genome treated with DNase and Lane 4 the PPURV genome treated with RNase. **B**. the PPUDV genome is a DNA phage. Lane 1 contains a 23 kb ladder and lane 2 contains the undigested PPUDV genome. Lane 3 contains PPUDV genome treated with DNase and Lane 4 PPUDV genome treated with RNase.

Furthermore, the PPURV RNA genome was confirmed to be a dsRNA, since as it was sensitive to RNase A digestion at low treatment, but was resistant to RNase A treatment at high NaCl concentration (**Figure 4.6A**). The PPUDV bacteriophage genome, on the other hand, was a ssDNA as confirmed by agarose gel. After 6 min boiling, it migrates to a similar distance as the un-boiled genome (**Figure 4.6B**).

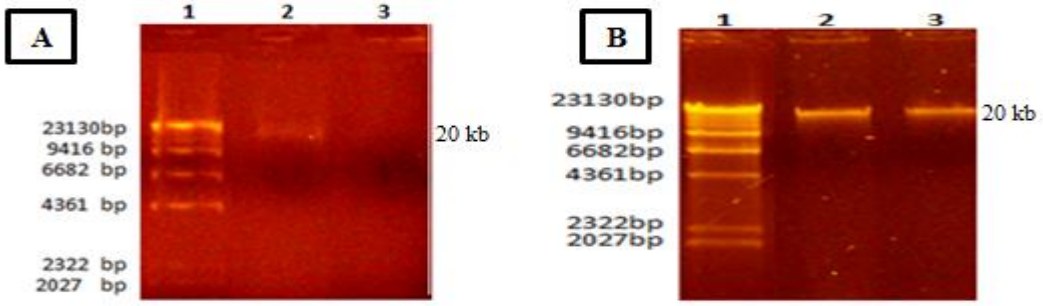

Figure 4.6. The PPURV was a dsRNA whereas PPUDV was a ssDNA phage. A. Lane 1 contains a 23 kb DNA Marker of lambda - Hind III, lane 2 contains the PPURV genome treated with RNase A in the presence of 0.4 M NaCl and lane 3 contains the PPURV genome treated with RNase A at 0.1 M NaCl. **B.** the PPUDV is a ssDNA phage. Lane 1 contains a 23 kb DNA Marker of lambda digested with *Hind* III, lane 2 contains the PPUDV genome incubated on ice and Lane 3 contains PPUDV genome after 6 min incubation in boiling water. All treatments and ladder were loaded on a 0.7% agarose gel.

4.5 One step growth curve of the isolated bacteriophages

Through monitoring agar plates subjected to double layer plaque assay, 4 plaques were detected in 48 h for the PPUDV phage. The PPURV phage showed 6 plaques in 36 h.

To reveal the nature of virus replication upon infection to host bacteria, one step growth curves were designed. It includes estimating the titers of bacteriophage stocks through using "Plaque Forming Assay" (PFU). This reflects how many particles an original infected cell release of viral progeny. Consequently, the titer calculation in PFU /mL were obtained through multiplying number of plaques with dilution factor then divided on the inoculum volume **(Mullan, 2002).** It relies first on constructing a table showing each titration with the resultant plaques. This helps in measuring the (PFU) as previously described as only the first titration is capable of forming plaques. Therefore, plaque numbers were monitored over time. As showing in (**Table 4.3**), for PPURV phage, 600 PFU were detected after 36 h, and then it increased to 700 PFU in 48 h and then remained constant until 96 h. For PPUDV, 400 PFU formed and increased to 600 PFU in 72 h, before it became constant until 96 h. (**Table 4.3**).

Table 4.3. Number of plaques detected in each tittered phage sample after monitoring incubated samples from 1-96 hour.

Time (h)	PFU /ml	
	PPUDV	**PPURV**
12	0	0
24	0	0
36	0	600
48	400	700
60	500	700
72	600	700
84	600	700
96	600	700

The data from the plaque assays were further analyzed. The number of PFU per bacterial cell was plotted on the Y axis against time required on the X axis. Consequently, for each bacteriophage, the latent, burst period and burst size were then determined (**Figure 4.7**). For PPURV phage, the latent period was about 24 h, burst period was 47 h and the burst size of about 700 PFU per infected cell. Whereas, the latent period for PPUDV was about 36 h with burst period of about 70 h and a burst size of about 600 PFU /cell. The overall one step growth curve results are summarized in **(Table 4.4**).

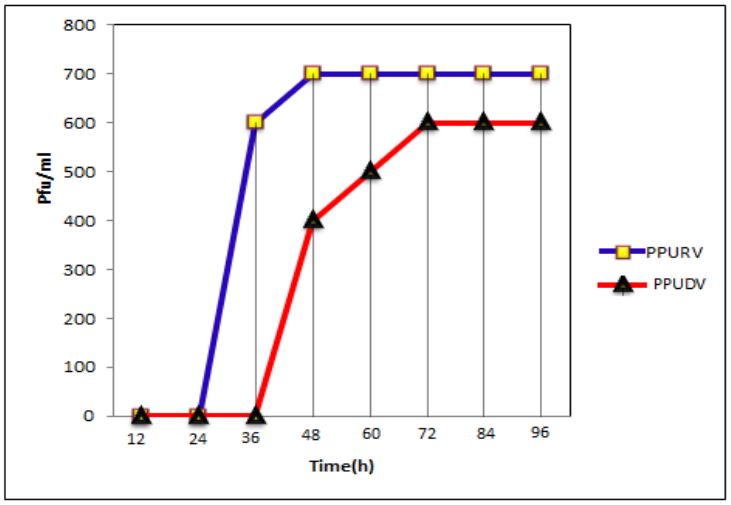

Figure 4.7. One step growth curves for PPUDV and PPURV bacteriophages. The curves showed the latent, burst period and burst size for each phage through its replication after bacteria intrusion. It is based on counting the number of formed plaques that were formed, then measuring PFU and comparing it against time. Results are the measure of three time trials.

Table 4.4. Latent period, burst time and burst size of bacteriophages

System / Phage	Latent period (h)	Burst Time (h)	Burst size (PFU / infective center)
PPURV	**24h**	**47 h**	700 PFU /ml
PPUDV	**36 h**	**70 h**	600 PFU /ml

4.6 SDS-PAGE analysis of the isolated bacteriophages

The phage total proteins were analyzed on SDS-PAGE. Samples were electrophoresed on a 10% polyacrylamide gel in the presence of SDS. Stained gels showed three distinct protein bands for PPUDV phage particles (**Figure 4.8**). The band sizes were estimated to approximately 27 KDa, 40 KDa and 45 KDa. On the other hand, there were two major bands (46 KDa, 6KDa) for PPURV genome.

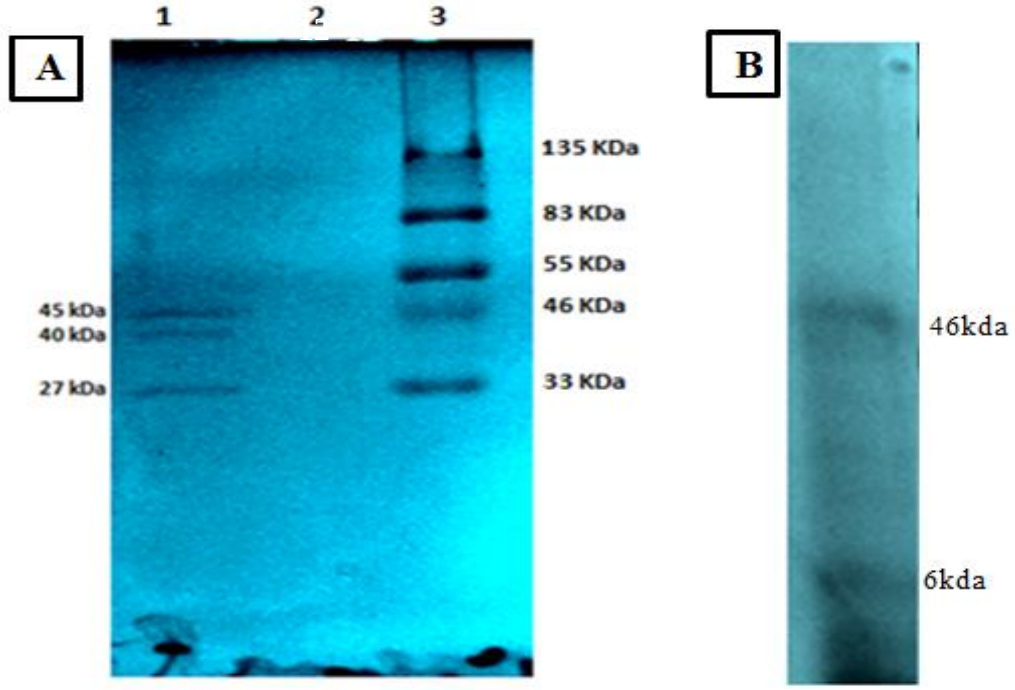

Figure 4.8. SDS-PAGE gel image of PPUDV and PPURV phage proteins. **A.** Lane 1 shows three bands and lane 2, empty well with no phage sample. Lane 3 contains: high molecular weight protein ladder (Sigma/ S8320). **B.** SDS-PAGE gel for PPURV phage proteins. As shown, at least two major bands (6 and 46 kDa) were detected.

CHAPTER 5.0 DISCUSSION

5.1 Characterization of bacteriophage host bacteria

In this study, molecular characterization was used to identify the two host bacteria that were susceptible to bacteriophage lysis. Two phylogenetic markers that are universally distributed in bacteria and commonly used in bacterial strain identifications, the *16S rDNA and recA* genes were targeted **(Collins et al. 1991; Torriani et al. 2001; Patil et al. 2010)**. The*16S rDNA* gene is used as a useful marker in bacterial characterization as it has conserved sequence and function among bacteria, still it shows a remarkable limitation due to its inability to differentiate between closely related species that share 99% or higher sequence identity, like in some lactic acid bacterial strains (**Torriani et al. 2001**). Such inability was clearly observed in the case of the *L. plantarum* and *L. pentosus* **(Torriani et al. 2001)**. Therefore, to further confirm the identity of the host bacteria in this study, the *recA* gene was also used as a marker commonly used in lactic acid bacterial identifications **(Torriani et al. 2001)**. In this regard, the *recA* gene has fundamental advantage over the *16S rDNA,* its ability to differentiate closely related species **(Torriani et al. 2001)**.

Sequence analysis and GenBank BLAST of the two marker genes used for the identification of BC4 and BC1 revealed their identities as *Bacillus amyloliquefaciens* for BC4 **(Figure 4.3A, B)** and *Lactobacillus helveticus* for the BC1 **(Figure 4.4A, B)**. The *16S rDNA gene* was less informative in determining the relativeness of the BC4 bacteria. Using the highest maximum identity score, it was initially difficult to determine whether BC4 was a close relative to *Bacillus vallismortis*, or *Bacillus subtilis*, or *Bacillus amyloliquefaciens.* The use of the *recA* gene easily confirmed the identity of the BC4 host to be more closely related to *Bacillus amyloliquefaciens* **(Figure 4.3)**. The *B. amyloliquefaciens*is is a gram positive bacterium, discovered and normally found in soil. It is considered a useful industrial microorganism, representing, for example, an ample source for producing amylase enzyme commercially used in starch hydrolysis **(Priest et al. 1987; Gangadharan et al. 2006)**. It is also a source of protease enzyme that catalyzes the breakdown of proteins and used in detergent industries **(Priest et al. 1987)**. There is no previous study to report the natural presence of the *B. amyloliquefaciens* in dairy products and that it plays a role in milk fermentation similar to lactic acid bacteria. However, it was

shown to be a useful source for a milk-clotting enzyme in some industrial diary processing fermentations **(Ding et al. 2012)** and in the fermentation of other food products, such as soybean-fermented food **(Joo et a. 2007)**. Otherwise, the explanation for its existence is to be a result of Jameed contamination with soil. During the process of Jameed drying, it is usually kept uncovered on the ground in sunny areas for a few days to reduce the moisture contents and it is possible that this strain is just a contaminant from the nearby soil. Milk contaminants belong mostly to the genus *Bacillus*, including the *B. amyloliquefaciens* and *B. cereus*, which produce a toxin that can cause diarrhea and another that causes vomiting (**Gonzáles et al. 2012**). *Bacillus cereus* spores are heat-resistant and may survive pasteurization. Very rare cases have been linked to dried milk and dried infant formulas (**Boor, 2001**). The source of contamination in dairy products could be multifarious, like soil, air and water **(Coorevits et al. 2008; Gonzáles et al. 2012)**. The dairy industries implement several strategies to reduce contaminations, such as pasteurization under high temperature, but even though, contamination still could occur due to some microbial heat resistant spores. Obviously, dairy processing that relies on traditional methods is subject to a higher possibility of contaminations. Traditional Laban Jameed production is dependent on personnel in milk transport and processing under mostly unsterile environment and storage.

The BC1 host was clearly identified to be a close relative to the *Lactobacillus helveticus* strains **(Figure 4.4)**. This strain is one of the common lactic acid producing gram positive bacteria **(**Slattery **et al. 2010; Marco et al. 2012)**. It helps in maintaining good food flavor and acidic conditions that inhibit the spoilage of milk products. Furthermore, the relationship of PPURV and BC1 host bacteria with other related species was determined by the phylogenetic tree, which was constructed using the highest identities as appeared by the two markers, *recA* and 16S rRNA sequence BLASTs. The tree includes the most common *Lactobacillus* bacteria that occupy the highest percentage of the microbial population; *L. pentosus, L. casei, L. fermentum, L. plantarum*and *L. paraplantarum,* **(Figures 4.3 & Figure 4.4)** while those with lower identities were masked and excluded.

5.2 Characterization of bacteriophages

Several studies reported the isolation and characterization of bacteriophages that can infect common bacteria in dairy products, but there was no report of phages isolated from Laban

Jameed. In this study, two Jameed milk PPURV dsRNA and PPUDV ssDNA bacteriophages were isolated and partially characterized.

In the process of phage purification and isolation, the addition of low amounts of chloroform to the suspension was needed to kill bacteria that were still alive and to liberate the phage particles from the cells. Chloroform causes denaturation of bacterial proteins and is believed to also cause minor denaturation to viral proteins, but viruses are usually still viable **(Mullan, 2001)**. To remove the cell debris, the suspension was centrifuged before filtration through a 0.45 µm membrane to retain bacterial cell debris, while allowing phage particles to pass through the membrane **(Mullan, 2001)**. Most importantly, the sensitivity and specificity of particle purification were established. Sensitivity was determined by comparing ten -fold phage titers (10^{-1} - 10^{-4}) upon host bacteria under the same test conditions which resulted in a clear circle plaque, a sign of bacteriophage lysis. Specificity was performed through a host range test, at which phage filtrates separately spotted onto different bacterial cultures. Bacteriophages are known to target specific host bacteria that could be only one host (**De Antoni et al. 2010**) or multiple ones **(Jensen et al. 1998; Lu et al. 2012)**. The isolated bacteriophages in this study were specific only to BC1 and BC4 host bacteria for PPUDV and PPURV bacteriophages, respectively **(Tables 4.1& Tables 4.2)**. No lysis effect was seen when the two phages were spotted on avian pathogenic *E.coli* strains, a clear indication of the high specificity of these viruses **(Tables 4.1 & 4.2)**. It is not known; however, if the two bacteriophages, PPURV and PPUDV could cause any effect on other pathogenic bacteria that are associated with dairy production including milk spoilage and milk associated pathogens. A common disease affecting dairy cattle is mastitis that is caused by *Staphylococcus aureus*, which causes inflammation for the cattle mammary gland and is may be highly resistant to antibiotics (**Erskin et al. 2004**). Their highly antibiotic resistance was clearly shown in strains isolated here in Palestine **(Adwan, 2006)**. Such diseases significantly affect the dairy industries mainly by lowering the milk quality **(Erskin et al. 2004)**. It is of great interest to test these two viruses on such pathogenic strains, but it was difficult during the term of this study to carry out these tests due to safety limitations.

The three APEC pathogenic strains tested in this study were obtained from the BRC stock and were isolated though a different project. These avian pathogens cause high mortality rates among birds and are considered detrimental to poultry industries in Palestine **(Qabajah and Ashhab 2012).**

5.3 One step growth curve

To identify the phases of bacteriophage infection, one step growth curves were constructed. These curves were performed using the double layer plaque assay, a commonly used and highly accurate, fast and easy to handle method (**Moce-Llivina et al. 2004).** Results are recorded as the mean of three trials; measures of PFUs were approximately conserved. This means the relationship between phage titer and phage infection on the related host cells are the same in the three trials. Both PPURV and PPUDV bacteriophages have long latent periods. This indicates that further conditions should be manipulated as to detect optimum bacteriophage activity after infecting their host bacteria. Thus, such conditions could be the culture media itself, as skim milk broth and agar were used. Other studies preferred the MRS broth media being used for the detection of dairy bacteriophages (**Abedon, 1989; Cann, 2005**).

5.4 Determination of Bacteriophage genome nucleic acid

We reported the isolation of two bacteriophages; a dsRNA PPURV and ssDNA PPUDV hosting on *Bacillus amyloliquefaciens* and *Lactobacillus helveticus,* respectively.

The majority of identified *Bacillus amyloliquefaciens* and *Lactobacillus helveticus* bacteriophage genomes belong to dsDNA **(Erickson and Young 1974; Sechaud et al. 1991; Villion and Moineau 2009; Zago et al. 2013)**, Little is known about dsRNA and ssDNA bacteriophages that feed *Bacillus amyloliquefaciens* or *Lactobacillus helveticus* strains, although one dsDNA *Lactobacillus helveticus* phage with genome size 36,566 bp from Grana Padano cheese product was isolated **(Zago et al. 2013).** Also, Sechaud et al. (1991) characterized 35 cheese whey *Lactobacillus helveticus* dsDNA bacteriophages, but without providing information on their genome size. Most lactic acid bacteriophages are believed to be dsDNA **(Villion and Moineau 2009; De Antoni et al. 2010).** In addition, the isolation of 235 bacteriophages affecting *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus* and L. *delbrueckii* subsp. *lactis* strains, all with dsDNA and belonged to Siphoviridae, Myoviridae and Podoviridae were also described **(Villion and Moineau 2009).**

There are no reports of dsRNA bacteriophages that host on *Bacillus amyloliquefaciens*, although a dsDNA PBA12 *Bacillus amyloliquefaciens* bacteriophage was isolated from soil (**Erickson and Young 1974**). While Qiao et al. (2010) described a

dsRNA bacteriophage from radish leaves with genome size of 12,684 bp hosting on *Pseudomonas syringae* bacteria.

To our knowledge, this study represents the first isolation of dsRNA phage that hosts on *Bacillus amyloliquefaciens and* ssDNA phage that hosts on *Lactobacillus helveticus* from dairy Jameed milk. The bacteriophage genomes were isolated under reducing condition to minimize degradation. The β-mercaptoethanol was added to inhibit the activity of DNase and RNase degrading enzymes by reducing their disulfide bonds **(Manasrah and Barghouthi 2012).**

To liberate phage genomes from DNA and RNA binding proteins, phage proteins were denatured and precipitated through the addition of NaOH and SDS and the phage genomes were precipitated from the supernatant through the addition of sodium acetate in the presence of β-mercaptoethanol as reported by other studies (**Edy et al. 1976; Chu and Westaway 1985).** The PPUDV phage was found to be a DNA genome as no bands appeared on the 0.7% agarose gel when treated with DNase. This is in contrast to PPURV phage which was resistant to DNase. Unlike PPUDV, it was responsive to RNase digestion as observed on the agarose gel (**Figure 4.5A, B**). Both PPUDV and PPURV genomes appeared to have similar sizes, approximately 20 kb ±1kb as observed on agarose gel with a 23 kb ladder. There are other methods that can be used to determine the sizes of phage genomes, which include the digestion of genomes with restriction enzymes (**De Antoni et al. 2010; Yang et al. 2010; Ownes et al. 2012).** DNA phages can be directly digested by several restriction endonucleases, such as *Pvu*I, *Mlu*I, and *Hind*III, whereas RNA genomes can be first converted to cDNAs before digestion **(Abedon, 1989; Qiao et al. 2010).** The sum of sizes of the digested fragments can give an indication of the phage genome size.

The nature of the bacteriophage genomes was further studied. The RNA genome was determined to be a dsRNA (**Figure 4.5A)**, whereas the DNA genome was a ssDNA genome (**Figure 4.5B)**. There are other assays, such as the hyperchromicity test, ds/ssRNase specific enzyme degradation used to differentiate between ds/ss genomes, but these methods were reported to possess low efficiency in working on small amounts of RNA **(Edy et al. 1976; Morris and Dodds 1979)**. In this study, the RNase A assay was preferred and used. The RNase A digests both ssRNA and dsRNA under low NaCl concentration (0.1 M), while it digests only ssRNA under high NaCl concentration (0.3 M) **(Edy et al. 1976; Morris and Dodds 1979; Chu and Westaway 1985; Westaway et al. 1999; Targett-Adams et al. 2008; Ablasser et al. 2009; Dayer et al. 2012).** A previous

study reported the resistance of dsRNA of viruses infected plant and fungi to RNase A treatment under 0.3 M NaCl even after 24 h incubation **(Morris and Dodds 1979)**. Following treatment with high salt concentration, clear bands were still visible, but disappeared under low (0.1 M) salt treatment **(Morris and Dodds 1979)**. In addition, ssRNA extracted from other viruses was also examined. It completely disappeared under 0.1 M and 0.3 M NaCl treatments (**Morris and Dodds 1979).** The PPUDV bacteriophage genome, on the other hand, was a ssDNA. The ssDNAs are usually smaller in size than dsDNA, thus having more fragile structure and are expected therefore to run much faster on agarose gels. The heated PPUDV genome migrated similarly to the unboiled PPUDV genome on the gel, thus confirming its ssDNA identity. The single strandedness entity was further confirmed by the resistance of genomes to restriction enzymes treatment that cut dsDNA, no bands were detected in both 8h and 16 h treatment using untreated genome as a positive control.

The ssDNA phage genomes are mostly related to either the Microviridae, circular ssDNA genomes with nonenveloped and isometric shapes, or Inoviridae families, circular ssDNA genomes with nonenveloped and filamentous shapes as appeared by morphological studies using the electron microscope **(Table 1.1) (Ackerman, 2011; ictvonline.org)**. Phages that belong to the Cystoviridae family are dsRNA and possess segmented genomes with enveloped capsids and spherical shapes for the phage genomes **(Table 1.1)**. However, up to 2009, 88% of completely sequenced genomes in GenBank phage databases are related to dsDNA, mainly distributed among the Caudoviridae orders. The PPUDV and PPURV bacteriophages are therefore possibly belong to the Microviridae, or Inoviridae (ssDNA nucleic acid) or Cystoviridae (dsRNA) family, respectively **(Table 1.1)**.

5.5 Phage protein analysis

Total phage protein extracts were loaded on SDS-PAGE. Three major protein bands appeared for PPUDV ssDNA bacteriophage with estimated molecular weights of approximately, 27, 40 and 45 kDa (**Figure 4.8**). For PPURV dsRNA bacteriophage, only two major protein bands of 6 and 46 kDa appeared (**Figure 4.8**). These results indicate that aggregations of several proteins accumulate at each band and/or that some proteins are repetitive copies encoded from the same gene.

5.6 Conclusions and future perspectives

Several studies demonstrated the isolation of dairy product bacteriophages, but previously no phages have been isolated from Jameed. Most of the isolated dairy bacteriophages were characterized as dsDNA. To our knowledge, this study reports the first isolation of ssDNA and dsRNA bacteriophages from a dairy product.

Moreover, as there is a significant increase in antibiotic resistance among bacteria, there is a notion to use phage therapy as an alternative for antibiotics. The isolated bacteriophages from Jameed milk may have the potential for future uses as alternatives for antibiotics if they prove to lyse pathogenic bacteria, particularly those that exist in close proximity to milk, such as those causing mastitis disease.

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APPENDICES

Appendix 1. List of chemicals and suppliers

Chemical	Supplier
Agarose	Sigma
Chloroform	Frutarom
Ethanol	Frutarom
European bacteriological Agar	Hy labs
Ethedium Bromide 1mg/ml	Hy labs
Dnase	Promega
NaCl	Frutarom
Ammonium Sulfate	Sigma
Rnase	Sigma
Skim milk	oxoid
Yeast Extract	Conda
Trypton	Neogen
Isoprpropanol	Frutarom
Sodium Dodecyl Sulfate (SDS)	Applichem
MgSO4	MP biomedicals
5M KAcetate	Riedel-de haen
3M Na Acetate	Riedel-de haen
NaOH	Frutarom
Mercaptoethanol	Sigma
Acrylamide Mix	Alfa Aesar
1.5 M tris –Hcl	Promega
Ammonium persulfate	Sigma
Tetramethylethylenediamine (TEMED)	Sigma Aldrich
Glycine	fluka
methanol	Frutarom
Glacial –Acetic Acid	sigma
Xylose lysine deoxycholate (XLD)	BD
Tris – Base	Promega

Bromphenolblue	Fluka
Xylen Cyanol	Amresco
EDTA	Fluka
Boric acid	Sigam
Acrylamide	Alfa Aesar
Ultra-pure water	Biological industries

Appendix 2. Buffers, Solutions and Media. All solutions were prepared with ultrapure water.

Name	Reagents
6x Loading Dye	25% (v/v) Ficoll, 1% (w/v) Orange G, 0, 5% (w/v) Bromphenolblue and 0.5% (w/v) Xylen Cyanol
Tris-borate-EDTA (TBE) (10x)	107, 8 g Tris 55 g Boric acid 40 ml 0, 5 M EDTA pH 8, 0
Agarose Gel (0.7-2%)	0.7-2 g agarose in 100 ml 1x TBE
SDS-PAGE Resolving Gel (10%, 6 ml)	2.477ml water, 1. 8ml acrylamide (30%), 60 µl Ammonium persulfate (10%), 1.6 ml TRIS-HCl (1.5 M, pH 8. 8), 60 µl SDS (10%) and 2.4 µl TEMED.
SDS-PAGE Stacking Gel (5%, 4ml)	2. 74 ml water, 670 µl acrylamide (30%), 40 µl Ammonium persulfate (10%), 500 µl TRIS-HCl (1.5 M, pH 8. 8), 40 µl SDS (10%) and 4 µl TEMED.
Running Bucffer, 10x	25 mM TRIS–base, 0.1% (w/v) SDS 250 mM Glycine (PH 8. 3), dd H2O
2X SDS Gel-loading buffer	100 mM Tris- Cl (pH 6. 8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 200 mM β-mercaptoethanol
Skim milk broth / 100 ml	5g Skim milk Powder, 100ml ddH2O

Skim milk agar / 100 ml	5g Skim milk Powder, 100ml ddH2O,1g (European agar for Bacteria)
Coomassie Brilliant Blue	0.25 g of Coomassie Brilliant Blue powder in 100 ml of methanol - acetic acid solution (5 methanol: 4 water: 1glacial acetic acid)
Luria-Bertani (LB) Broth /100ml	1g Trypton, 1g NaCl, 1g Yeast extract, 100 ml ddH2O.
SDS Fixing Solution	(1 glacial acetic acid: 2 methanol: 7 water).
Xylose Lysine deoxycholate (XLD)	XLD, ddH2O

Appendix 3. The sequence results of the *16S rDNA* and *recA* genes partial sequences of BC4 bacteria

A Bacteria colony 4 (BC4) *16s rDNA* >

CCCAAACTGGGCCGGCGTGCCCTTAATACATGCCAAGTCGAGCGGACAGAATGGGTAGCT
TGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACT
GGGAT AACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGA
CATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC AC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG
G ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTC
TGTTGTTAGGGAAGAACAAGCGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAG
A AAGCCACGGCTAACTACGTGCCCAGCAGCCGCGACAATAACGTACGAGGCAAACCGTTG
TCCCGCAAT TACTTGCGCGAAAAGGGCTCGAAGGCGGTTTCTTTAAGACTGAA GTGAAAG
CCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGA
GTGG AATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG
GCGACTCTCTGGTCTGTAACTGACGCGA GGAGCGAAAGCTTGGACCAAAGAA

B Bacteria colony 4(BC4) recA gene >

TTCATTGATGCTGAGCACGCTCTTGATCCTGTTTACGCGCAAAAGCTCGGTGTCAATATCG
AAGAGCTTCTGCTTTCTCAGCCGGATACGGGAGAGCAGGCGCTAGAGATTGCTGAAGCGC
TGGTGCGAAGCGGAGCTGTTGATATCGTAGTCGTTGACTCTGTTGCGGCGCTTGTTCCAAA
AGCTGAAATTGAAGGTGACATGGGTGATTCACACGTCGGTTTACAGGCGCGTCTCATGTGTGC
A GGCGCTCCGTAAGCTTCCGGCGCTATCAATAAA TGTAAAACAATCGCCAATCCTTTA
TTACCCAGATTCGGGAAAAAATTCGGGCCTATTTTTTGAGG .

Appendix 4. The sequence results of the *16S rDNA* and *recA* genes partial sequences of BC1 bacteria

A Bacteria colony 1 (BC1) *16s rDNA* >

GCGCTGCGGCGTGCCTATACATGCAGTCGACGAGCAGACCCCAGATTTATTTCGGTTGT
G AC GCTGGGGACTCGAGCGGGGGGGGGGAGAGTAACACGTGGGGAACCTGCCCCAT
A CTGTGGGATCCCTCTTGAACACAGGCGCTAATACCGGATAAGAAAGCATATCGCATGA
TCAGCTTATAAAGGGCGGCGTACGCTGTCTTTATGGGAAGGCCCCGGGGCGCATTATCT
A GGTGGGAGGGAAACGGTTTACCAGGGCAGTGATGCATAGCCGATTTGAGACAGTGAT
CGGACACAT TGACACTGACACACGGCCCACTCTCCTACGGGAGGCAGCAATAGGGAAT
T TTCCACAGTGGACGCAAGTGTGATGGAGCAACGCCGCGTGAGAGAAGAGTGTTTTCA
GATCGTAAATCTGTGGTGGTGGAGAAAAAAGATAGAGGTAATATGTGCTCTATATTTGAC
G GAAATAAAACAAAAAGTCGCGGATAACTACGCGCCAGCACGCGGGGTTATACGTATG
TGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAATAATAAGTC
TGATGTGAAAGCCCTCGGCTCAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGTG
CAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAA
CACC AGTGGCGAAGGCGGACTCTCTGGTTCTGCAACTGACGCGAGGTCCGAAAGGC T
TGGTTTGGGAGGG.

B Bacteria colony 1(BC1) recA gene >

TTTTATTGATGCAGAACATGCAATGGATCCAGCATATTGCTGAAGCATTAGGTGTAGAC
ATTGATTCACTTATTTTATCTCAACCTAATACAGGTGAAGAAGGTTTACAAATTGCTGATA
CTTTGATTTTTAGTGGTGCCATTGATATTGTGGTAGTTGGCTCTGTTGCAGCGTTAGTTCCA
CGTGCTGAAATTGAAGGCGAGTTGGGGGATGCTCATCTTGGTAAAGCAAGCTTTAGCCAT
TATTCAGG ATTTAAGTTAATTTTTGGTGGTTGTCAAGCCTAAATCCCAACATTCTGATAAA
CTAATAGAATAAACGGCAAAACCATG

Appendix 5. The 16S rRNA (A) and *recA* (B) alignments for BC4 with the highest maximum identity score.

A

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus vallismortis strain ZZB08 16S ribosomal RNA gene, partial sequence	1243	1243	97%	0.0	97%	JQ765433.1
Bacillus sp. L010 16S ribosomal RNA gene, partial sequence	1242	1242	97%	0.0	97%	KC153301.1
Bacillus amyloliquefaciens strain HA-01 16S ribosomal RNA gene, partial sequence	1242	1242	97%	0.0	97%	JX915740.1
Bacillus subtilis strain B43 16S ribosomal RNA gene, partial sequence	1242	1242	97%	0.0	97%	JX548222.1
Bacillus sp. MBEA29 gene for 16S rRNA, partial sequence	1242	1242	97%	0.0	97%	AB733525.1
Uncultured bacterium gene for 16S rRNA, clone: Acja3, isolated from Apis cerana japonica gut	1242	1242	97%	0.0	97%	AB668064.1
Bacillus amyloliquefaciens subsp. plantarum gene for 16S rRNA, partial sequence, strain: M20J	1242	1242	97%	0.0	97%	AB735995.1
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: M19F	1242	1242	97%	0.0	97%	AB735994.1
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: M6K	1242	1242	97%	0.0	97%	AB735984.1
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: M5K	1242	1242	97%	0.0	97%	AB735983.1
Bacillus amyloliquefaciens strain 48MoSuero 16S ribosomal RNA gene, partial sequence	1242	1242	97%	0.0	97%	JQ973602.1
Bacillus amyloliquefaciens subsp. plantarum YAU B9601-Y2 complete genome	1242	12367	97%	0.0	97%	HE774679.1
Lactobacillus murinus strain AU06 16S ribosomal RNA gene, partial sequence	1242	1242	97%	0.0	97%	JN987182.1
Bacillus amyloliquefaciens strain SWM1 16S ribosomal RNA gene, partial sequence	1242	1242	97%	0.0	97%	JN851189.1

B

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus amyloliquefaciens subsp. plantarum UCMB5036 complete genome	534	534	93%	2e-148	97%	HF563562.1
Bacillus amyloliquefaciens strain MJ7-66 RecA gene, partial cds	534	534	93%	2e-148	97%	JN813533.1
Bacillus amyloliquefaciens strain MJ5-48 RecA gene, partial cds	534	534	93%	2e-148	97%	JN813532.1
Bacillus amyloliquefaciens strain MJ3-6 RecA gene, partial cds	534	534	93%	2e-148	97%	JN813531.1
Bacillus amyloliquefaciens strain MJ1-4 RecA gene, partial cds	534	534	93%	2e-148	97%	JN813530.1
Bacillus amyloliquefaciens Y2, complete genome	534	534	93%	2e-148	97%	CP003332.1
Bacillus amyloliquefaciens subsp. plantarum YAU B9601-Y2 complete genome	534	534	93%	2e-148	97%	HE774679.1
Bacillus amyloliquefaciens strain MJ5-41 recombinase protein (recA) gene, partial cds	534	534	93%	2e-148	97%	JN048426.1
Bacillus amyloliquefaciens FZB42, complete genome	534	534	93%	2e-148	97%	CP000560.1
Bacillus amyloliquefaciens recA gene, strain FZB42	534	534	93%	2e-148	97%	AJ841295.1
Bacillus amyloliquefaciens IT-45, complete genome	529	529	93%	7e-147	97%	CP004065.1
Bacillus amyloliquefaciens subsp. plantarum AS43.3, complete genome	529	529	93%	7e-147	97%	CP003838.1
Bacillus amyloliquefaciens subsp. plantarum CAU B946 complete genome	529	529	93%	7e-147	97%	HE617159.1
Bacillus sp. fiply 3A RecA (recA) gene, partial cds	529	529	93%	7e-147	97%	GQ900665.1

Appendix 6. The 16S rRNA (A) and *recA* (B) sequence alignments for BC1with the highest maximum identity score.

Alignments Download GenBank Graphics Distance tree of results

A

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Lactobacillus helveticus strain MGA30-2 16S ribosomal RNA gene, partial sequence	913	913	97%	0.0	89%	HM057914.1
Lactobacillus helveticus strain MGA8-8 16S ribosomal RNA gene, partial sequence	913	913	97%	0.0	89%	HM057854.1
Uncultured bacterium clone IMAU 153 16S ribosomal RNA gene, partial sequence	913	913	97%	0.0	89%	GQ267896.1
Lactobacillus helveticus strain MGA30-5 16S ribosomal RNA gene, partial sequence	909	909	97%	0.0	88%	HM057915.1
Lactobacillus helveticus gene for 16S rRNA, partial sequence, strain: NBRC 15019	907	907	97%	0.0	88%	AB680751.1

B

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Lactobacillus helveticus CNRZ32 recombinase A (recA) gene, complete cds	374	374	67%	3e-100	97%	DQ826134.1
Lactobacillus helveticus partial recA gene for recombinase A, strain ATCC 15009T	374	374	67%	3e-100	97%	AJ621645.1
Lactobacillus helveticus R0052, complete genome	368	368	67%	2e-98	97%	CP003799.1
Lactobacillus helveticus H10, complete genome	368	368	67%	2e-98	97%	CP002429.1
Lactobacillus helveticus DPC 4571, complete genome	368	368	67%	2e-98	97%	CP000517.1
Lactobacillus helveticus strain BCRC 12936 recombinase A gene, partial cds	344	344	61%	3e-91	98%	JN226839.1